Molecular epidemiology of mycobacteria: Development and refinement of innovative molecular typing tools to study mycobacterial infections

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Markus Hilty

aus

Vaduz (Liechtenstein)

Basel, 2006

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel auf Antrag der

Herren Prof. Dr. Marcel Tanner, Prof. Dr. Glyn Hewinson und PD. Dr. Jakob Zinsstag

Basel, den 14 Februar 2006

Prof. Dr. Hans-Jakob Wirz

Dekan

Table of contents

Acknowledgments **iii**

Summary **v**

Zusammenfassung **vii**

Résumé **ix**

Abbreviations **xi**

Chapter I: Introduction **1**

1.1. Burden and epidemiology of human tuberculosis 2

1.2 Diagnosis of *Mycobacterium tuberculosis* complex 3

1.3. Molecular epidemiology of *Mycobacterium tuberculosis* 4

1.3.1 Spoligotyping 5

1.3.2 Variable Number of Tandem Repeats Typing 6

1.3.3. IS*6110*-RFLP and ligation-mediated PCR 7

1.4 Burden and epidemiology of *M. bovis* with particular reference to Africa 7

1.5 Molecular epidemiology of *M. bovis* 9

1.6 Evolution and ecotypes of the *Mycobacterium tuberculosis* complex 9

1.7 Disease burden caused by *Mycobacterium ulcerans* 11

1.8 Using molecular typing tools to study *M. ulcerans* transmission 11

1.9. Rationale and research frame work 12

1.10 References of Introduction 13

Chapter II: Goals and objectives **17**

2.1. Goal 18

2.2. Objectives 18

Chapter III: Molecular characterization and drug resistance testing of *Mycobacterium tuberculosis* isolates from Chad **19**

Chapter IV: *Mycobacterium bovis* Isolates from Tuberculous Lesions in Chadian Zebu Carcasses **35**

Chapter V: Evaluation of the discriminatory power of Variable Number Tandem Repeats typing of *Mycobacterium bovis* strains **49**

Chapter VI: Population structure of *Mycobacterium bovis* from a high incidence country: Implications for molecular epidemiology and design of diagnostic candidates **61**

Chapter VII: Genetic diversity in *Mycobacterium ulcerans* isolates from Ghana revealed by a newly identified locus containing a variable number of tandem repeats **69**

Chapter VIII: Comparative Nucleotide Sequence Analysis of Polymorphic Variable-Number Tandem-Repeat Loci in *Mycobacterium ulcerans* **83**

Chapter IX: General discussion and conclusions **95**

9.1 Abstract ... 96
9.2 Features of molecular epidemiological typing tools ... 97
9.2.1 Discriminatory power of IS6110 RFLP, spoligotyping and MIRU-VNTR ... 97
9.2.2 Molecular clock ... 97
9.2.3 Low heterogeneity and Convergence: the need for higher discriminatory power ... 99
9.3 Practical usage of molecular epidemiological results with special consideration of Africa ... 100
9.3.1 Reinfection versus relapse or mixed infection versus micro evolution: the ‘correct’ diagnosis ... 100
9.3.2 Degree of ongoing transmission, global mycobacterial population structure and outbreak investigations ... 101
9.3.3 Linking epidemiological and social science studies ... 102
9.3.4 Inter animal species transmission ... 103
9.3.5 Zoonotic transmission ... 104
9.4 Genotyping in *M. ulcerans* ... 105
9.5 Ten key messages and recommendations of this thesis ... 105
9.6 References of conclusion ... 107

Appendix 1: Variable host-pathogen compatibility in *Mycobacterium tuberculosis* **111**

Appendix 2: Species identification of non-tuberculous mycobacteria from humans and cattle of Chad **127**

Appendix 3: Methods **139**

1. Ligation mediated PCR ... 139
2. Spoligotyping ... 141
3. MIRU- and ETR-VNTR typing ... 144
4. IS6110-Restriction Fragment Length Polymorphism typing ... 146

Curriculum vitae **153**

Acknowledgments

The present PhD project was kindly funded by the NCCR North-South and was undertaken within a network of collaborations in Switzerland, Chad, Mauritania, United Kingdom and Ghana. Numerous people were involved in many different ways and without whom this work would never have been possible.
First and foremost my thanks go to my supervisor at the Swiss Tropical Institute, PD Dr. Jakob Zinsstag. He was very approachable and easy to work with. I also wish to acknowledge the members of Jakob Zinsstag's group (Borna Mueller, Daniel Weibel, Salome Duerr, Moustapha Ould-Taleb and Rea Tschopp) with whom I had great exchanges. Special thanks go also to Esther Schelling, who gave great support in the initial planning stages and in getting things started.
Many thanks must go to the people who work in Chad. Thank you to Colette Diguimbaye-Djaibe for helping me with the isolation of Mycobacteria in N'Djaména and for the friendly exchanges we had. I could not have done the typing work without the isolates from Chad. Richard Ngandalo is acknowledged for accompanying me on a field trip for collecting samples from clinically suspected tuberculosis cases and Sambo Guemgo for his work on infant tuberculosis in N'Djaména. I also received great support from the employees of the CSSI/ITS under the supervision of Dr. Daugla.
I would also like to mention and acknowledge the great support I received from Dr. Franca Baggi and her team at the National Centre for Mycobacteria, Zurich during the first 18 months of my thesis.
I especially thank Prof. Gerd Pluschke for enabling and supervising my work on *Mycobacterium ulcerans.* This topic counts as one of the most exciting ones during my thesis. Concerning the work on *M. ulcerans*, I also want to acknowledge Dorothy Yeboah-Manu who contributed a lot to making the *M. ulcerans* project a success and also the other students and members of Prof. Pluschke's group for a constructive working atmosphere.
From the Swiss Tropical Institute (STI) I further acknowledge Prof. Marcel Tanner, director of the STI and head of the Individual Project 4 within the NCCR North South, who made this thesis possible. He was also a great motivator during my time spent at the institute. My thanks go also to Prof. Mitchell Weiss, head of 'Gesundheitswesen und Epidemiologie', Bianca Plüss for her internship within our group, Stefan Dürr, who did his civil service in Chad and all the other students from the GWE.

I want to address another big thank you to Dr. Steven V. Gordon, Prof. Glyn Hewinson and Dr. Noel Smith from the Veterinary Laboratory Agencies (VLA), Weybridge, for allowing me to come and work at the VLA. I had very stimulating scientific exchanges and greatly enjoyed the working atmosphere. From this group I thank also Dr. Carmen Garcia-Pelayo, Dr. Javier Nunez-Gracia, Melissa Okker and Si Palmer for helping me with the micro array and spoligotyping work.
However, this thesis would not have been possible without the private support I received too. Thanks go to my sister, my grand mother, other family members and friends. Thank you to Dr. Susanne Pfenninger and Dr. Heinz Lüscher for stimulating discussions.
Finally, and above all, I want to thank my mother Judith Hilty for her never ending support and my girlfriend Andrea Drury. Andrea, you really helped me a lot.

Summary

One approach of molecular epidemiology of mycobacteria is the genotyping and comparison of DNA of infectious strains in order to monitor the transmission pathways of diseases. It is based on the assumption that patients infected with clustered strains are epidemiologically linked. Such results may help in understanding the modes of transmission and therefore in putting in place an adapted control strategy. To perform molecular epidemiological studies appropriate genotyping tools are a basic requirement. For *M. tuberculosis* they are well developed but their appropriateness has to be evaluated in the geographical area of interest. Like *M. tuberculosis*, *M. bovis* is also a member of the *M. tuberculosis* complex (MTC) and causes bovine tuberculosis in cattle, humans and a wide variety of other hosts. However, compared to *M. tuberculosis*, it is generally much more homogenic which renders the choice of an appropriate genotyping tool much more challenging. *M. ulcerans* appears to be even less diverse as, so far, strains have only been differentiated between but not within continents (with the exception of Australia).

Therefore the overall aim of this study was to contribute to the development and refinement of innovative molecular typing tools in order to study *Mycobacterium tuberculosis*, *bovis* and *ulcerans* infections.

Variable Number Tandem Repeats (VNTR) typing is a genotyping tool which evaluates the number of repeats at different loci distributed throughout the genome. We performed VNTR typing of 12 Mycobacterial Interspersed Repetitive Units (MIRU) and 3 Exact Tandem Repeats (ETR) for 40 *M. tuberculosis* strains from Chad. This revealed a similar discriminatory power to spoligotyping, which evaluates the presence or absence of 43 spacer DNA sequences between the 36 bp direct repeats (DRs) in the genomic DR region. Therefore, VNTR typing for *M. tuberculosis* is as valid a genotyping tool as spoligotyping. However, in contrast to spoligotyping, VNTR typing could also be useful in evaluating mixed infections within different members of the *M. tuberculosis* complex members in the future. Additionally, the use of both spoligotyping and VNTR typing could provide additional valuable information for future micro-epidemiological studies of the possible highly virulent Cameroon family clone. This clone is most prevalent in Nigeria, Cameroon and Chad, and is defined by the lack of spoligo spacers 23-25 and by the loss of characteristic chromosomal deletions.

We also performed spoligotyping and VNTR typing based on 16 known loci (12 MIRUs, 3 ETRs and VNTR 3232) for 67 *M. bovis* strains collected sequentially at the slaughterhouse of N'Djaména, Chad. The strains originated from two different zebu breeds of which the Mbororo was found to be more susceptible than the Arabe breed.

Genotyping of Chadian *M. bovis* strains confirmed the usual characteristically high homogenetic population structure of *M. bovis*. We could even identify that the 67 strains are members of only 2 clones. The clones were defined by spoligotyping (lack of spacer 30 vs. lack of spacers 20-22) and the finding of characteristic chromosomal deletions, indicating that the strains derived from two ancestral, single cells in the past. However, ETR A, B, C and MIRU 26, 27 were most appropriate for first line typing of *M. bovis* strains from Chad and superior than spoligotyping. This finding could help in identifying risk factors for inter animal and also zoonotic transmission and therefore have important public health implications.

As VNTR-typing is very attractive for *M. tuberculosis* complex members, attempts for using VNTR typing for *M. ulcerans* have also recently been made. However, the presented resolution was not higher than other genotyping tools. During this thesis, we identified a new VNTR locus, designated ST1, which did not have any orthologues in the *M. tuberculosis* genome. In combination with a previously published MIRU locus, we were able to identify three different genotypes within Ghanaian *M. ulcerans* strains and therefore demonstrate diversity in African strains for the first time. We further showed that DNA sequencing of the different VNTR loci can refine the discriminatory power if the loci are analyzed separately but, if analyzed commonly, doesn't improve the overall discriminatory power. In the latter, agarose gel electrophoresis of the amplification products of all polymorphic VNTR loci is normally sufficient and sequencing does not result in further refinement.

Zusammenfassung

In der Molekularen Epidemiologie der Mycobakterien können mit Hilfe der DNA Genotypisierung Übertragunswege infektiöse Bakterienstämme verfolgt werden. Sie basiert auf der Annahme, dass Patienten, welche mit gleichen (clustered) Stämmen infiziert sind, eine epidemiologische Verbindung haben. Die Analyse genotypischer Ähnlichkeit kann helfen zu besseren Bekämpfungsstrategien beizutragen. Um molekular epidemiologische Studien überhaupt durchführen zu können sind angepasste Genotypisierungsmethoden eine Grundvoraussetzung. Im Falle von *M. tuberculosis* sind sie gut entwickelt aber ihre Eignungen müssen in den jeweiligen geographischen Gebieten evaluiert werden. Wie *M. tuberculosis* ist auch *M. bovis* ein Mitglied des *Mycobacterium tuberculosis* Komplexes (MTC) und verursacht die bovine Tuberkulose in Rindern, Menschen und in einer grossen Bandbreite von anderen Wirten. Verglichen mit *M. tuberculosis* ist *M. bovis* jedoch im allgemeinen viel homogener und deshalb ist die Wahl der geeigneten Genotypisierungsmethode viel herausfordernder. *Mycobacterium ulcerans* scheint gar noch homogener zu sein, da Stämme bis jetzt nur zwischen aber nicht innerhalb der Kontinente unterschieden wurden (mit der Ausnahme von Australien).

Deshalb war es das übergeordnete Ziel dieser Doktorarbeit zu Entwicklung und Verbesserung von innovativen, molekularen Typisierungsmethoden beizutragen um die Infektion von *M. tuberculosis*, *M.bovis* und *M.ulcerans* zu studieren.

Das Typisieren von VNTR (Variable Number Tandem Repeats) ist eine Genotypisierungsmethode welche die Anzahl von Repetitionen an verschiedenen, über das ganze Genom verteilten Orten, evaluiert. Wir führten die Typisierung von VNTR an 12 MIRUs (Mycobacterial Interspersed Repetitve Units) und 3 ETRs (Exact Tandem Repeats) für 40 *M. tuberculosis* Stämme vom Tschad durch. Es resultierte ein ähnlicher Unterscheidungsgrad wie für das Spoligotyping, welches das Vorkommen von 43 ‚Spacer' Sequenzen untersucht welche sich zwischen 36 Basenpaaren langen und direkten Repetitionen in der DR (Direct repeat) befinden. Aus diesem Grund ist das Typisieren von VNTRs als Genotypisierungsmethode genauso wertvoll wie das Spoligotyping. Das Typisieren von VNTRs könnte jedoch, im Gegensatz zum Spoligotyping, für die Zukunft nützlich werden um Mischinfektionen zwischen verschiedenen Mitgliedern des MTC zu evaluieren. Zusätzlich könnte der Gebrauch von beiden Methoden, spoligotyping und VNTRs, zusätzliche und wertvolle Informationen für zukünftige mikro-epidemiologische Studien des möglicherweise sehr virulenten Klon der Kamerun Familie liefern. Dieser Klon

weist eine sehr hohe Prävalenz in Nigeria, Kamerun und Tschad auf und ist definiert durch den Verlust der Spacer Sequenzen 23-25 und charakteristischen chromosomalen Löschungen.
Ebenfalls führten wir das Spoligotyping und das Typisieren der VNTRs anhand von 16 bekannten Orten (12 MIRUs, 3 ETRs und dem VNTR 3232) für 67 *M. bovis* Stämme durch, welche sukzessive von Proben des Schlachthofs von N'Djaména, Tschad, erhalten wurden. Die Stämme stammten von 2 verschiedenen Rinderrassen von welchen die Mbororo gegenüber *M. bovis* empfänglicher war als die Arabe Rasse.
Das Genotypisieren von tschadischen *M. bovis* Stämmen bestätigte die üblicherweise hohe homogene Populationsstruktur von *M. bovis*. Wir konnten sogar zeigen dass alle diese 67 Stämme Mitglieder von nur 2 Klonen sind. Diese Klone wurden definiert durch das Spoligotyping (Verlust von Spacer Sequenzen 30 für den einen und Verlust von 20-22 für den anderen Klon) und den Verlust von charakteristischen, chromosomalen Löschungen, was darauf hinweisen könnte, dass alle Stämme von nur zwei einzelnen Zellen aus der Vergangenheit abstammen. Abgesehen davon zeigte das Typisieren, dass ETR A, B, C und MIRU 26 27 am geeignetsten für ein erstes, grobes Typisieren von *M. bovis* Stämmen von Tschad ist und dem Spoligotyping überlegen ist. Dieser Befund könnte in der Zukunft helfen Risikofaktoren für die zoonotische aber auch zwischen verschiedenen Tieren stattfindende Übertragungswege zu identifizieren und könnte deshalb wichtige Konsequenzen für das öffentlich Gesundheitswesen haben.
Da das Typisieren der VNTR für MTC Mitglieder sehr attraktiv ist, wurde erst kürzlich Versuche gemacht dieses auch für *M. ulcerans* zu etablieren. Die präsentierte Auflösung war jedoch nicht besser als diejenige von anderen Genotypisierungsmethoden. Während dieser Doktorarbeit, haben wir einen VNTR identifiziert, welcher ST1 genannt wurde und keine Analogien im *M. tuberculosis* Genom hatte. Im gemeinsamen Gebrauch mit einem bereits beschriebenen und publizierten MIRU gelang es uns drei verschiedene Genotypen innerhalb von ghanaischen Stämmen zu unterscheiden. So konnten wir zum ersten Mal Heterogenität innerhalb von Afrikanischen Stämmen nachweisen. Des weiteren zeigten wir, dass das Sequenzieren verschiedener VNTR die Auflösung verfeinern kann, wenn die polymorphen VNTR separat aber nicht gemeinsam analysiert werden. Im letztgenannten Fall ist die Agarose-Gel-Elektrophorese der amplifizierten, polymorphen VNTR Produkte normalerweise ausreichend und das Sequenzieren ermöglicht keine weitere Verfeinerung.

Résumé

L’approche d'épidémiologie moléculaire des mycobactéries permet d’analyser et de comparer l'ADN de souches infectieuses afin de suivre les voies de transmission des maladies. Elle est basée sur la supposition que les patients infectés de mycobactéries génotypiquement identiques sont liés épidémiologiquement. Ces résultats peuvent aider à comprendre les voies de transmission et contribuer à adapter les stratégies de lutte.

Pour effectuer des études d’épidémiologie, des outils appropriés pour le typage d’ADN sont une condition de base. Pour *M. tuberculosis*, ils sont bien développés mais doivent être évaluées pour chaque zone géographique d'intérêt. Comme *M. tuberculosis*, *M. bovis* est aussi un membre du complexe de *M. tuberculosis* (MTC) et cause la tuberculose chez le bétail, l’homme et une large variété d'autres hôtes. Cependant, comparé à *M. tuberculosis*, l’ADN de *M. bovis* est généralement beaucoup plus homogène et donc le choix de l'outil approprié pour le typage est beaucoup plus complexe. *M. ulcerans* semble d’être encore moins variable car jusque là, les souches ont pu être différenciées entre les continents, mais pas à l’intérieur des continents (à l'exception de l'Australie).

Donc le but final de cette thèse était de contribuer au développement et au perfectionnement d'outils moléculaires innovateurs pour le typage des mycobactéries pathogènes (*M. tuberculosis*, *M. bovis* et *M. ulcerans)* et pour étudier l'infection causée par ces derniers.

Le typage par VNTR (variable number tandem repeats) est un outil moléculaire qui évalue le nombre de répétitions à des sites différents répartis dans le génome. Nous avons effectué le typage par VNTR de 12 MIRU (Mycobacterial Interspersed Repetitve Units) et 3 ETR (Exact Tandem Repeats) pour 40 souches de *M. tuberculosis* du Tchad. La pouvoir de discrimination était semblable au spoligotyping, qui évalue la présence ou l'absence de 43 séquences de la région génomique DR (direct repeat) de l’ADN. Le typage de VNTR pour *M. tuberculosis* est aussi valable comme outil d’épidémiologie moléculaire que le spoligotyping. Dans l’avenir le typage par VNTR serait utile dans l'évaluation d'infections mixtes par les différents membres de MTC. L’utilisation des deux méthodes : spoligotyping et typage par VNTR, pourrait fournir des informations complémentaires de valeur pour des études futures sur la micro épidémiologie du clone de la famille camerounaise. Ce clone, qui pourrait être fortement virulent, est très répandu au Nigeria, au Cameroun et au Tchad et est défini par l’absence des séquences DR 23-25 et par des délétions chromosomiques caractéristiques.

Nous avons aussi effectué le spoligotyping et le typage par VNTR basés sur 16 locus connus (12 MIRUS, 3 ETRS et VNTR 3232) pour 67 isolats de *M. bovis*, collectés à l'abattoir de N'Djaména, Tchad. Les souches proviennent de deux races différentes de zébus dont le zébu de race Mbororo est plus susceptible que le zébu de race Arabe.

Le typage moléculaire de souches tchadiennes de *M. bovis* a confirmé encore une fois la structure fortement homogène de la population de *M. bovis*. Les 67 souches analysées semblent être membres de seulement deux clones. Les clones ont été définis par le spoligotyping (le manque de la séquence 30 pour l'un et des séquences 20-22 pour l'autre) et par la découverte de délétions chromosomiques caractéristiques, indiquant que les souches descendent de deux seules cellules ancestrales. De plus, ETR A, B, C et MIRU 26, 27 étaient les plus appropriés pour un premier typage approximatif des souches de *M. bovis* du Tchad et supérieurs au spoligotyping. Cela permettrait d'identifier les facteurs de risques pour la transmission entre différents animaux, mais aussi la transmission zoonotique et pourrait donc avoir des implications importantes pour la santé publique.

Comme l'utilisation de typage par VNTR est très attractive pour les membres MTC, on a essayé récemment de l'utiliser aussi pour *M. ulcerans* mais, la résolution obtenue n'était pas meilleure aux 'autres outils moléculaires.

Dans le cadre de cette thèse, nous avons identifié un nouveau locus VNTR, désigné ST1, qui n'avait pas de séquences similaires dans le génome de *M. tuberculosis*. Par la combinaison avec un locus MIRU précédemment publié nous étions capables d'identifier trois génotypes différents dans les souches ghanéennes de *M. ulcerans* et donc de trouver pour la première fois une diversité parmi les souches africaines. Entre autre, nous avons pu montré que le séquençage d'ADN des différents VNTRs peut raffiner le pouvoir discriminatoire si les VNTR polymorphes sont analysés séparément, mais pas s'ils sont inclus ensemble pour l'analyse. Et enfin,, l'électrophorèse par gel d' agarose des produits amplifiés de tous les VNTR polymorphes est suffisante et le séquençage ne contribue pas à une meilleure résolution.

Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
AFB	Acid Fast Bacilli
BCG	Bacillus Calmette-Guèrin
BU	Buruli Ulcer
bp	base pairs
BTB	Bovine Tuberculosis
CSSI	Centre de Support en Santé International
dNTP	Deoxyribonucleosidetriphosphate
DOTS	Direct Observed Treatment Strategy
DNA	Deoxyribonucleic Acid
DR	Direct Repeat
ETR	Exact Tandem Repeats
HGRTN	Hôpital Général de Référence Nationale du Tchad
HIV	Human Immunodeficiency Virus
IS	Insertion Sequence
LJ	Löwenstein Jensen
LRVZ/V	Laboratoire de recherches vétérinaires et zootechniques de Farcha
LSP	Large Sequence Polymorphism
MIRU	Mycobacterial interspersed repetitive units
MLST	Multilocus Sequence typing
MTC	*Mycobacterium tuberculosis* complex
NALC	N-Acetyl-L-Cystéine
NTM	Non tuberculosis mycobacteria
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
PRPA	PCR-restriction Profile Analysis
RD	Region of Diversity
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
STI	Swiss Tropical Institute
TB	Tuberculosis

VNTR	Variable Number Tandem Repeats
WHO	World Health Organization
ZN	Ziehl Neelsen

Chapter I: Introduction

1.1. Burden and epidemiology of human tuberculosis

Despite the availability of anti-tuberculosis antibiotics, the disease burden of human tuberculosis remains a very serious and wide-spread public health problem. At present, approximately a third of the world population is infected with *Mycobacterium tuberculosis,* which is a member of the *Mycobacterium tuberculosis* complex (MTC) and the main causative organism for human tuberculosis. Today we consider that 2 million deaths and 8 million new human infections occur every year (11). Many of the 22 most affected countries identified by the WHO are developing countries (Fig. 1) (13). There are various reasons why tuberculosis control strategies have not yet succeeded:

- Resistance to antibiotics used in the treatment of tuberculosis. In different countries, between 0 and 54 % of tuberculosis cases are multi drug resistant (15).
- Poverty connected to the problems of unemployment, access to good quality sanitary services and urbanization

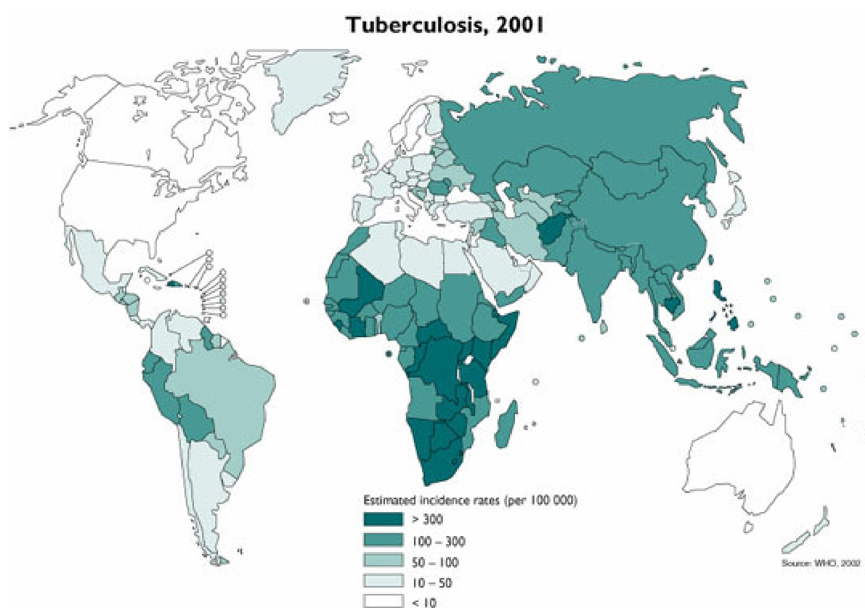

Fig. 1: Estimated global incidence rates of tuberculosis (2001). (Source: World Health Organization (WHO) 2003)

- Exponential increase of journeys and migration
- Co-existence of the Human Immunodeficiency Virus (HIV) fuels the epidemic of tuberculosis on a large scale (11). Worldwide, 70.1 % (25.3 millions) of HIV positive people live in sub-Saharan Africa (WHO / CDS / TB / 2002.296). In 1997, new cases of TB totalled an estimated 7.96 million, including 3.52 million cases (44%) of infectious pulmonary disease (smear-positive), with 16.2 million existing cases of disease. An estimated 1.87 million people died of TB and the global case fatality rate was 23% but exceeded 50% in some African countries with high HIV rates (11).

Infection with HIV favours a new infection with mycobateria; however, it can also reactivate a latent infection.

- DOTS (Directly Observed Treatment short course), a strategy promoted by the World Health Organization (WHO) is either not implemented, ineffective or not feasible in various countries.
- Although *M. tuberculosis* most often causes pulmonary tuberculosis, it is also the causative agent for extra pulmonary tuberculosis. This form of tuberculosis is often underdiagnosed.
- *Mycobacterium bovis*, another member of the MTC is also known to cause clinically undistinguishable tuberculosis in humans. Its zoonotic importance for the burden of human tuberculosis is unknown and currently under research (see also chapter on *M. bovis*).

1.2 Diagnosis of *Mycobacterium tuberculosis* complex

M. tuberculosis, which is the main pathogen for human tuberculosis, has some specific characteristics which diagnostics can take advantage of. As for all mycobacteria, *M. tuberculosis* is a gram positive and rod shaped bacterium which posseses a thick lipid-rich cell wall. This allows the acid fast staining of clinical specimens or cultures with Carbol fuchsin in the presence of acetic alcohol or fluorescent auramine-rhodamine dyes. However, some important antigens are specific for MTC only including: purified protein derivative (PPD), old tuberculin (OT) and cord factor (http://www.life.umd.edu/classroom/bsci424/PathogenDescriptions/Mycobacterium.htm). The building of cords of MTC can be observed in MTC positive, liquid cultures with a light microscope (Fig. 2) (25). Within the MTC complex, members can be differentiated through a biochemical test. As this is not very reliable, different PCR approaches are in use, of which the Hain test is best known (http://www.hain-lifescience.de). However, despite the performance ability of PCR, culturing remains the golden standard and cannot be omitted.

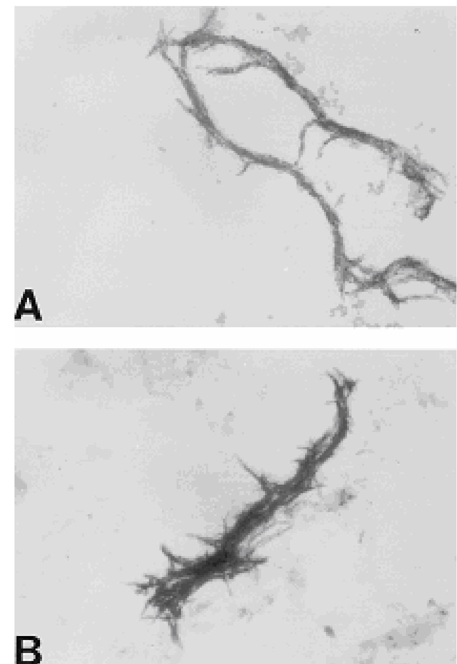

Fig. 2. Microscopic morphology of *Mycobacterium* species grown in BACTEC 12B broth and stained with Kinyoun acid-fast stain. (A) *M. tuberculosis*, exhibiting serpentine cording. (B) *Mycobacterium* species other than *M. tuberculosis* that exhibit loose aggregates, referred to as pseudocording (Source: McCarter *et al.*, *J. Clin. Microbiol.* 1998)

1.3. Molecular epidemiology of *Mycobacterium tuberculosis*

Molecular epidemiology is a powerful approach for monitoring infectious diseases (32). It is particularly important in the study of chronic diseases such as tuberculosis, where patients with recurrent tuberculosis can be chronically infected with a given strain and relapse due to reactivation of that strain or, in contrast, can be reinfected by a different strain after cure (42). A correct distinction between these alternatives is essential for accurate estimation of the success rates of tuberculosis programs (5). Moreover, it can give unique insights into the international dissemination dynamics of *M. tuberculosis* by the comparison of isolates from widespread geographic areas and allows one to analyze evolutionary changes of pathogen populations (38). Molecular studies of *M. tuberculosis* are made extensively in industrialized but only few developing countries. Molecular epidemiological results from developed countries often show high polymorphism in the genetic patterns of *M. tuberculosis* complex strains (4,19,44). This is explained by two factors (43): The relatively high percentage of cases in low-incidence areas due to endogenous reactivation and the large proportion of cases in these areas found amongst non-native populations originating from different geographical origins, which introduce exotic strains not known in these areas.

However, in interpreting the proportion of clustered strains found in a study, knowledge of the proportion of tuberculosis cases in the community included in the study is important. A high number of tuberculosis cases analyzed in a community can overestimate the proportion of recent transmission. On the other hand, a low number of samples can underestimate the proportion of recent transmission because the percentage of clustered strains is known to increase sharply at the beginning of a study till a certain number of cases is reached. Furthermore, molecular epidemiological studies should give information on the study setting, duration of study, the recruitment period and the definition of clustering used. The data on clustering should be disaggregated at the very least by age, sex and immigration status (16).

If we consider Africa, apart from studies carried out in Tunisia and Egypt, where most of the *M. tuberculosis* strains only belonged to a few genotype families (20), results have also been obtained from the countries of Botswana (24) and South Africa. Wilkinson *et al*. (47) found a high clustering rate of patterns (45%) in a rural area of KwaZulu Natal, South Africa. In contrast, quite a low clustering rate was found in Botswana (24) and in the communities of Ravensmead and Uitsig, Cape Town, South Africa (46). These contradicting results from high incidence countries show how little is known when it comes to molecular epidemiology of TB in Africa. In addition, many countries, like Chad, completely lack data from similar studies suggesting that further research in these countries is urgently needed. In order to

perform molecular epidemiological studies, one or more appropriate genotyping tools are necessary. Nowadays, there exists a number of different ‘working’ tools, which are used routinely or for special occasions:

1.3.1 Spoligotyping

This method is based on the evaluation of the presence or absence of 43 spacer DNA sequences between the 36 bp direct repeats (DRs) in the genomic DR region of MTC strains (Fig. 3). Spoligotyping pattern are obtained by PCR amplification, containing a non-and biotinylated Primer, of the DR region and hybridisation of the amplification products on the 43 spacer DNA containing membrane. The visualizing of the pattern is obtained by a second antibody which leads to a fluorescent emission (21). The lack of certain spacers can be helpful for diagnosing certain *M. tuberculosis* strain families and different *M. bovis* strains (Table 1).

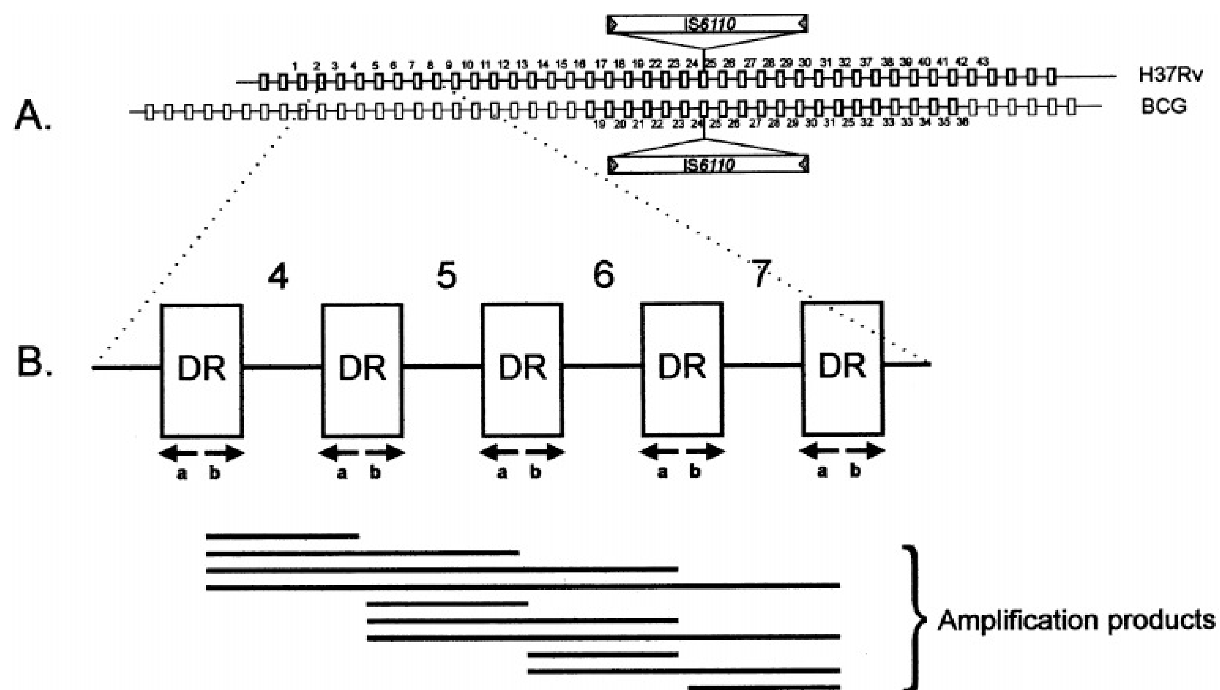

FIG. 3: (A) Structure of the DR locus in the mycobacterial genome. *M. tuberculosis* H37Rv and *M. bovis* BCG contain 48 and 41 DRs, respectively (depicted as rectangles), which are interspersed with unique spacers varying in length from 35 to 41 bp. The (numbered) spacers used correspond to 37 spacers from *M. tuberculosis* H37Rv and 6 from *M. bovis* BCG. The site of integration of insertion element IS*6110* is depicted.
(B) Principle of in vitro amplification of the DR region by PCR. Any DR in the DR region may serve as a target for these primers; therefore, the amplified DNA is composed of a mixture of a large number of different-size fragments. Shown is the combination of fragments that would be produced by in vitro amplification of a DR target containing only five contiguous DRs. (Source: Kamerbeek *et al*., *J. Clin. Microbiol.* 1997)

Table 1: Diagnostic spoligo spacer missing for *M. tuberculosis* family members (12), *M. africanum* and host adapted *M. bovis* strains (35).

M. tb family members	Spacer lacking
M. tb (Beijing)	1-34
M. tb (Haarlem)	31, 33-36
M. tb (Latin America)	21-24, 33-36
M. tb (East African India)	29-32, 34
M. tb (Central Asia)	4-7, 23-34
M. tb (Cameroon)	23-25, 33-36
M. africanum (Type I)	9, 39
M. bovis (antelope)	9, 16, 39
M. bovis (seal/vole)	3, 9, 16, 39-43
M. bovis (caprine)	3, 9, 16, 39-43
M. bovis (cattle)	3, 9, 16, 39-43
M. bovis BCG	3, 9, 16, 39-43

1.3.2 Variable Number of Tandem Repeats Typing

This method evaluates the number of repeats at different loci distributed throughout the genome. PCR amplification and comparison of the product sizes with a molecular size marker on an agarose gel is normally sufficient as the size differences are within a range of 30-100 base pairs. There are different types of VNTR. Mycobacterial interspersed repetitive units (MIRUs) in DNA elements are often found as tandem repeats and dispersed in intergenic regions of the genomes. The M. tuberculosis H37Rv reference strain contains 41 MIRU loci, of which 12 are polymorphic and therefore appropriate for VNTR typing (Fig. 4) (39).

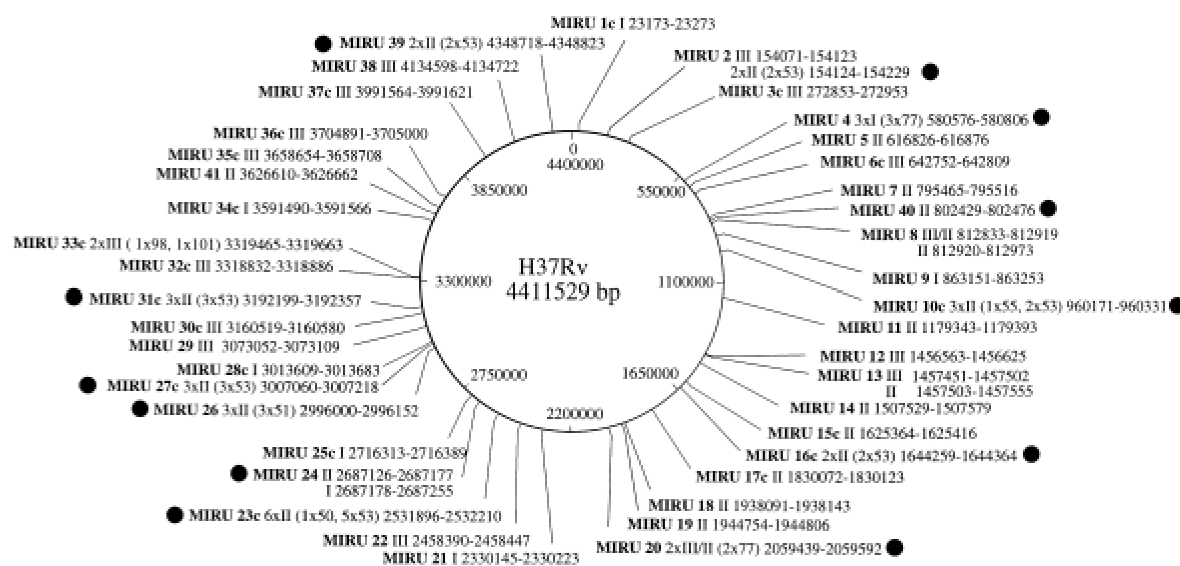

Fig. 4. Position of the 41 MIRU loci on the *M. tuberculosis* H37Rv chromosome. Arabic numbers in bold specify the respective MIRU locus numbers. The \`c' designates that the corresponding MIRUs are in the reversed orientation to that defined by Cole *et al.* (1998). Roman numbers give the type of MIRU (type I, II or III). The exact positions of the MIRU loci are given in arabic numbers after the type numbers. The 12 loci containing variable numbers of MIRUs among the 31 analysed strains are indicated by black dots (Source: Supply *et al.*, *Mol. Microbiol.* 2000).

In a different analysis of eleven tandem repeat loci, six exact tandem repeat (ETR) loci contained large DNA repeats with identical sequences in adjacent repeats and are therefore also appropriate for VNTR typing (Fig. 5) (14). Recently a number of different VNTRs have been presented (31,34), which are mostly used for *M. bovis* typing for it is not normally as polymorphic as *M. tuberculosis*.

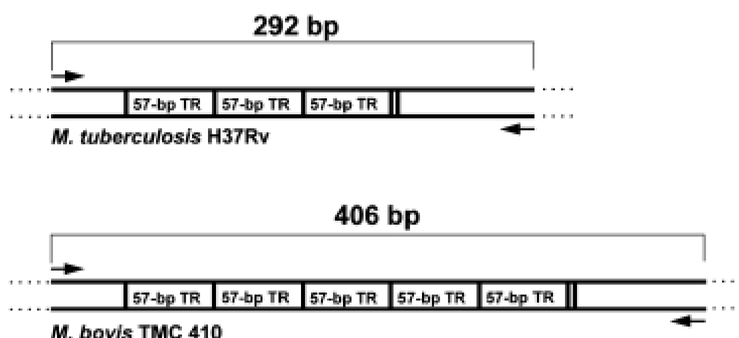

Fig. 5: Example of a VNTR locus. The figure shows genomic DNA at the ETR-B locus in *M. tuberculosis* H37Rv and *M. bovis* TMC 410. Amplification of this locus using PCR primers complementary to flanking DNA (arrows) resulted in receiving the respective 292 and 406 bp PCR products. *M. tuberculosis* H37Rv DNA contains three complete copies of the 57-bp tandem repeat, plus eight additional bases corresponding to the beginning of another tandem repeat. *M. bovis* TMC 410 DNA has five complete copies plus the same eight additional bases (Source: Frothingham *et al*., *Microbiol.* 1998)

1.3.3. IS*6110*-RFLP and ligation-mediated PCR

IS*6110*-RFLP is the current golden standard in DNA fingerprinting of *M. tuberculosis* complex members. The technique exploits the variability in both the number and genomic position of IS6110 to generate strain-specific patterns (41). However, the need for extensive strain cultivation, the high cost, the long handling procedure and the difficulty of comparing results between different laboratories are considerable drawbacks for this method.

Ligation-mediated PCR uses one primer specific for IS*6110* and a second specific for a linker ligated to SalI-restricted genomic DNA (30). In contrast to IS*6110*-RFLP, it is a rapid screening method and relatively cheap.

1.4 Burden and epidemiology of *M. bovis* with particular reference to Africa

Bovine tuberculosis (BTB), a disease characterised by progressive development of specific granulomatous lesions or tubercles in lung tissue, lymph nodes or other organs, is caused by *Mycobacterium bovis* and cattle are considered to be the primary hosts (3). However, the pathogen seems to have one of the broadest host ranges (27) as it is also isolated in many

different host species such as wild boar, deer (33) badgers, goats, sheep, rabbits and pigs (8). But while some species (wild boar, deer and badgers) are considered to be maintenance hosts and therefore are dangerous natural reservoirs, others (goats, sheep, rabbits and pigs) act only as a spillover for *M. bovis*. These hosts are infected with host adapted *M. bovis* substrains rather than with classical *M. bovis* strains e.g. caprae (see 1.6 Evolution and ecotypes of MTC).

In many industrialized countries, like Switzerland, bovine tuberculosis has been eradicated due to elimination programs and milk pasteurization (29). However, the burden of disease is still considerable in other industrialized countries such as the U.S. and U.K as the presence of natural reservoirs (badgers, deer) makes eradication difficult. Although a vaccination exists, the currently available BCG is not effective enough to completely prevent infection and interferes with the PPD test and there are assumptions that the vaccination of cattle is not deemed financially profitable (J. Zinsstag, personal communication). Indeed, during the 4th *M. bovis* conference in 2005, policy makers agreed to vaccinate wild life reservoirs rather than cattle.

In developing countries, the incidence of animal TB is especially high as control measures are not at all or only partially applied (3). Additionally, some of them, such as the test and slaughter policy are not feasible due to the lack of financial compensation (23). In Africa, bovine TB represents a potential health hazard to both animals and humans, as nearly 85 % of cattle and 83 % of the human population live in areas where the disease is prevalent (3).

M. bovis is additionally of particular interest from a public health perspective as man is also susceptible to infection. The burden of tuberculosis in humans caused by *M. bovis* is largely unknown or underdiagnosed due to the lack of adequate laboratory equipment but its presence has been proven and infections due to *M. bovis* are described in various African countries (9). Clinically, tuberculosis caused by *M. bovis* is not different from that caused by *M. tuberculosis,* but *M. bovis* is resistant to the antibiotic pyrazinamide, which is a first line drug in the treatment against human tuberculosis within the program of DOTS. In developing countries consumption of unpasteurised milk, poorly heat-treated meat and close contact with infected animals represent the main sources of infection for humans (3).

In conclusion there are three main reasons why eradication of bovine TB is recommended (3):

- loss in productivity due to infected animals
- animal market restrictions
- the risk of infection to the human population

1.5 Molecular epidemiology of *M. bovis*

Different studies on *M. bovis* are carried out in order to improve the traceability of the *M. bovis* infections and identification of the origin of the outbreak Haddad N. *et al.* (18) genotyped 1266 *M. bovis* isolates in France and observed an apparently high level of heterogeneity of 161 different clusters and a low frequency of the two main spoligotypes clusters. In contrast, similar molecular studies in island countries like Great Britain (7) or Australia (10) showed a low level of heterogeneity and a high frequency of the main spolygotype clusters.

Again, very few such studies have been carried out in developing countries in Africa. Some studies in Cameroon (26) and Tanzania (22) have shown similar results to those made in Great Britain or Australia with a high homogeneity and thereby indicate a high recent transmission rate. There have been no molecular epidemiological studies of *M. bovis* in Chad before this PhD thesis.

There are also studies which look at transmission pathways from *M. bovis* between different animal species, from animal to human (zoonotic) and from human to human. Serraino *et al*. (33) report spoligotype clusters which include 9 strains isolated from wild boar and 11 strains isolated from cattle, thus confirming the possibility of transmission between the two animal species. V. Soolingen *et al*. (45) show clusters containing *M. bovis* isolated from humans and cattle using the combination of the RFLP methods IS6110 and PGRS. One of the first results indicating but not proving *M. bovis* zoonotic transmission between cattle and humans in Africa is shown in a study from Tanzania, where the same *M. bovis* spoligotype was isolated from man and cattle (23). Moreover, molecular epidemiological studies by Guerrero *et al*. (17) showed the transmission of *M. bovis* MDR tuberculosis between HIV-1-positive patients. It is suggested that transmission of *M. bovis* took place within hospitals and that advanced HIV-1 immunosuppression was associated with the development of MDR tuberculosis.

As with *M. tuberculosis*, molecular epidemiology can also develop a better understanding of the sources and modes of *M. bovis* transmission thereby enabling more effective control measures to be implemented in bovine eradication programs.

1.6 Evolution and ecotypes of the *Mycobacterium tuberculosis* complex

Human and animal tuberculosis are caused by different members of the *Mycobacterium tuberculosis* complex (MTC), of which *M. tuberculosis* and *M. bovis* are best known and share 99.9 % of the same genome.

Brosch *et al.* 2002 (6) described a new evolutionary scenario for MTC members which concluded that all animal adopted *M. tuberculosis* complex strains differ to human adopted *M. tuberculosis* strains by the absence of a specific chromosomal region (RD9; Fig. 6). These results contradict the often presented hypothesis that *M. tuberculosis* evolved from *M. bovis*. It suggests that it is more likely that the common ancestor of the tubercle bacilli was *M. tuberculosis* or *M. canettii* alike and may already have been a human pathogen (6).

The RD9 deleted lineage, which is almost phenotypically homogenous, excludes *M. tuberculosis* as well as *M. canettii*, but includes *M. africanum* (found in humans), *M. microti* (voles, wood mice and shrews), *M. pinnipedii* (marine mammals), *M. caprae* (goats) and *M. bovis* (associated with cattle).

A recent study suggested using phylogenetically informative spacers, in combination with previously identified single nucleotide mutations and chromosomal deletions to identify different clades in the RD9 deleted lineage each with a separate host preference (Fig. 7) (35). It is therefore suggested that the MTC is rather described as a series of host-adapted ecotypes than attributed a broad host range of distinct members of the MTC, like it was the case for a long time for *M. bovis* (27).

The vaccine strain (*M. bovis* BCG) differs from the pathogenic *M. bovis* strain by the absence of RD1 (Fig. 6). Consequently, RD1 is associated with virulence and is of great interest in current research (6).

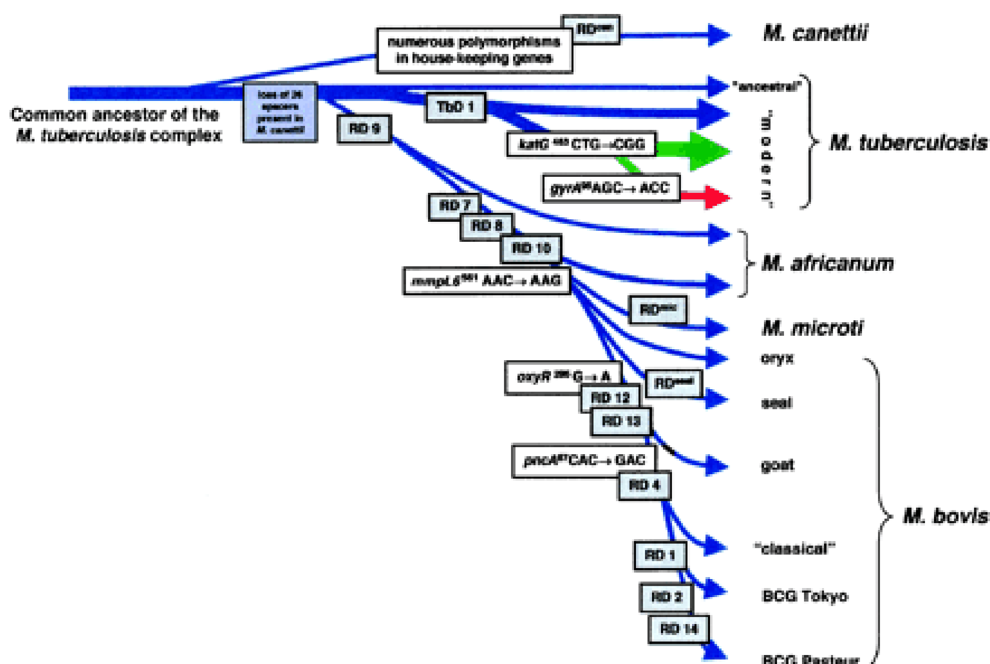

Fig. 6: Scheme of the proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (grey boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes (Source: Brosch *et al.*, *PNAS*, 2002)

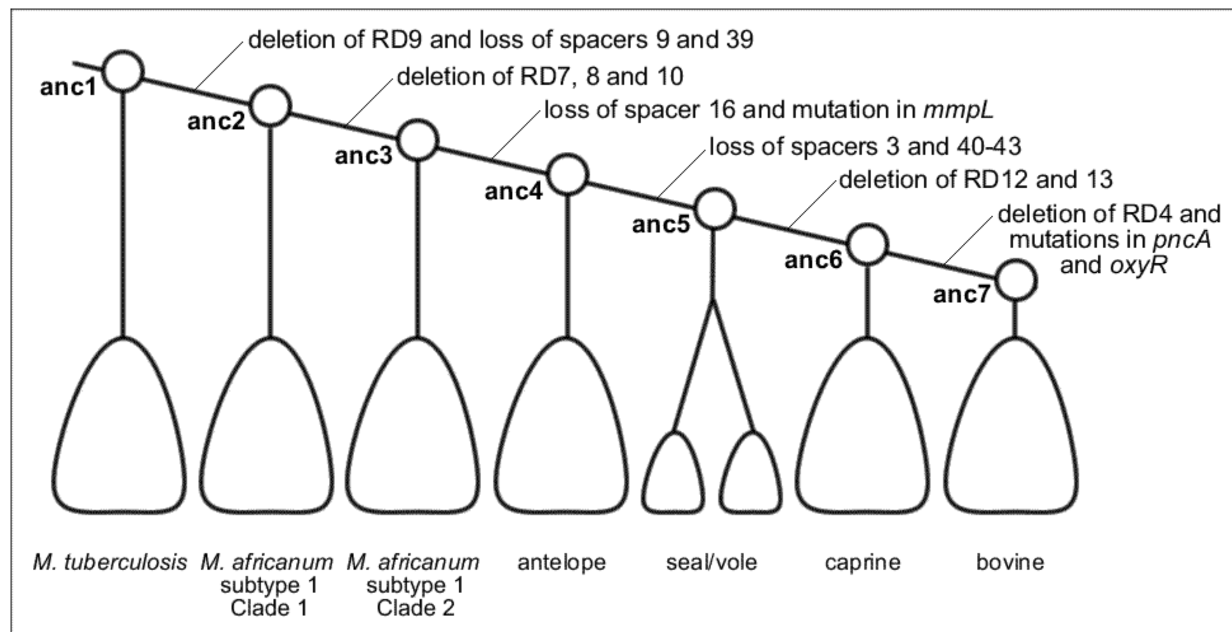

Fig. 7 (bottom): The phylogeny of the RD9 deleted lineage and *M. tuberculosis* showing the informative deletions (RD deletions), single nucleotide mutations and deleted spoligotype spacers used to define the clades. The hypothetical ancestors, anc1–anc7, are shown as open circles (Source: Smith *et al., J. Theor. Biol.* 2005)

1.7 Disease burden caused by *Mycobacterium ulcerans*

Mycobacterium ulcerans, the causative agent of Buruli ulcer (BU), is an emerging pathogen particularly in Sub-Saharan African countries, and is also found in tropical and sub-tropical regions of Asia, the Western Pacific and Latin America (2). However, reported incidences probably do not give a complete picture as under-reporting has to be assumed. More than 30,000 cases have been estimated in West Africa. After *M. tuberculosis* and *M. leprae*, *M. ulcerans* is the third most frequent mycobacterium causing infections in humans. BU is characterized by chronic, necrotic lesions of subcutaneous tissues. Due to the lack of an established effective antimicrobial therapy, surgical excision and skin grafting is currently the recommended treatment (40).

1.8 Using molecular typing tools to study *M. ulcerans* transmission

While it is known that proximity to slow flowing or stagnant water bodies is a risk factor for *M. ulcerans* infection, the exact mode of transmission is unknown. Molecular typing methods such as multi-locus sequence typing, 16S rRNA sequencing (28), restriction fragment length polymorphism, the 2426 PCR analysis (36), IS2404-Mtb2 PCR (1) and VNTR typing (37) have revealed a remarkable lack of genetic diversity of *M. ulcerans* and a clonal population structure within given geographical regions. The discriminatory power of all these methods is

particularly insufficient to differentiate between African isolates. Innovative molecular genetic fingerprinting methods are therefore required for local epidemiological studies aiming to reveal transmission pathways and environmental reservoirs of *M. ulcerans*.

1.9. Rationale and research frame work

While a number of molecular epidemiological studies of *M. tuberculosis* are performed in industrialized countries, data from similar works from the African continent, where incidence rates are high, are rare. However, this data is needed as it could prove useful in the tuberculosis control strategy of the different countries. In Chad, the results of molecular epidemiological studies could help in proposing new and innovative control strategies, showing for example risk factors for recent transmission of drug sensitive and resistance strains and researching the degree of mixed infections. Furthermore, it could help in evaluating the percentage of human tuberculosis infections due to *M. bovis* and in finding the sources of infections.

Appropriate genotyping tools are a prerequisite for performing molecular epidemiological studies of *Mycobacterium tuberculosis* complex and *M. ulcerans* strains. For *M. tuberculosis*, these tools are well established and their degrees of appropriateness may only vary slightly depending on geographical area. *M. bovis*, despite also being a member of the *M. tuberculosis* complex, is generally much more homogenetic and the discriminatory power of the different tools has to be evaluated with great care. Even less genomic diversity seems to be attributed to *M. ulcerans* for which no typing tool was able to discriminate strains within the African continent.

In an attempt to develop and evaluate innovative genotyping tools for the *M. tuberculosis* complex in Chad and *M. ulcerans* strains in Ghana, a scientific partnership was established between the Noguchi Memorial Institute for Medical Research of Legon and the Tema Municipal Health Directorate of Tema in Ghana and the Laboratoire de recherches vétérinaires et zootechniques de Farcha (LRVZ) and Centre de Support en Santé International (CSSI) in Chad. An evaluation of VNTR for genotyping the *M. tuberculosis* complex and *M. ulcerans* and its potential to enable micro epidemiological studies in the near future is presented.

This thesis is co-funded by the NCCR North-South

1.10 References of Introduction

1. **Ablordey, A., R. Kotlowski, J. Swings, and F. Portaels**. 2005. PCR amplification with primers based on IS2404 and GC-rich repeated sequence reveals polymorphism in Mycobacterium ulcerans. J.Clin.Microbiol. **43**:448-451.

2. **Asiedu, K., R. Scherpbier, and M. Raviglione**. 2000. Buruli ulcer - *Mycobacterium ulcerans* infection. WHO document WHO/CDS/CPE/GBUI/2000.1.

3. **Ayele, W. Y., S. D. Neill, J. Zinsstag, M. G. Weiss, and I. Pavlik**. 2004. Bovine tuberculosis: an old disease but a new threat to Africa. Int.J.Tuberc.Lung Dis. **8**:924-937.

4. **Bauer, J., Z. Yang, S. Poulsen, and A. B. Andersen**. 1998. Results from 5 years of nationwide DNA fingerprinting of Mycobacterium tuberculosis complex isolates in a country with a low incidence of M. tuberculosis infection. J.Clin.Microbiol. **36**:305-308.

5. **Bloom, B. R. and C. J. L. Murray**. 1992. Tuberculosis - Commentary on A Reemergent Killer. Science **257**:1055-1064.

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

7. **Clifton-Hadley, R. S., J. Inwald, S. Hughes, N. Palmer, A. R. Sayers, K. Sweeney, J. D. A. van Embden, and R. G. Hewinson**. 1998. Recent advances in DNA fingerprinting using spoligotyping - Epidemiological applications in bovine TB. Journal of the British Cattle Veterinary Association **6**:79-82.

8. **Corner, L. A.** 2005. The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: How to assess the risk. Vet.Microbiol.

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

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

11. **Dye, C., S. Scheele, P. Dolin, V. Pathania, and R. C. Raviglione**. 1999. Global burden of tuberculosis - Estimated incidence, prevalence, and mortality by country. Jama-Journal of the American Medical Association **282**:677-686.

12. **Ferdinand, S., G. Valetudie, C. Sola, and N. Rastogi**. 2004. Data mining of Mycobacterium tuberculosis complex genotyping results using mycobacterial interspersed repetitive units validates the clonal structure of spoligotyping-defined families. Res.Microbiol. **155**:647-654.

13. **Floyd, K., L. Blanc, M. Raviglione, and J. W. Lee**. 2002. Resources required for global tuberculosis control. Science **295**:2040-2041.

14. **Frothingham, R. and W. A. Meeker-O'Connell**. 1998. Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats. Microbiology-Uk **144**:1189-1196.

15. **Gleissberg, V.** 1999. The threat of multidrug resistance: is tuberculosis ever untreatable or uncontrollable? Lancet **353**:998-999.

16. **Glynn, J. R., J. Bauer, A. S. de Boer, M. W. Borgdorff, P. E. Fine, P. Godfrey-Faussett, and E. Vynnycky**. 1999. Interpreting DNA fingerprint clusters of Mycobacterium tuberculosis. European Concerted Action on Molecular Epidemiology and Control of Tuberculosis. Int J.Tuberc.Lung Dis. **3**:1055-1060.

17. **Guerrero, A., J. Cobo, J. Fortun, E. Navas, C. Quereda, A. Asensio, J. Canon, J. Blazquez, and E. GomezMampaso**. 1997. Nosocomial transmission of Mycobacterium bovis resistant to 11 drugs in people with advanced HIV-1 infection. Lancet **350**:1738-1742.

18. **Haddad, N., A. Ostyn, C. Karoui, M. Masselot, M. F. Thorel, S. L. Hughes, J. Inwald, R. G. Hewinson, and B. Durand**. 2001. Spoligotype diversity of Mycobacterium bovis strains isolated in France from 1979 to 2000. Journal of Clinical Microbiology **39**:3623-3632.

19. **Heldal, E., H. Docker, D. A. Caugant, and A. Tverdal**. 2000. Pulmonary tuberculosis in Norwegian patients. The role of reactivation, re-infection and primary infection assessed by previous mass screening data and restriction fragment length polymorphism analysis. Int.J.Tuberc.Lung Dis. **4**:300-307.

20. **Hermans, P. W. M., F. Messadi, H. Guebrexabher, D. Vansoolingen, P. E. W. Dehaas, H. Heersma, H. Deneeling, A. Ayoub, F. Portaels, D. Frommel, M. Zribi, and J. D. A. Vanembden**. 1995. Analysis of the Population-Structure of Mycobacterium-Tuberculosis in Ethiopia, Tunisia, and the Netherlands - Usefulness of Dna Typing for Global Tuberculosis Epidemiology. Journal of Infectious Diseases **171**:1504-1513.

21. **Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. Van Embden**. 1997. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J.Clin.Microbiol. **35**:907-914.

22. **Kazwala, K. D., Sinclaire, J. Challans, D. M. Kambarage, J. M. Sharp, J. Van Embden, C. J. Daborn, and J. Nyange**. 1997. Zoonotic importance of Mycobacterium tuberculosis complex organisms in Tanzania: a molecular biology approach. Actes Editions, Rabat, Morocco 199-204.

23. **Kazwala, R. R., L. J. Kusiluka, K. Sinclair, J. M. Sharp, and C. J. Daborn**. 2005. The molecular epidemiology of Mycobacterium bovis infections in Tanzania. Vet.Microbiol.

24. **Lockman, S., J. D. Sheppard, C. R. Braden, M. J. Mwasekaga, C. L. Woodley, T. A. Kenyon, N. J. Binkin, M. Steinman, F. Montsho, M. Kesupile-Reed, C. Hirschfeldt, M. Notha, T. Moeti, and J. W. Tappero**. 2001. Molecular and conventional epidemiology of Mycobacterium tuberculosis in Botswana: A population-based prospective study of 301 pulmonary tuberculosis patients. Journal of Clinical Microbiology **39**:1042-1047.

25. **McCarter, Y. S., I. N. Ratkiewicz, and A. Robinson**. 1998. Cord formation in BACTEC medium is a reliable, rapid method for presumptive identification of Mycobacterium tuberculosis complex. J.Clin.Microbiol. **36**:2769-2771.

26. **Njanpop-Lafourcade, B. M., J. Inwald, A. Ostyn, B. Durand, S. Hughes, M. F. Thorel, G. Hewinson, and N. Haddad**. 2001. Molecular typing of Mycobacterium bovis isolates from Cameroon. J.Clin.Microbiol. **39**:222-227.

27. **O'Reilly, L. M. and C. J. Daborn**. 1995. The epidemiology of Mycobacterium bovis infections in animals and man: a review. Tuber.Lung Dis. **76 Suppl 1**:1-46.

28. **Portaels, F., P. A. Fonteyne, H. DeBeenhouwer, P. DeRijk, A. Guedenon, J. Hayman, and W. M. Meyers**. 1996. Variability in 3' end of 16S rRNA sequence of Mycobacterium ulcerans is related to geographic origin of isolates. Journal of Clinical Microbiology **34**:962-965.

29. **Pritchard, D. G.** 1988. A century of bovine tuberculosis 1888-1988: conquest and controversy. J.Comp Pathol. **99**:357-399.

30. **Prod'hom, G., C. Guilhot, M. C. Gutierrez, A. Varnerot, B. Gicquel, and V. Vincent**. 1997. Rapid discrimination of Mycobacterium tuberculosis complex strains by ligation-mediated PCR fingerprint analysis. J.Clin.Microbiol. **35**:3331-3334.

31. **Roring, S., A. Scott, D. Brittain, I. Walker, G. Hewinson, S. Neill, and R. Skuce**. 2002. Development of variable-number tandem repeat typing of Mycobacterium bovis: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. J.Clin.Microbiol. **40**:2126-2133.

32. **Savine, E., R. M. Warren, G. D. van der Spuy, N. Beyers, P. D. van Helden, C. Locht, and P. Supply**. 2002. Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 loci in serial isolates of Mycobacterium tuberculosis. Journal of Clinical Microbiology **40**:4561-4566.

33. **Serraino, A., G. Marchetti, V. Sanguinetti, M. C. Rossi, R. G. Zanoni, L. Catozzi, A. Bandera, W. Dini, W. Mignone, F. Franzetti, and A. Gori**. 1999. Monitoring of transmission of tuberculosis between wild boars and cattle: genotypical analysis of strains by molecular epidemiology techniques. J.Clin.Microbiol. **37**:2766-2771.

34. **Skuce, R. A., T. P. McCorry, J. F. McCarroll, S. M. M. Roring, A. N. Scott, D. Brittain, S. L. Hughes, R. G. Hewinson, and S. D. Neill**. 2002. Discrimination of Mycobacterium tuberculosis complex bacteria using novel VNTR-PCR targets. Microbiology-Sgm **148**:519-528.

35. **Smith, N. H., K. Kremer, J. Inwald, J. Dale, J. R. Driscoll, S. V. Gordon, D. van Soolingen, H. R. Glyn, and S. J. Maynard**. 2005. Ecotypes of the Mycobacterium tuberculosis complex. J.Theor.Biol.

36. **Stinear, T., J. K. Davies, G. A. Jenkin, F. Portaels, B. C. Ross, F. Oppedisano, M. Purcell, J. A. Hayman, and P. D. R. Johnson**. 2000. A simple PCR method for rapid genotype analysis of Mycobacterium ulcerans. Journal of Clinical Microbiology **38**:1482-1487.

37. **Stragier, P., A. Ablordey, W. M. Meyers, and F. Portaels**. 2005. Genotyping Mycobacterium ulcerans and Mycobacterium marinum by using mycobacterial interspersed repetitive units. J.Bacteriol. **187**:1639-1647.

38. **Supply, P., S. Lesjean, E. Savine, K. Kremer, D. van Soolingen, and C. Locht**. 2001. Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. Journal of Clinical Microbiology **39**:3563-3571.

39. **Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht**. 2000. Variable human minisatellite-like regions in the Mycobacterium tuberculosis genome. Molecular Microbiology **36**:762-771.

40. **van der Werf, T. S., T. Stinear, Y. Stienstra, W. T. A. van der Graaf, and P. L. Small**. 2003. Mycolactones and Mycobacterium ulcerans disease. Lancet **362**:1062-1064.

41. **van Embden, J. D., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick, and .** 1993. Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. J.Clin.Microbiol. **31**:406-409.

42. **Van Rie, A., R. Warren, M. Richardson, T. C. Victor, R. P. Gie, D. A. Enarson, N. Beyers, and P. D. van Helden**. 1999. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. New England Journal of Medicine **341**:1174-1179.

43. **van Soolingen, D.** 2001. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. Journal of Internal Medicine **249**:1-26.

44. **van Soolingen, D., M. W. Borgdorff, P. E. de Haas, M. M. Sebek, J. Veen, M. Dessens, K. Kremer, and J. D. van Embden**. 1999. Molecular epidemiology of tuberculosis in the Netherlands: a nationwide study from 1993 through 1997. J.Infect.Dis. **180**:726-736.

45. **van Soolingen, D., P. E. W. Dehaas, J. Haagsma, T. Eger, P. W. M. Hermans, V. Ritacco, A. Alito, and J. D. A. Vanembden**. 1994. Use of Various Genetic-Markers in Differentiation of Mycobacterium-Bovis Strains from Animals and Humans and for Studying Epidemiology of Bovine Tuberculosis. Journal of Clinical Microbiology **32**:2425-2433.

46. **Warren, R., J. Hauman, N. Beyers, M. Richardson, H. S. Schaaf, P. Donald, and P. van Helden**. 1996. Unexpectedly high strain diversity of Mycobacterium tuberculosis in a high-incidence community. S.Afr.Med.J. **86**:45-49.

47. **Wilkinson, D., M. Pillay, J. Crump, C. Lombard, G. R. Davies, and A. W. Sturm**. 1997. Molecular epidemiology and transmission dynamics of Mycobacterium tuberculosis in rural Africa. Trop.Med.Int.Health **2**:747-753.

Chapter II: Goals and objectives

2.1. Goal

To contribute to the development and refinement of innovative molecular typing tools for the study of *Mycobacterium tuberculosis*, *bovis* and *ulcerans* infections.

2.2. Objectives

- Evaluation and analysis of the population structure of drug sensitive and resistant *Mycobacterium tuberculosis* isolates from Chad

- Finding of possible human to animal transmission of MTC strains in Chad

- Evaluation and analysis of the population structure of *Mycobacterium bovis* in the varyingly susceptible mbororo and arabe cattle breeds from Chad

- Evaluation of the most discriminative and appropriate typing tool to study *Mycobacterium bovis* transmission in Chad

- Development of Variable Number of Tandem Repeats typing to study *Mycobacterium ulcerans* infection

- Evaluation of the sequencing of different VNTR loci to enhance the discriminatory power within *M. ulcerans*.

Chapter III: Molecular characterization and drug resistance testing of *Mycobacterium tuberculosis* isolates from Chad

Colette Diguimbaye,[1] Markus Hilty,[2] Richard Ngandolo,[1] Hassane H. Mahamat,[1] Gaby E. Pfyffer,[3] Franca Baggi,[4] Marcel Tanner,[2] Esther Schelling,[2] and Jakob Zinsstag [2]

[1] Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha, N'Djaména, Chad

[2] Swiss Tropical Institute, Basel, Switzerland

[3] Department of Medical Microbiology, Kantonsspital Luzern, Switzerland

[4] National Centre for Mycobacteria, University of Zurich, Switzerland

Modified and published in *Journal of Clinical Microbiology* 2006 Apr;44(4):1575-7

Abstract

The establishment of a new mycobacteriology unit at the National Veterinary Laboratory of Farcha, Chad, allowed us to identify the first cultures of *Mycobacterium tuberculosis* from human patients in Chad. Of the 40 isolates obtained, thirty-three were tested for their susceptibility to five drugs: streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide. Thirteen (39%) were resistant to at least one of the drugs tested with resistance to isoniazid, a first line drug in Chad, as most frequent (27%).

The use of spoligo- and MIRU/ETR typing for the strains' molecular characterization identified 13 isolates (32.5%) that all lacked Direct Repeat spacers 23-25 and therefore were members of the "Cameroon family". Members of this family are therefore endemic in Chad as in Cameroon and Nigeria. Using microarray-based comparative genomics, two unique deletions were identified and can be used for easy diagnostic strain identification by PCR and to epidemiologically trace back this clone. Furthermore, spoligo-and MIRU/ETR typing identified members of the Haarlem family, which may be inherently isoniazid resistant. The added value and feasibility of performing modern, molecular typing techniques in resource-poor settings is discussed.

Keywords: *Mycobacterium tuberculosis,* drug resistance, Cameroon family, spoligotyping, VNTR-typing, microarray- based comparative genomics, Chad

Introduction

In Chad, the annual incidence rate of pulmonary tuberculosis was estimated at 60-120/100,000 in 1990 (24), but increased to 370/100,000 in 2000 (39) making Chad a high incidence country. Together with the HIV/AIDS epidemic, tuberculosis became a major public health problem (34). The current gold standard for diagnosis, recommended by the WHO, is culture confirmation of *Mycobacterium tuberculosis*, the causative agent. However, in Chad, the routine detection of *M. tuberculosis* by cultures has not been done due to the lack of an adequate laboratory. Direct smear microscopy of sputum was the only method used and false-positive, and false-negative classifications of tuberculosis cases must be assumed. The WHO recommended treatment strategy for patients with open and extra-pulmonary tuberculosis is directly observed chemotherapy (DOTS) and is adopted in most African countries and specifically in Chad. An increase of drug resistances is feared due to non-compliance during treatment, however, the lack of baseline data on drug resistance from these countries makes monitoring difficult.

Next to drug resistance testing, it became routine practice to characterize and fingerprint *M. tuberculosis* complex members with molecular typing tools and various reasons justify this. Molecular typing is particularly recommended in the study of chronic diseases such as tuberculosis, where patients with recurrent tuberculosis can be chronically infected with a given strain and relapse due to reactivation of that strain or, patients could be reinfected by a different strain after cure (37). A correct distinction between these two options is essential for accurate estimation of the success rates of tuberculosis treatment programs (1). Furthermore, typing data assists in identification of the source of infection and can serve as a laboratory quality control for cross-contamination. Finally, fingerprinting data provides unique insights in the national and international dissemination dynamics of *M. tuberculosis* by comparison of isolates from different geographic areas and also allows to analyze evolutionary changes of pathogen populations (32).

Recently, a variety of different molecular genetic typing tools for *M. tuberculosis* complex isolates have been developed (38) with the most widely-used, IS*6110* typing, as the gold standard. However, spoligo-(16) and MIRU/ETR-typing (33) have shown advantages as they are more cost-effective and easier to perform and to compare results between laboratories.

Most recently, microarray-based comparative genomic analysis of the *M. tuberculosis* complex has defined a set of chromosomal deletions that are unique polymorphisms marking all descendants of an ancestral strain (3,15,17,25,27,35). While these comparative studies

initially made use of genome sequence information, the microarrays allow the screening of a high number of *M. tuberculosis* strains for genomic deletions. This screening identified 'diagnostic' deletions which nowadays facilitate the unequivocal placing of an isolate in a strain family, e.g. using genome level informed PCR (GLIP) (27,31).

In 2000, a mycobacteriology unit at the National Veterinary Laboratory of Farcha (Laboratoire de Recherches Vétérinaires et Zootechniques) in Chad was setup. This unit cultures, characterizes and tests for drug resistance of mycobacteria and is at the moment the only one to do so in Chad. The outcome of the drug resistance tests of the first *M. tuberculosis* isolates and the implications for public health and treatment control are shown and discussed in this study. Furthermore, we show how fingerprinting and genome level informed PCR (GLIP) can quickly provide information about drug resistance and other epidemiologically important strains.

Materials and Methods

1. Clinical Specimens

Between March and July 2001, and February and October 2002, a total of 357 sputum and 282 urine samples were collected from tuberculosis patients at the National Reference Hospital (Hôpital Général de Référence Nationale- HGRNT) in the Chadian capital N'Djaména and at four rural health centres that were 50 to 300 kilometres away from N'Djaména (Figure 1).

In the laboratory of the Reference Hospital, patient's specimens (sputum and urine) were collected with the patient's consent and smears were processed twice per week. At the rural health centres, a questionnaire was filled in with patients that were suspected to be tuberculosis positive by the head of the health centre and specimens were collected with the patient's consent. Specimens were transported to the LRVZ on ice. The collection of specimens in Nigeria and the isolation of strains used in this study was previously described (5).

2. Specimen processing and cultivation of acid fast bacilli AFB

All specimens (sputum and urine) were decontaminated with N-acetyl-L-cysteine sodium hydroxide (0.5% NALC in 2% NaOH) (18) and inoculated onto two Löwenstein–Jensen (LJ) slants, one containing 0.75% glycerol and the other containing 0.6% sodium pyruvate. In addition, liquid Middlebrook 7H9 medium containing OADC and PANTA (polymyxin, amphotericin B, nadilixic acid, trimethoprim, azlocillin) was used in the latter parts of the

study. The inoculated media were incubated at 37°C without CO_2 for 8 weeks. Smears were made from the sediment and were stained by the Ziehl-Neelsen method (18).

3. Identification, spoligotyping, and MIRU/ETR analysis of mycobacterial isolates

Growth of mycobacteria was confirmed by smear. AFB-positive colonies were subcultured on 3 LJ slants and a Middlebrook 7H10 agar plate. Three biochemical tests (nitrate, niacin, and 68°C catalase) (18) were used to identify *M. tuberculosis* complex from non-tuberculous mycobacteria (NTM). The Lebeek test was used as an additional phenotypical test to distinguish between the complex members (14).

The standard method for molecular identification of *Mycobacterium tuberculosis* complex members was performed by real time PCR as described previously (19). Genotyping and identification of *M. tuberculosis* isolates was done by spoligotyping (16) and obtained spoligotypes were compared to the international database (SpolDB3.0) (10).

The reaction mixture for MIRU and ETR typing contained 1x *Taq* PCR buffer, deoxynucleoside triphosphates (0.2 mM each), 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), a 0.5 µM concentration of the primer pairs and mycobacterial DNA in a final volume of 20 µl. 12 MIRU and 3 ETR primer pairs were used (6,12). The reactions were carried out as previously described (14).

4. Microarray analysis and sequencing

DNA of two Cameroon family isolates (29) from Chad with spoligotypes 852 and 838 (SpolDB3.0) were applied to an *M. tuberculosis* amplicon-array and fluorescence scanned with an Affymetrix 428 scanner as described (13). Data were analysed using GeneSpring 5.0 (Silicon Genetics, Redwood City,CA) and Mathematica (Wolfram Research). A cut-off for the normalised test/control ratio of <2.0 was used and results were entered into gene deletion lists.

PCR-Products of the deletions B and E were received as described (14) with the primers:

BF 5' AACTAGTTGGGGCAGAAAGAAC / BR 5' CTGAGTGCCCTTACCTCCAAG

EF 5' AGCAAAAACATTGCTAGGTTCG / ER 5' GGGTGGTGCTCTATTTGCAC

PCR products were sequenced directly with an ABI Prism 310 Genetic Analysis System.

The flanking sequences for Deletion B and E for two *M. tuberculosis* strains has been entered in the EMBL database under accession numbers AM063041/AM063042 and AM063039/AM063040, respectively.

5. Drug susceptibility test

Drug susceptibility testing was performed in the BACTEC MGIT 960 instrument (BD Biosciences, Sparks, Md., USA): isoniazid (INH) 0.1µg/ml, rifampicin (RMP) 1µg/ml,

ethambutol (EMB) 5µg/ml and pyrazinamide (PZA) 100µg/ml. Streptomycin (SM) 2µg/ml and 10µg/ml was tested by the agar proportion method (18).

6. Data analysis

Cluster analysis was done with SAS (Statistical Analysis Systems Inc., Cary, USA, Version 8.02 Proc cluster) using the UPGMA algorithm.

Results and Discussion

Culturing of mycobacteria

In 2001 and 2002, a total of 357 sputum specimens and 282 urine samples were transported to the mycobacteriology unit of the LRVZ (Table 1). 169 cultures from 123 sputum and 46 urine samples showed growth of acid-fast bacilli on at least one medium. Additionally, one culture was obtained from an extra-pulmonary sample. Subsequent performing of real time PCR identified 34/123 (27.6 %) isolates from sputum, 5/46 (10.6 %) from urine and the extra pulmonary sample as *M. tuberculosis* complex (MTC). Few of the remaining non-tuberculosis mycobacteria (NTM) could be further characterized (8) while others were considered to be environmental contaminants. A very high contamination rate with NTM was observed for cultures obtained in 2001 when only solid media (Löwenstein) was used. Consequently, a liquid media with antibiotic supplements (PANTA) was introduced which led to higher proportions of MTC cultures. Still, of the samples collected in rural health centres only few MTC in comparison to NTM isolates were obtained (Table 1; Fig. 1). Considering the high number of clinically positive tuberculosis cases in these areas, this relative paucity of MTC positive-cultures may be explained by inadequate sample collection, long transport times and insufficient decontamination, rather than lower occurrence of the disease.

Diagnosis of *M. tuberculosis*

In this study, real time PCR and spoligotyping were used as a reference method for diagnosing MTC and *M. tuberculosis* isolates, respectively. As these methods are not yet established in the Chadian mycobacteria laboratory we compared the PCR-based results to those obtained with the biochemical tests (catalase, nitrate, niacin) and Lebeek media for agreement assessment. With real time PCR, 40 MTC-positives were identified and subsequent performing of spoligotyping resulted in 40 *M. tuberculosis* isolates. In comparison, 35/40 strains showed a biochemical pattern of *M. tuberculosis complex* (heat-resistant catalase negative), with 22/40 characterized as *M. tuberculosis* (heat-resistant catalase negative, nitrate positive, and niacin positive). All of these isolates were aerophilic

on Lebeek media (indicating *M. tuberculosis* rather than *M. bovis* or *M. africanum*). Thus 18/40 *M. tuberculosis* isolates were not detected with biochemical testing. Additionally, a number of isolates were false classified as MTC by biochemical testing compared to real time PCR, too (data not shown). Possible interference of NTM might explain the low predictive value of the biochemical tests in our study and caution in interpreting biochemical tests is suggested if comparison to other reference methods are lacking.

Considering the geographic location of Chad (Central Africa), we were surprised that spoligotyping failed to reveal any *M. africanum* type I and II in our strain collection. *M. africanum* I (lack of spoligo spacer 37-39) is very frequent in West Africa and found in neighbouring Cameroon. However, it was suggested that there has been a decreasing trend of *M. africanum* type I transmission in the last three decades (29). *M. africanum* II (lack of spacer 40) is predominantly isolated in Uganda (28) and although we obtained 6 spoligotypes lacking spacer 40 all of ours were aerophilic on Lebeek media which is not coherent with *M. africanum*. Indeed, a recent study (26) on genomic analysis has not been able to differentiate *M. africanum* sub-type II from modern *M. tuberculosis,* casting doubt on whether sub-type II should be considered as separate to *M. tuberculosis*.

Molecular characterization of *M. tuberculosis* and identification of the Cameroon family clone

In total, spoligotyping identified 26 different spoligotypes (Fig. 2). When compared with the international database (SpolDB3.0) 8/26 spoligotypes are described for the first time (T1-T8) (Figure 2). Twenty-one (52.5 %) isolates were clustered within 7 spoligotypes (DB 50, 52, 848, 244, 61, 838 and T1) while 19 spoligotypes were unique. With a total of 25 different genotypes, of which 7 and 18 were clustered and unique, respectively, MIRU/ETR-VNTR typing showed a similar degree of discrimination of *M. tuberculosis* isolates. Thirteen strains (DB 61, 838, T1 and T6) had the Cameroon family spoligotype with lacking DR 23 – 25 (29). This family was first described in Cameroon (29) to be an endemic strain family and its presence in Chad is not surprising since everyday transborder movements of people are frequent. Within this clone, clustering of the VNTR types differs the clusters received by spoligotyping (Fig. 2). Therefore combined spoligo- and MIRU/ETR typing data could provide valuable additional information for future micro-epidemiological studies of this clone. Additionally, one VNTR cluster (V3) and three spoligotypes (T1, T6, T7) have not yet been previously described in Cameroon (30) and possibly indicate that sub-clones are spreading in Chad and in Cameroon (Fig. 2).

Microarray-based comparative genomics showed 5 deletions (Table 2, A-E) for two *M. tuberculosis* isolates from the Cameroon family. While deletions A, C and D are already published in a comparable form in previous studies but with a different nomenclature (2,3,11,15,17,27,35) the Deletions B and E are described only partly (15,35) or for the first time here, respectively. Deletion B was found to be split in two sub-deletions of 1941 bp and 1381 bp with a conserved, but inversed, 240 bp region within Rv1674 (Table 2); sequence characterization of Deletion E revealed to be 1749 bp, removing Rv3486 and parts of Rv3485 and Rv3487. All 13/13 from Chad and 14/14 test strains from Nigeria were positive while 27/27 control strains from Chad were negative for the deletions. These findings facilitate a diagnostic PCR which can be used for improved back-tracing of this epidemiologically important clone in Central Africa. From a public health perspective to trace back this clone and try to interrupt its transmission pathways could prove effective in the tuberculosis control strategy if proven that the high frequency of these strains arose because of the biological differences in the organism. However, as for the Beijing strains (22), this remains to be identified but could be investigated with a population-based assessment of transmission of this clone related to the underlying levels of drug resistance, clinical and socio-economic characteristics of human tuberculosis.

Demographic and resistance data of the *M. tuberculosis* isolates

Most *M. tuberculosis* isolates (23/40; 57 %) originated from male patients of the HGRNT in N'Djaména (Figure 1 and 2). Drug susceptibility testing of 33 *M. tuberculosis* isolates showed that 20 (60.6%) were susceptible to all drugs, while 13 (39.4%) were resistant to at least one drug (Figure 2). Resistance to isoniazid was the most frequent (9 patients [27.3 %]; associated with resistance to ethambutol in 3 patients [9.1 %]). Resistance to ethambutol was observed in four isolates (12.1%) and to pyrazinamide in 3/30 isolates (10 %). We did not find any isolate that was resistant to rifampicin or streptomycin.

Looking at the resistance data, the level of resistance to INH is alarming (9/ 33; 27.3%) when one considers that INH is used as a first line drug in Chad. Primary resistance to INH in other African countries is usually lower: 8.3% in Ethiopia (4), 12.5% in Equatorial Guinea (36), 12.1% in Western province of Cameroon (20), and 6.6% in Northern Nigeria (9). However, we did not find any strains resistant to both streptomycin and rifampicin. Usually, resistance to these drugs is very common in Africa, except in Guinea Bissau (resistance to INH only; (7) and in Congo (no RMP-resistant isolates; (21). The rate of resistance of the other drugs was low compared to INH.

Interestingly only 1/9 tested Cameroon family isolates showed resistance to commonly used anti-mycobacterials. This indicates that resistance to drugs does not necessarily contribute to the high frequency of this family. In contrast, the Haarlem family members (lack of spacer 31) showed resistance to INH. This could confirm data on Haarlem strains from Tunisia which were also associated with resistance (23). However, our sample size is too small to draw any general conclusion at this point.

Concluding remarks

This study makes several recommendations on how to culture and diagnose Mycobacterium complex strains in a setting with infrastructural constraints and a high prevalence of not only *M. tuberculosis* but also non-tuberculosis mycobacteria (NTM). First drug resistance results of Chadian patients infected with *M. tuberculosis* show a high level of resistance to isoniazid, a front-line drug for treatment. Molecular characterization revealed the presence of an endemic clone (Cameroon family) which is based on the absence of spacer 23-25 and two specific large genomic deletions and it seems that this clone has high prevalences in Chad, Cameroon and Nigeria. Whether the dominance of these strains in this area is because of biological differences in the mycobacterium remains to be identified but could become important in the tuberculosis control strategy in the future. However, Microarray analysis led to the proposition of a single deletion PCR that can be used in resource-poor countries for easy detection of the Cameroon family strains

The new mycobacterial unit of the laboratory in Farcha will allow generating crucial information to improve clinical care for TB patients and a basis for planning a National Tuberculosis Program in Chad in the future.

Acknowledgements

We thank the technicians of the Swiss National Centre for Mycobacteria, the Swiss Tropical Institute, and the "Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha" who have contributed to the project. We thank Stephen Gordon for advice and discussion and Javier Nunez-Garcia (VLA Weybridge) for help with the microarray work. The NCCR North-South (IP4 Health and Well-being) of the Swiss National Science Foundation and the Swiss Agency for Development and Cooperation is acknowledged for financial support.

Table 1: Specimens collected in 2001 and 2002, proportion of AFB positive smears and positive cultures at least one medium

Site and year of collection	Nature of clinical specimen	Number of specimens	N° of AFB-positive smear (%)	N° of AFB-positive culture (%)
2001				
N'Djaména	Sputum	87	17 (19.5)	26 (29.8)
	Urine	51	3 (5.8)	5 (09.8)
Am Doback	Sputum	73	7 (9.5)	26 (35.6)
	Urine	78	0	22 (28.2)
Dourbali	Sputum	23	2 (8.7)	9 (39.1)
	Urine	23	0	1 (4.3)
2002				
N'Djaména	Sputum	91	20 (21.9)	49 (53.8)
	Urine	45	4 (08.8)	9 (20.0)
Massaguet	Sputum	44	0	4 (9.1)
	Urine	47	0	5 (10.6)
Mandalia	Sputum	39	2 (5.1)	9 (23.1)
	Urine	38	2 (5.2)	4 (10.5)
Total	Sputum	357	48 (13.4)	123 (34.4)
	Urine	282	9 (3.2)	46 (16.3)

Table 2: Genomic divergence of *M. tuberculosis* Cameroon family strains relative to tuberculosis H37Rv. Nomenclature of deletion are described in +(3), ++(17), *(35), and **(2). + and – indicate presence and absence of regions.

Deletion number	Region	ORF/ Gene name		H37Rv / Cameroon family	Sequence confirmed
A	RD3[+]	Rv-1573	Probable phiRV1 phage protein	+ / –	No
		Rv-1574	Probable phiRV1 phage related protein	+ / –	
		Rv-1575	Probable phiRV1 phage protein	+ / –	
		Rv-1576c	Probable phiRV1 phage protein	+ / –	
		Rv-1577c	Probable phiRv1 phage protein	+ / –	
		Rv-1578c	Probable phiRv1 phage protein	+ / –	
		Rv-1579c	Probable phiRv1 phage protein	+ / –	
		Rv-1580c	Probable phiRv1 phage protein	+ / –	
		Rv-1581c	Probable phiRv1 phage protein	+ / –	
		Rv-1582c	Probable phiRv1 phage protein	+ / –	
		Rv-1584c	Possible phiRv1 phage protein	+ / –	
		Rv-1585c	Possible phage phiRv1 protein	+ / –	
		Rv-1586c	Probable phiRv1 integrase	+ / –	
B1	150*	Rv-1672c	Probable conserved integral membrane transport protein	+ / –	1897543-1899483 (deletion) 1899484- 1899723 (inversion) 1899724-1901104 (deletion)
		Rv-1673c	Conserved hypothetical protein	+ / –	
		Rv-1674c	Probable transcriptional regulatory protein	+ / –	
B2	New (CamFa1)	CDC-1713	hypothetical protein	+ / –	
		Rv-1675c	Probable transcriptional regulatory protein	+ / –	
C	152* DS6 (12) DS6L (12)[++] RD14 (17)[+]	Rv-1758	Probable cutinase cut1	+ / –	No
		Rv-1759c	PE-PGRS family protein	+ / –	
		Rv-1760	conserved hypothetical protein	+ / –	
		Rv-1761c	hypothetical exported protein	+ / –	
		Rv-1762c	hypothetical protein	+ / –	
D	MTCDC1551 MiD3**	Rv-3343c	PPE family protein (PPE 54)	+ / –	No
		Rv-3345c	PE-PGRS family protein (PE-PGRS 50)	+ / –	
		Rv-3347c	PPE family protein (PPE 55)	+ / –	
		Rv-3348	Probable transposase	+ / –	
		Rv-3349c	Probable transposase	+ / –	
E	New (CamFa2)	Rv-3485c	Probable short-chain type dehydrogenase/reductase	+ / –	3904958-3906706 (deletion)
		Rv-3486	conserved hypothetical protein	+ / –	
		Rv-3487c	Probable esterase/lipase lipf	+ / –	
6	RvD1[+]	AF-1785c		– / +	No
		AF-1786		– / +	
		AF-1787		– / +	
7	RvD2[+]	AF-2048c		– / +	No
		AF-2049c		– / +	

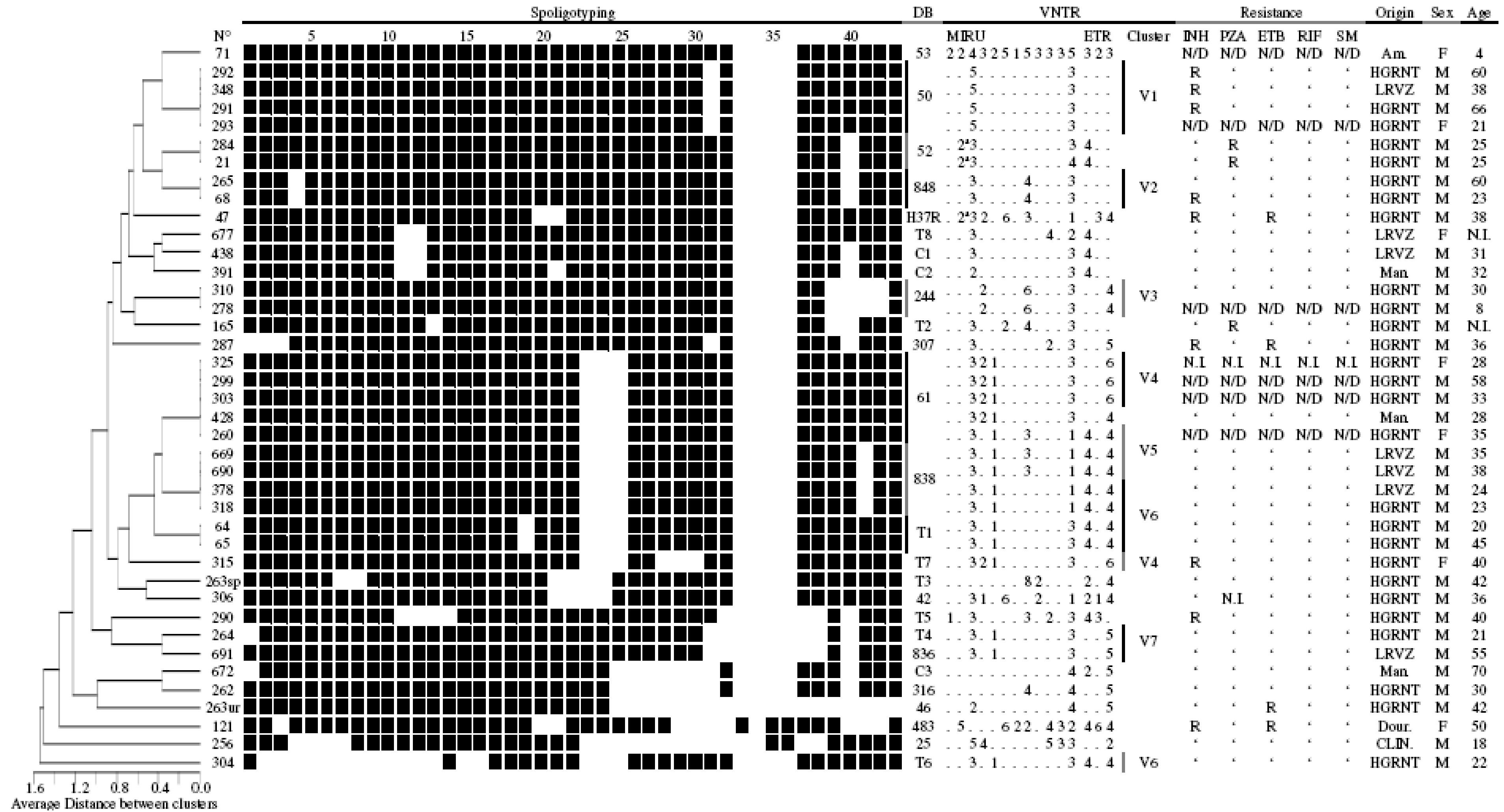

Figure 2: Molecular characteristics and results of drug resistance testing of 40 isolates

SP: sputum; UR: urine; T_{1-8}: novel spoligotype described in this study; C1-3: unique spoligotype first described in Cameroon; [a]: lacking 53 bp repeat at MIRU 4 (2nd column); MIRU 4 and MIRU 31 (10th column) correspond to ETR D and ETR E (12); INH, PZA, ETB, RIF and SM: Isoniazid, Pyrazinamide, Ethambutol, Rifampicin and Streptomycin respectively; ‘: susceptible; R: resistant. N.I.: not identified; N/D: not done; HGRNT, LRVZ and Clin: Hospital, laboratory, and Clinique of N’Djaména; respectively. Man.: Mandelia; Dour.: Dourbali; Am: Am Dobak. M: male; F: female

Reference List

1. **Bloom, B. R. and C. J. L. Murray**. 1992. Tuberculosis - Commentary on A Reemergent Killer. Science **257**:1055-1064.

2. **Brodin, P., K. Eiglmeier, M. Marmiesse, A. Billault, T. Garnier, S. Niemann, S. T. Cole, and R. Brosch**. 2002. Bacterial artificial chromosome-based comparative genomic analysis identifies Mycobacterium microti as a natural ESAT-6 deletion mutant. Infect.Immun. **70**:5568-5578.

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

4. **Bruchfeld, J., G. Aderaye, I. B. Palme, B. Bjorvatn, S. Ghebremichael, S. Hoffner, and L. Lindquist**. 2002. Molecular epidemiology and drug resistance of Mycobacterium tuberculosis isolates from Ethiopian pulmonary tuberculosis patients with and without human immunodeficiency virus infection. J.Clin.Microbiol. **40**:1636-1643.

5. **Cadmus, S., S. Palmer, M. Okker, J. Dale, K. Gover, N. Smith, K. Jahans, R. G. Hewinson, and S. V. Gordon**. 2006. Molecular Analysis of Human and Bovine Tubercle Bacilli from a Local Setting in Nigeria. Journal of Clinical Microbiology **44**:29-34.

6. **Cowan, L. S., L. Mosher, L. Diem, J. P. Massey, and J. T. Crawford**. 2002. Variable-number-tandem repeat typing of mycobacterium tuberculosis isolates with low copy numbers of IS6110 by using mycobacterial interspersed repetitive units. Journal of Clinical Microbiology **40**:1592-1602.

7. **Dias, F., S. G. Michael, S. E. Hoffner, L. Martins, R. Norberg, and G. Kallenius**. 1993. Drug susceptibility in Mycobacterium tuberculosis of a sample of patients in Guinea Bissau. Tuber.Lung Dis. **74**:129-130.

8. **Diguimbaye, C., V. Vincent, E. Schelling, M. Hilty, R. Ngandolo, HH. Mahamat, G. Pfyffer, F. Baggi, M. Tanner, and J. Zinsstag**. 2006. Species identification of non-tuberculous mycobacteria from humans and cattle of Chad. Schw.Arch.Tierheilkunde **148**(5):251-6.

9. **Fawcett, I. W. and B. J. Watkins**. 1976. Initial resistance of Mycobacterium tuberculosis in Northern Nigeria. Tubercle. **57**:71-73.

10. **Filliol, I., J. R. Driscoll, D. van Soolingen, B. N. Kreiswirth, K. Kremer, G. Valetudie, D. A. Dang, R. Barlow, D. Banerjee, P. J. Bifani, K. Brudey, A. Cataldi, R. C. Cooksey, D. V. Cousins, J. W. Dale, O. A. Dellagostin, F. Drobniewski, G. Engelmann, S. Ferdinand, D. Gascoyne-Binzi, M. Gordon, M. C. Gutierrez, W. H. Haas, H. Heersma, E. Kassa-Kelembho, M. L. Ho, A. Makristathis, C. Mammina, G. Martin, P. Mostrom, I. Mokrousov, V. Narbonne, O. Narvskaya, A. Nastasi, S. N. Niobe-Eyangoh, J. W. Pape, V. Rasolofo-Razanamparany, M. Ridell, M. L. Rossetti, F. Stauffer, P. N. Suffys, H. Takiff, J. Texier-Maugein, V. Vincent, J. H. de Waard, C. Sola, and N. Rastogi**. 2003. Snapshot of moving and expanding clones of Mycobacterium tuberculosis and their global distribution assessed by spoligotyping in an international study. J.Clin.Microbiol. **41**:1963-1970.

11. **Fleischmann, R. D., D. Alland, J. A. Eisen, L. Carpenter, O. White, J. Peterson, R. DeBoy, R. Dodson, M. Gwinn, D. Haft, E. Hickey, J. F. Kolonay, W. C. Nelson, L. A. Umayam, M. Ermolaeva, S. L. Salzberg, A. Delcher, T. Utterback, J. Weidman, H. Khouri, J. Gill, A. Mikula, W. Bishai, J. W. Jacobs, Jr., J. C. Venter, and C. M. Fraser**. 2002. Whole-genome comparison of Mycobacterium tuberculosis clinical and laboratory strains. J.Bacteriol. **184**:5479-5490.

12. **Frothingham, R. and W. A. Meeker-O'Connell**. 1998. Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats. Microbiology-Uk **144**:1189-1196.

13. **Garcia-Pelayo, M. C., K. C. Caimi, J. K. Inwald, J. Hinds, F. Bigi, M. I. Romano, D. van Soolingen, R. G. Hewinson, A. Cataldi, and S. V. Gordon**. 2004. Microarray analysis of Mycobacterium microti reveals deletion of genes encoding PE-PPE proteins and ESAT-6 family antigens. Tuberculosis.(Edinb.) **84**:159-166.

14. **Hilty, M., C. Diguimbaye, E. Schelling, F. Baggi, M. Tanner, and J. Zinsstag**. 2005. Evaluation of the discriminatory power of variable number tandem repeat (VNTR) typing of Mycobacterium bovis strains. Vet.Microbiol. **109**:217-222.

15. **Hirsh, A. E., A. G. Tsolaki, K. DeRiemer, M. W. Feldman, and P. M. Small**. 2004. Stable association between strains of Mycobacterium tuberculosis and their human host populations. Proc.Natl.Acad.Sci.U.S.A **101**:4871-4876.

16. **Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. Van Embden**. 1997. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J.Clin.Microbiol. **35**:907-914.

17. **Kato-Maeda, M., J. T. Rhee, T. R. Gingeras, H. Salamon, J. Drenkow, N. Smittipat, and P. M. Small**. 2001. Comparing genomes within the species Mycobacterium tuberculosis. Genome Res. **11**:547-554.

18. **Kent, P. T. and G. P. Kubica**. 1985. Public health mycobacteriology- a guide for the level III laboratory. U.S.Departement of health and human Services publication, Atlanta, Ga.

19. **Kraus, G., T. Cleary, N. Miller, R. Seivright, A. K. Young, G. Spruill, and H. J. Hnatyszyn**. 2001. Rapid and specific detection of the Mycobacterium tuberculosis complex using fluorogenic probes andreal-time PCR. Mol.Cell Probes **15**:375-383.

20. **Kuaban, C., R. Bercion, J. Noeske, P. Cunin, P. Nkamsse, and N. S. Ngo**. 2000. Anti-tuberculosis drug resistance in the West Province of Cameroon. Int.J.Tuberc.Lung Dis. **4**:356-360.

21. **M'Boussa, J., M. Bendo, F. Yala, R. Kounkou, E. Kaoudi, and F. Portaels**. 1998. Etude préliminaire sur la sensibilité des mycobactéries au Centre Hospitalier Universitaire de Brazzaville. Bulletin OCEAC **31**.

22. **Malik, A. N. and P. Godfrey-Faussett**. 2005. Effects of genetic variability of Mycobacterium tuberculosis strains on the presentation of disease. Lancet Infect.Dis. **5**:174-183.

23. **Mardassi, H., A. Namouchi, R. Haltiti, M. Zarrouk, B. Mhenni, A. Karboul, N. Khabouchi, N. C. Gey van Pittious, E. M. Streicher, J. Rauzier, B. Gicquel, and K. Dellagi**. 2005. Tuberculosis due to resistant Haarlem strain, Tunisia. Emerg.Infect.Dis. **11**:957-961.

24. **Massenet, D. and O. N. Djemadji**. 1994. [Chad: bibliographic review of reported cases]. Med.Trop.(Mars.) **54**:179-188.

25. **Mostowy, S., D. Cousins, J. Brinkman, A. Aranaz, and M. A. Behr**. 2002. Genomic deletions suggest a phylogeny for the Mycobacterium tuberculosis complex. J.Infect.Dis. **186**:74-80.

26. **Mostowy, S., A. Onipede, S. Gagneux, S. Niemann, K. Kremer, E. P. Desmond, M. Kato-Maeda, and M. Behr**. 2004. Genomic analysis distinguishes Mycobacterium africanum. J.Clin.Microbiol. **42**:3594-3599.

27. **Nguyen, D., P. Brassard, D. Menzies, L. Thibert, R. Warren, S. Mostowy, and M. Behr**. 2004. Genomic characterization of an endemic Mycobacterium tuberculosis strain: evolutionary and epidemiologic implications. J.Clin.Microbiol. **42**:2573-2580.

28. **Niemann, S., S. Rusch-Gerdes, M. L. Joloba, C. C. Whalen, D. Guwatudde, J. J. Ellner, K. Eisenach, N. Fumokong, J. L. Johnson, T. Aisu, R. D. Mugerwa, A. Okwera, and S. K.**

Schwander. 2002. Mycobacterium africanum subtype II is associated with two distinct genotypes and is a major cause of human tuberculosis in Kampala, Uganda. J.Clin.Microbiol. **40**:3398-3405.

29. **Niobe-Eyangoh, S. N., C. Kuaban, P. Sorlin, P. Cunin, J. Thonnon, C. Sola, N. Rastogi, V. Vincent, and M. C. Gutierrez**. 2003. Genetic biodiversity of Mycobacterium tuberculosis complex strains from patients with pulmonary tuberculosis in Cameroon. J.Clin.Microbiol. **41**:2547-2553.

30. **Niobe-Eyangoh, S. N., C. Kuaban, P. Sorlin, J. Thonnon, V. Vincent, and M. C. Gutierrez**. 2004. Molecular characteristics of strains of the cameroon family, the major group of Mycobacterium tuberculosis in a country with a high prevalence of tuberculosis. J.Clin.Microbiol. **42**:5029-5035.

31. **Rajakumar, K., J. Shafi, R. J. Smith, R. A. Stabler, P. W. Andrew, D. Modha, G. Bryant, P. Monk, J. Hinds, P. D. Butcher, and M. R. Barer**. 2004. Use of genome level-informed PCR as a new investigational approach for analysis of outbreak-associated Mycobacterium tuberculosis isolates. J.Clin.Microbiol. **42**:1890-1896.

32. **Supply, P., S. Lesjean, E. Savine, K. Kremer, D. van Soolingen, and C. Locht**. 2001. Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. Journal of Clinical Microbiology **39**:3563-3571.

33. **Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht**. 2000. Variable human minisatellite-like regions in the Mycobacterium tuberculosis genome. Molecular Microbiology **36**:762-771.

34. **Tosi, C. H., M. N. Ngangro, N. Djimadoum, and V. Richard**. 2002. [Study of HIV seroprevalence in patients with pulmonary tuberculosis in 1999 in Chad]. Med.Trop.(Mars.) **62**:627-633.

35. **Tsolaki, A. G., A. E. Hirsh, K. DeRiemer, J. A. Enciso, M. Z. Wong, M. Hannan, Goguet de la Salmoniere YO, K. Aman, M. Kato-Maeda, and P. M. Small**. 2004. Functional and evolutionary genomics of Mycobacterium tuberculosis: insights from genomic deletions in 100 strains. Proc.Natl.Acad.Sci.U.S.A **101**:4865-4870.

36. **Tudo, G., J. Gonzalez, R. Obama, J. M. Rodriguez, J. R. Franco, M. Espasa, P. R. Simarro, G. Escaramis, C. Ascaso, A. Garcia, and M. T. Jimenez De Anta**. 2004. Study of resistance to anti-tuberculosis drugs in five districts of Equatorial Guinea: rates, risk factors, genotyping of gene mutations and molecular epidemiology. Int.J.Tuberc.Lung Dis. **8**:15-22.

37. **Van Rie, A., R. Warren, M. Richardson, T. C. Victor, R. P. Gie, D. A. Enarson, N. Beyers, and P. D. van Helden**. 1999. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. New England Journal of Medicine **341**:1174-1179.

38. **van Soolingen, D.** 2001. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. Journal of Internal Medicine **249**:1-26.

39. **World Health Organisation (WHO)**. 2004. Tuberculosis.Fact Sheet N° 104.

Chapter IV: *Mycobacterium bovis* Isolates from Tuberculous Lesions in Chadian Zebu Carcasses

Diguimbaye-Djaibé C.[1*], Hilty M.[2*], Ngandolo R.[1], Mahamat HH.[1], Pfyffer G. E.[3] , Baggi F.[4], Hewinson G.[5], Tanner M.[2], Zinsstag J.[2] and Schelling E.[2]

[1] Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha, N'Djaména, Chad

[2] Swiss Tropical Institute, Basel, Switzerland

[3] Department of Medical Microbiology, Kantonsspital Luzern, Luzern, Switzerland

[4] National Centre for Mycobacteria, University of Zurich, Switzerland

[5] Veterinary Laboratories Agency, Weybridge, United Kingdom

Modified and published in *Emerging Infectious Diseases* 2006;12(5):769-771

*Contributed equally

Abstract

During a prospective study (July to August 2002) at the slaughterhouse in N'Djaména, Chad, meat inspectors have condemned 727/10'000 cattle carcasses due to tuberculosis-like lesions. A significantly higher proportion of Mbororo than Arab cattle carcasses were entirely declared unfit in comparison to partial condemnation of carcasses (33% versus 9%, p = 0.002). Microbiological examination of 201 lesions from 75 Mbororo zebu and 124 Arab zebu carcasses confirmed bovine tuberculosis for 55 animals by isolation of *Mycobacterium bovis*. *M. bovis* was more often cultured from specimens of Mbororo than of Arab cattle (p = 0.004). Spoligotypes of 53 out of 55 (96.4%) isolates showed lack of the spacer 30 as has been described for isolates from Cameroon. Our strains were isolated from a slaughterhouse with a bovine tuberculosis prevalence of 7% and 92.7% of strains were clustered. This indicates a high recent transmission rate.

Keywords: *Mycobacterium bovis*, zebu, Arab breed, Mbororo breed, slaughterhouse, spoligotyping, Chad

Introduction

M. bovis is the causative agent of bovine tuberculosis in livestock such as cattle and farmed deer. The disease is among these, which may affect trade between countries and is under OIE (Office International des Epizooties) List B diseases i.e. transmissible diseases that are considered to be of socio-economic and/or public health importance within countries and that are significant in the international trade of animals and animal products. According to OIE records for bovine tuberculosis of the past five years (1998-2003), 31 out of 50 African countries have reported the occurrence of the disease in their respective countries (1). Developing countries do not have the same means available for control and elimination as industrialized countries had few decades ago. However, control measures have been put in place in 35 of the 50 African countries reporting to OIE.

Furthermore, *M. bovis* is also recognized as a zoonotic pathogen that infects many people, particularly in the developing world with highest prevalences of bovine tuberculosis and HIV/AIDS co-infection (2).

In order to improve the traceability of the *M. bovis* infections and identify the origin of the outbreak, different genotyping studies were made. An apparently high level of heterogeneity of individual strains by the use of spoligotyping was observed in France (3). In contrast, similar molecular studies in island countries as Great Britain (4) and Australia (5) showed a low level of heterogeneity suggesting a high recent transmission rate between livestock. For developing countries, particularly for Africa, few molecular genotyping studies - as prevalence and incidence surveys in general - have been undertaken. Studies in Cameroon (6) and Tanzania (7) show low heterogeneity of isolated strains as in Great Britain and Australia.

The slaughterhouse of Farcha (Société Moderne des Abattoirs) in N'Djaména is the largest one in Chad. Among the 50'000 animals that are slaughtered annually, the main species are cattle followed by small ruminants (8). The cattle population of Chad was estimated to be 5'595'000 in 2000 and is mainly composed of the zebu breeds (*Bos indicus*) Arab, Peul, Mbororo, with the Toupouri and Kouri breeds (*Bos taurus*) less common. It is estimated that 90% of all slaughtered cattle are of the Arab breed, with 7% Mbororo and 3% Kouri (9). Several studies in slaughterhouses have demonstrated that tuberculosis is an important cause of condemnation, causing approximately 9% of all inspected carcasses to be condemned (10). A retrospective study on causes of condemnation after meat inspection at the slaughterhouse of Farcha showed that (i) most carcasses with tuberculous lesions were detected between the

months of July and November, and (ii) more Mbororo cattle than animals of other breeds had tuberculosis-like lesions (42/60 versus 132/1539) (11).

Tuberculin-positive cattle have been detected in Chadian cattle herds (12;13). At the slaughterhouse, the diagnosis of tuberculosis is mainly based on the typical macroscopic lesions of the organs rather than on Ziehl-Neelsen stained smears. Confirmation of a suspected diagnosis of bovine tuberculosis after meat inspection has so far not been confirmed by the isolation of *M. bovis* in Chad.

This study was aimed at isolating *M. bovis* from specimens of Mbororo and Arab cattle in Chad, at characterizing the strains with molecular methods, and at comparing the isolates with *M. bovis* strains from Cameroon (14).

Materials and Methods

1. Carcasses and specimens

At the Farcha slaughterhouse 727/10'000 cattle carcasses (7397 zebu Arab, 2596 zebu Mbororo and 7 Kouri cattle) were condemned due to tuberculous lesions upon meat inspection between July 1st and August 31st 2002. A sample from approx. every fourth condemned carcass was collected between July 11th and August 29th 2002. Specimens from 201 affected organs (lymph nodes, lungs, and liver) from 199 carcasses were transported on ice to the Chadian National Veterinary and Animal Husbandry Laboratory (Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha) and stored there at -20 °C prior to processing. For each specimen, the following information was collected by two trainees: breed, sex, partial or total condemnation of the carcass, date of collection, and nature of specimen (15).

2. Specimen processing and cultivation of acid-fast bacilli (AFB)

Specimens were washed three times with sterile, distilled water. Tissue samples were cut into 5 or 6 pieces and put in a sterile plastic bag containing 10 ml of sterile saline for homogenization. Samples were homogenized in a blender (type STOMACHER 80; Seward Laboratory Systems, Bristol, U.K.) for 1 min, and repeated three time. Ten millilitres of the suspension were transferred into a 50 ml conic FALCON® tube for decontamination. Homogenized suspensions were decontaminated with N-acetyl-L-cysteine sodium hydroxide (0.5% NALC- 2% NaOH) (16) and inoculated on two Löwenstein-Jensen (LJ) slants containing a) glycerol (0.75%) and b) pyruvate (0.6%), but no glycerol. In addition,

Middlebrook 7H9 medium containing OADC and PANTA (polymyxin, amphotericin B, nadilixic acid, trimethoprim, azlocillin) was prepared. Inoculated media were incubated at 37°C (without CO_2) for 8 weeks. Smears were prepared with one drop of the sediment after centrifugation of the homogenized suspensions for detection of AFB by microscopy.

3. Identification of mycobacterial isolates

Growth of mycobacteria was confirmed by smear (stained by the Ziehl-Neelsen method). AFB-positive colonies were subcultured on 3 LJ slants and a Middlebrook 7H10 agar plate.

Three biochemical tests (nitrate, niacin, and 68°C catalase) (17) were used to identify mycobacteria and to distinguish between *M. tuberculosis* complex (MTC) and non-tuberculous mycobacteria (NTM).

Species identification was performed by real time PCR (16) to confirm MTC isolates.

4. Genotyping of MTC strains

Genotyping of *M. tuberculosis* complex (MTC) strains was done at the National Centre for Mycobacteria (NCM) by spoligotyping (18) and IS*6110*-based analysis of restriction fragment length polymorphism (RFLP) (19). The latter was carried out with 50 % of spoligotypes. Spoligotyping of all strains was repeated at the Veterinary Laboratories Agency, Weybridge, to confirm the results of the first spoligotyping round.

Additionally, the presence of spacers 14 and 15 for 15 strains with weak or absent signals in spoligotyping was confirmed by a PCR reaction with a primer-pair bridging the DNA region from spacer 14 (3’ gtgtgatgcggatggtcggctc 5’) to 22 (5’ tgtctcaatcgtgccgtctgcgg 3’) (20). The PCR reaction mixture contained 1x *Taq* PCR buffer, deoxynucleoside triphosphates (0.2 mM each), 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), a 0.5 µM concentration of the primer pair and mycobacterial DNA to a final volume of 20 µl. After 10 min at 95°C, the PCR was performed for 40 cycles of 0.5 min at 94°C, 0.5 min at 65°C and 1 min at 72°C. The reactions were terminated after an incubation of 10 min at 72°C. PCR fragments were analyzed by agarose gel electrophoresis using 2 % NuSieve agarose. The size of the amplicons was compared with a positive control of spacers 14 and 15.

5. Statistical analysis

A Chi-square test was used to analyze the co-variables (condemnation, culture growth) between breeds. A multivariate regression model with *M. bovis* isolation as the outcome was adjusted for co-variables. Cluster analysis was done with SAS (Version 8.02 Proc cluster, USA Statistical Analysis Systems Inc., Cary, NC/ USA). The relationship of clusters to geographical origin of animals, breed and type of condemnation was done by the Fisher test (SAS, proc freq).

Results

The overall prevalence of suspect lesions was 7.3%. A significantly higher ($p = 0.04$) prevalence was found among Mbororo (8.2%; 212/2596) than Arab cattle (7%; 515/7397) (15). Lesions were mainly found in the lymph nodes and lungs (Table 1). At the slaughterhouse and in the sub-sample of 199 animals, entire condemnation of the carcass in comparison to partial condemnation was observed more often among Mbororo than Arab cattle ($p \leq 0.001$ and $p = 0.002$) (Table 2). This difference between the two breeds was even more accentuated in female cattle.

The proportion of positive specimens smears was relatively low (21.6%), with was no difference evident between the two breeds (Table 3). Most AFB-positive smears originated from lymph nodes (18%) and lungs (26%), while liver specimens (n=5) were always AFB-negative. Of 201 specimens which were inoculated onto three types of media, 132 (65.7%) showed growth of mycobacteria on at least one medium, whereas 55 (27.3%) remained culture negative. Fourteen (7%) cultures were contaminated (Table 4). Ninety-eight of 161 (61%) AFB smear-negative specimens became culture positive.

Culture morphology and biochemical tests identified 58 MTC and 26 Non-Tuberculosis Mycobacteria (NTM) strains. Real-time PCR confirmed that 56 strains belonged to MTC and 28 strains were NTM. 55 MTC strains were of the *M. bovis* spoligotype, while 1 failed to give a spoligotype pattern. Overall, *M. bovis* was isolated from more than a fourth of tissues in which tuberculosis had been suspected and in 42% of all positive cultures. There were significantly more *M. bovis* strains isolated from Mbororo zebu (30/75) than from Arab zebu (26/124) ($p = 0.004$) (Table 4). The difference remained statistically significant when including the type of condemnation and type of organ in a multivariate logistic regression model.

In total, twelve different spoligotypes were found among the 55 *M. bovis* isolates, with only four spoligotypes were unique. Eight clusters of spoligotypes were identified. The number of strains per cluster varied between 22 and 2 (Figure 1). We found that 5/8 clusters were composed of strains which have been isolated from Mbororo and Arab zebus. The distribution of the two breeds (Mbororo and Arab) within cluster differ significantly ($p < 0.01$).

All strains lacked spacers 3, 9, 16, 39-43 which is a characteristic of *M. bovis*. In addition, 53/55 strains did not have spacer 30. Upon RFLP analysis, the cluster 5 identified by RFLP

was distinct in its spoligotypes (SP5 & 6); however, other RFLP clusters could not be further distinguished by spoligotypes (Figure 2). For clusters RFLP1, 3b, 5 and 6a, a second band was visible, while a second band was missing for RFLP2, 3a, 4 and 6b.
All Chadian strains showed a different RFLP pattern when compared with the BCG reference strain of the NCM Zurich (clinical *M. bovis* BCG isolate after BCG vaccination at the paediatric hospital of Zurich in 1999).

Discussion

For the first time, *M. bovis* has been isolated in specimens from Chad. The prevalence of tuberculin-positive cattle was 0.8% (95% confidence interval 0.2- 1.4%) in the East (Ouaddaï region) (12) and 16.9% (95% CI 10.4 – 23.5%) in the West of Chad (Chari- Baguirmi and Kanem regions) (13). The latter study has been continued with 476 additional cattle in 34 herds and a prevalence of 11.5% (95% CI 6.9 – 18.5%) was found when considering the herds as a random effect in the model. More tuberculin reactors were found among Mbororo than Arab zebus ($p = 0.02$).
Usually, cattle breeds are not restricted to specific geographical zones in Chad; however, a high proportion of Mbororo cattle was found in the West of the country (21). At Farcha, the most frequently slaughtered breeds were Mbororo and Arab zebus. Mbororo zebu showed generalized tuberculosis more often than Arab zebus which was reflected in the data through a higher proportion of these animals having their entire carcass condemned. Similarly, a higher susceptibility of Mbororo cattle to tuberculosis infection was also observed in Cameroon (14). It would be interesting to understand the immunological basis of this susceptibility in greater depth since it may have a bearing on the development of an improved livestock vaccine.
Sixty-five percent of specimens with tuberculosis lesions were culture-positive; however, only one fifth (21%) were smear positive. In this study, the proportion of smear-positive specimens was low in comparison to Sudan (53%; (22). Spoligotyping was used as a diagnostic tool, but also yielded important insight into the epidemiology of *M. bovis.* The spectrum of spoligotype clusters differed between Mbororo and Arab zebus, but not between the type of condemnation. Spoligotype clusters could not be related to geographical origin. However, most of the cattle were bought at local markets and their geographical origin was not known. The finding that 51/55 isolates (92.7%) were in 8 clusters indicates a substantial

degree of recent transmission, an observation that is underlined when the prevalence of tuberculosis lesions at the slaughterhouse is considered (7%).

Similar to Cameroonian *M. bovis* strains, our isolates most often lacked spacer 30, the only exception being SP11 (Figure 2). A possible explanation for this observation is the cross border movement of Chadian cattle to Cameroon. As in the study conducted by Njanpop-Lafourcade et al. in Cameroon (14), the pre-dominant spoligotype in our study is SP1 (Figure 1) with a cluster of 22 strains (40%). SP1 corresponds to the pattern of cluster C1 in the Cameroonian study and two other clusters described in Cameroon were found in Chad (C1 an C5, similar respectively to SP2 and SP4 in this work). Certain Cameroonian clusters (C7, C8, C9 and C10; (14) were only detected in the Adamaoua region but not in northern Cameroon or in Chad (our study). Apparently, the established measures of the Cameroonian government to prevent movement of cattle between the Adamaoua and the two regions of the North are effective. As to the other neighbouring countries, we have not found any publications related to molecular typing of *M. bovis* strains.

The comparison of the patterns found in Chad with the *M. bovis* spoligotype database (www.mbovis.org) showed that SP1, 2, 4 and 10 have already been described. All other patterns have never been described (www.bovis.org). All strains lacking spacers 27 and 28 (Figure 1 and Table 5) were isolated from Arab cattle. No other characteristics were observed within the spoligotypes for strains isolated from Arab and Mbororo cattle. Fifteen strains (8 from Arab and 7 from Mbororo zebu) were typed with the IS*6110* method, of which 11 and 4 isolates contained 2 or 1 band, respectively. Therefore, Chadian *M. bovis* strains belong to low IS*6110* copy number strains. Strains lacking spacer 30 had a band at 1.9 kB, in accordance to the findings in Cameroon (14). Cousins *et al.* (5) found that 85 % of *M. bovis* strains showed one band which was mainly at 1.9 kB. No association was found between the number of bands and the cattle breed. IS*6110* typing revealed 6 clusters and, thus, is of lower discriminatory power than spoligotyping. However, spoligopatterns were differentiated by RFLP due to the number of visible bands. Earlier, Zumarraga *et al.* (23) concluded that spoligotyping alone is not sensitive enough for the discrimination of *M. bovis* strains for in-depth epidemiological study. It would be interesting to see whether VNTR-typing (24) can improve strain discrimination.

The recent establishment of the first mycobacterial laboratory in Chad allowed a confirmation of the presence of bovine tuberculosis in Chadian herds. Future molecular epidemiology studies are needed to shed light on the transmission of *M. bovis* between Chadian and

Cameroonian cattle. Furthermore the observed higher susceptibility of Mbororo than Arab zebus to *M. bovis* disease should be followed-up by immunological investigations.

Acknowledgements

We thank the technicians of the National Centre for Mycobacteria, the Swiss Tropical Institute, and the "Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha" who have contributed to the project. The Swiss National Science Foundation is acknowledged for financial support. This work received support from the NCCR North-South IP-4. We thank Véronique Vincent, Institute Pasteur, Paris, for complimentary analyses and Steve Gordon (VLA Weybridge) for advice and discussion

Table 1: Specimens collected at the main slaughterhouse of N'Djaména, Chad, and specifications of the condemned carcasses

Organ/ Tissue	n	Condemnation		Breed		Sex	
		Entire	Partial	Arab	Mbororo	Male	Female
Lymph nodes	116	17	99	67	49	8	108
Lungs	75	13	62	51	24	1	74
Lungs and lymph nodes	2	0	2	2	0	0	2
Liver	5	0	5	4	1	0	5
Miliary tuberculosis	1	0	1	0	1	0	1
Total	199	30	169	124	75	9	190

Table 2: Slaughterhouse data of investigated zebu carcasses at the slaughterhouse of N'Djaména, Chad

Condemnation	Breed		Total	p
	Arab	Mbororo		
Partial	113	56	169	
Entire	11	19	30	0.002
Total	124	75	199	

Table 3 Microscopy results of specimens from Chadian Mbororo and Arab cattle

AFB-smear	Breed		Total	p
	Arab	Mbororo		
Positive	26	17	43	
Negative	100	58	158	0.734
Total	126	75	201	

Table 4 *M. bovis* and NTM cultures from Chadian Mbororo and Arab cattle

Cultures	Breed		Total	p
	Arab	Mbororo		
M. bovis	26	30	56	0.004
NTM	55	21	76	0.592
Negative	37	18	55	
Contaminated	8	6	14	
Total	126	75	201	

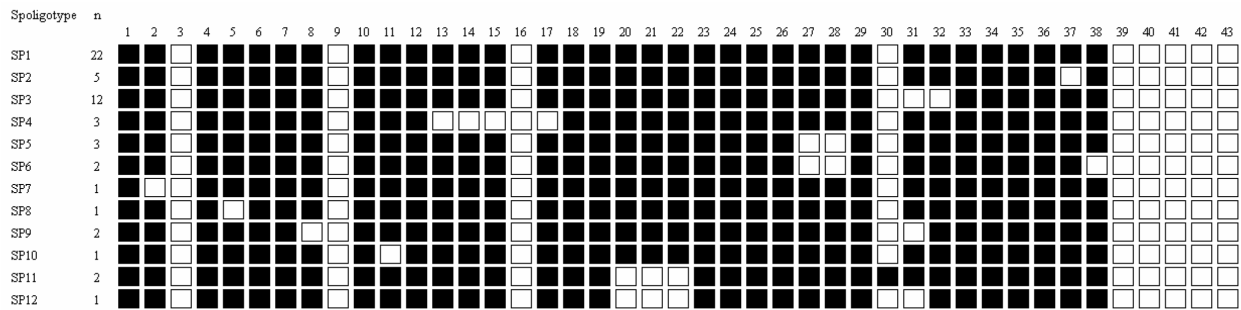

Figure 1 Spoligotypes obtained from 55 *M. bovis* isolates from Chadian zebus.

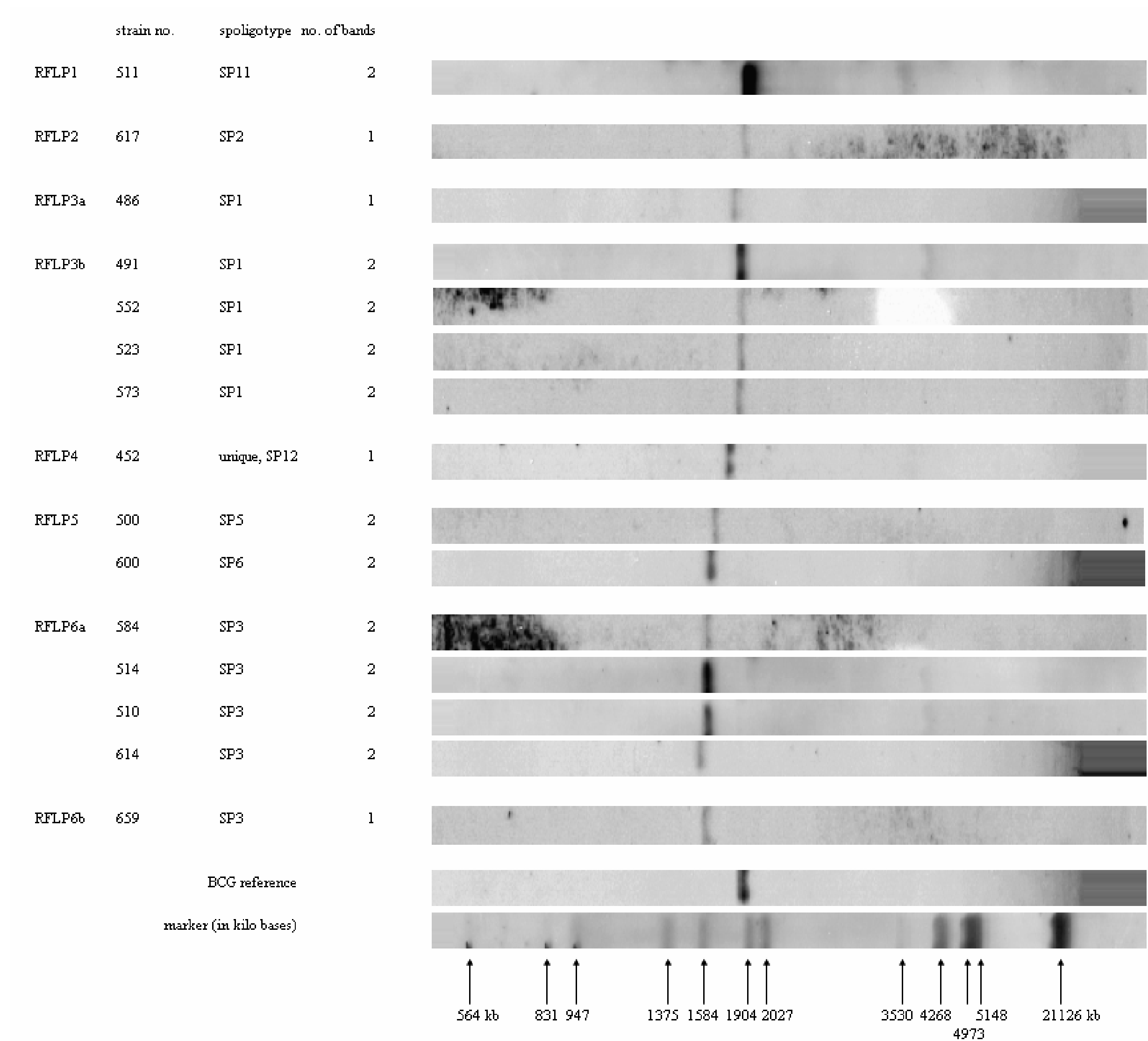

Figure 2 RFLP patterns of 15 Chadian *M. bovis* strains of which 14 were within spoligotype Clusters

Reference List

1. Office International des Epizooties. http://www oie int/hs2/report asp. 2005.

2. Cosivi O, Grange JM, Daborn CJ, Raviglione MC, Fujikura T, Cousins D et al. Zoonotic tuberculosis due to Mycobacterium bovis in developing countries. Emerg Infect Dis. 1998;4:59-70.

3. Haddad N, Ostyn A, Karoui C, Masselot M, Thorel MF, Hughes SL et al. Spoligotype diversity of Mycobacterium bovis strains isolated in France from 1979 to 2000. Journal of Clinical Microbiology. 2001;39:3623-32.

4. Clifton-Hadley RS, Inwald J, Hughes S, Palmer N, Sayers AR, Sweeney K et al. Recent advances in DNA fingerprinting using spoligotyping - Epidemiological applications in bovine TB. Journal of the British Cattle Veterinary Association. 1998;6:79-82.

5. Cousins D, Williams S, Liebana E, Aranaz A, Bunschoten A, Van Embden J et al. Evaluation of four DNA typing techniques in epidemiological investigations of bovine tuberculosis. J Clin Microbiol. 1998;36:168-78.

6. Njanpop-Lafourcade BM, Inwald J, Ostyn A, Durand B, Hughes S, Thorel MF et al. Molecular typing of Mycobacterium bovis isolates from Cameroon. J Clin Microbiol. 2001;39:222-27.

7. Kazwala KD, Sinclaire, Challans J, Kambarage DM, Sharp JM, Van Embden J et al. Zoonotic importance of Mycobacterium tuberculosis complex organisms in Tanzania: a molecular biology approach. Actes Editions, Rabat, Morocco. 1997;199-204.

8. Direction de l'Elevage et des Ressources Animales (DERA). Rapport annuel d'Activités 2000. N'Djaména. 2001;1-36.

9. Ministère de l'Elevage. Rapport national sur les ressources zoo génétiques du Tchad. N'Djaména, Tchad. 2003;1-196.

10. Maho A, Mbakasse RN, Boulbaye N. Causes de saisies aux abattoirs du Tchad oriental. LRVZ/F In: Actes des IIIèmes Journées Agro-Sylvo-Pastorales, 29/11 au 03/12/1997, N'Djaména, Tchad. 1999.

11. Maho A, Bornarel P, Hendrix P. Rapport technique: Abattage et motifs de saisie (dominantes pathologiques) aux abattoirs du Tchad: cas de N'Djaména, Ati, Bol, Mongo et Oum Hadjer. LRVZ/F, N'Djaména, Tchad. 1994;1-17.

12. Delafosse A, Goutard F, Thebaud E. Epidémiologie de la tuberculose et de la brucellose des bovins en zone péri-urbaine d'Abéché, Tchad. Rev Elev Med Vet Pays Trop. 2002;55:5-13.

13. Schelling E, Diguimbaye C, Daoud S, Daugla DM, Bidjeh K, Tanner M et al. La tuberculose causée par *Mycobacterium bovis* : résultats préliminaires obtenus chez les pasteurs nomades Foulbés et Arabes dans le Chari-Baguirmi au Tchad. Sempervira. 2000;44-55.

14. Njanpop-Lafourcade BM, Inwald J, Ostyn A, Durand B, Hughes S, Thorel MF et al. Molecular typing of Mycobacterium bovis isolates from Cameroon. J Clin Microbiol. 2001;39:222-27.

15. Doutoum AM, Toko MA. Mycobactérioses bovines et saisies à l'abattoir de Farcha. Institut Universitaire des Sciences et Techniques d'Abéché (IUSTA). 2002.

16. Kraus G, Cleary T, Miller N, Seivright R, Young AK, Spruill G et al. Rapid and specific detection of the Mycobacterium tuberculosis complex using fluorogenic probes andreal-time PCR. Mol Cell Probes. 2001;15:375-83.

17. Kent PT, Kubica GP. Public health mycobacteriology- a guide for the level III laboratory. U S Departement of health and human Services publication, Atlanta, Ga. 1985.

18. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J Clin Microbiol. 1997;35:907-14.

19. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B et al. Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993;31:406-9.

20. van Embden JD, van Gorkom T, Kremer K, Jansen R, Der Zeijst BA, Schouls LM. Genetic variation and evolutionary origin of the direct repeat locus of Mycobacterium tuberculosis complex bacteria. J Bacteriol. 2000;182:2393-401.

21. Nfi AN, Ndi C. Bovine tuberculosis at the Animal Research Antenna (ARZ) Bangangte, Western province, Cameroon. Bull Anim Hlth Prod Afri. 1997;45:1-3.

22. Sulieman MS, Hamid ME. Identification of acid fast bacteria from caseous lesions in cattle in Sudan. J Vet Med B Infect Dis Vet Public Health. 2002;49:415-18.

23. Zumarraga MJ, Martin C, Samper S, Alito A, Latini O, Bigi F et al. Usefulness of spoligotyping in molecular epidemiology of Mycobacterium bovis-related infections in South America. J Clin Microbiol. 1999;37:296-303.

24. Sola C, Filliol I, Legrand E, Lesjean S, Locht C, Supply P et al. Genotyping of the Mycobacterium tuberculosis complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infect Genet Evol. 2003;3:125-33.

Chapter V: Evaluation of the discriminatory power of Variable Number Tandem Repeats typing of *Mycobacterium bovis* strains

Markus Hilty,[1] Colette Diguimbaye, [1,2] Esther Schelling,[1] Franca Baggi,[3] Marcel Tanner[1], and Jakob Zinsstag[1]

Swiss Tropical Institute[1], 4002 Basel, Switzerland,
Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha[2], N'Djaména, Chad and
Swiss National Centre for mycobacteria[3], Zürich, Switzerland

Published in *Veterinary Microbiology* 2005 Aug 30;109(3-4):217-22

Abstract

The discriminatory power of Variable Number of Tandem Repeats (VNTR) typing based on 16 known loci (12 MIRUs, 3 ETRs and VNTR 3232) was assessed for *Mycobacterium bovis* strains collected sequentially at the slaughterhouse of N'Djaména, Chad. Of 67 *M. bovis* strains analysed, 67 % were clustered. In this study, VNTR typing was highly discriminative with an overall allelic diversity ($h_{o.a}$) of 0.922. We defined five loci (ETR A, B, C and MIRU 26, 27) as highly ($h > 0.25$), two loci (MIRU 4, and VNTR 3232) as moderately ($0.11 < h < 0.25$) and three loci (MIRU 16, 20, 31) as poorly ($0.01 < h < 0.11$) discriminative. Six loci (MIRU 2, 10, 23, 24, 39, and 40) showed no polymorphism at all. VNTR typing of the five highly discriminative loci ($h = 0.917$) proved to be most appropriate for first line typing of *M. bovis* strains of Chad and superior than spoligotyping ($h_{sp.} = 0.789$). In contrast to *M. tuberculosis* strains, a consensus on VNTR loci needs to be found for *M. bovis* strains. The selection of a generally agreed set of VNTR loci for molecular discrimination of *M. bovis* in different geographical settings is discussed.

Keywords: *Mycobacterium bovis;* VNTR typing; spoligotyping

Introduction

Bovine tuberculosis is caused by a member of the *Mycobacterium tuberculosis* complex, *Mycobacterium bovis*. The disease has one of the broadest host ranges (O'Reilly and Daborn, 1995) and leads to economic losses in livestock production and agriculture in many countries. In addition, *M. bovis* is a classical zoonosis and was the main reason for the heavy promotion of pasteurization in the early 20th century (Pritchard, 1988). Today, bovine tuberculosis affects mainly people in developing countries but its role in the human TB epidemic fostered by HIV/AIDS is not known (Cosivi et al., 1998). This is mainly due to the lack or inabilities of laboratories in developing countries to isolate and diagnose *M. bovis*. In addition, only few epidemiological studies aiming at identifying the proportion of *M. bovis* infection among tuberculosis patients have been conducted in developing countries (Cosivi et al., 1995).
The promotion of prevention of transmission of *M. bovis* is often hampered by the lack of epidemiological data. With the recent description of molecular epidemiological tools, the possibilities for finding answers to key questions like the importance and risk factors of inter-bovine transmission and the role of wild animals as reservoirs are improved.

Insertion sequence *6110* restriction fragment length polymorphism (IS*6110* RFLP) analysis is the current "gold standard" and the most widely applied typing method for molecular epidemiology of the *M. tuberculosis* complex (van Soolingen, 2001). However, this typing method has important drawbacks as the method requires previous extensive strain cultivation is technically demanding and expensive and results are difficult to compare between laboratories because comparison of profiles requires sophisticated software for image analysis and well trained users. Furthermore, the discrimination of strains with a low number of IS*6110* copies is insufficient, and this is especially true for *M. bovis* strains (Serraino et al., 1999 and Kremer et al., 1999).

The spoligotyping ("spacer oligonucleotide typing") method describes the presence or absence of 43 spacer DNA sequences between direct repeats (DR) in the DR region of tuberculosis complex (TBC) strains. This well established PCR-based method does not require extensive culturing is more discriminative than RFLP for strains with no or few copies of IS*6110* and classifies the members of *M. tuberculosis* complex with a high level of confidence (Kamerbeek et al., 1997).

The variable number tandem repeat (VNTR) typing is a recently developed PCR-based method without requiring large quantities of DNA. Sequences including exact tandem repeats (ETR) (Frothingham and Meeker-O'Connell, 1998), mycobacterial interspersed repetitive units (MIRUs) (Supply et al., 2000 and Mazars et al., 2001) and two sets of Queen's University Belfast (QUB) VNTRs (Roring et al., 2002 and Skuce et al., 2002) were presented. The two main advantages of VNTR typing are: (i) the good stability of markers, and (ii) results are easily comparable between laboratories (digital expression of results). VNTR typing is highly discriminative for *M. tuberculosis* and has also the potential to be the method of choice for *M. bovis* typing. However, it is not yet standardized and allelic diversity of loci can vary from country to country and between *M. tuberculosis* complex species.

In this study, different VNTRs were used on a panel of 67 *M. bovis* strains isolated from 2000 to 2002 in Chad (Diguimbaye, unpublished data). The objective was to asses the discriminatory power of each polymorphic locus and in combination with others to evaluate the most appropriate combination of VNTRs for molecular-epidemiological studies of *M. bovis* in Chad.

Materials and methods

Bacterial strains and spoligotyping

Sixty-seven *M. bovis* strains were isolated from the continuously collected samples from the slaughterhouse in N'Djaména, Chad. Tuberculous lesions of carcasses and organs were from two different zebu (*Bos indicus*) cattle breeds: Arab and Mbororo. Strains (8, 6 and 53) were isolated in 2000, 2001 and 2002, respectively. All 67 strains were characterized by spoligotyping (Kamerbeek et al., 1997).

VNTR–PCR analysis

The reaction mixture for all loci contained 1× *Taq* PCR buffer, deoxynucleoside triphosphates (0.2 mM each), 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), a 0.5 µM concentration of the primer pairs and mycobacterial DNA in a final volume of 20 µl. 12 MIRU, 3 ETR and VNTR 3232 primer pairs were used (Cowan et al., 2002 and FMO'Connell, 1998'Connell, 1998; Roring et al., 2002).

The reaction was carried out with a Perkin-Elmer 9600 cycler starting at a denaturing step of 10 min at 95 °C. After denaturation, the PCR was performed for 40 cycles of 0.5 min at 94 °C, 0.5 min at 65 °C and 1 min at 72 °C. The reactions were terminated by an incubation of 10 min at 72 °C. PCR fragments were analyzed by agarose (Sigma) gel electrophoresis

using 2% NuSieve agarose. The size of the amplicons was estimated by comparison with Size Marker VIII (Roche Diagnostics).

Allelic diversity

The allelic diversities (h) of VNTRs, individually and in combination were calculated using the following equation: $h = 1 - \sum x_i^2 [n/(n-1)]$, where n is the number of isolates and x_i the frequency of the ith allele at the locus (Selander et al., 1986). We considered $h > 0.25$ as highly, $0.11 < h < 0.25$ as moderately and $0.01 < h < 0.11$ as poorly discriminative.

Results

PCR products of 16 published loci (12 MIRUs, 3 ETRs and VNTR 3232) of all 67 *M. bovis* strains isolated were analyzed by agarose gel electrophoresis and repeated copy numbers were determined (not shown). Allelic diversities (h) differed greatly for individual loci and ranged from 0.00 (MIRU 2, 10, 23, 24, 39 and 40) to 0.74 (ETR B). Five loci (ETR A, B, C and MIRU 26, 27) were highly ($h > 0.25$), two loci (MIRU 4, and VNTR 3232) moderately ($0.11 < h < 0.25$) and three loci (MIRU 16, 20, 31) poorly ($0.01 < h < 0.11$) discriminative (Table 1). These allelic diversities were compared to a *M. bovis* study from Northern Ireland and to studies on *M. tuberculosis* complex strains from USA, France and South Africa (Table 2).

Based on all VNTRs, a total clustering rate of 67.2% and 33 different types ($h = 0.922$) were identified whereof 22 unique and 11 clustered. Clusters ranged from 2 ($n = 6$) to 12 ($n = 1$) identical strains (Table 3, set no. 4). The 12 MIRUs (Table 3, set no. 1) identified 18 different types ($h = 0.754$) and regrouped a large cluster of 30 strains.

With solely 3 ETRs (A, B, C) we received 22 types ($h = 0.906$, set no. 2) thus this set was already more distinctive than MIRUs or spoligotyping. Spoligotyping alone resulted in 16 types ($h = 0.789$, set no. 5) but increased the power of discrimination ($h = 0.944$, set no. 6) when compared to VNTRs alone ($h = 0.922$, set no 4).

By adding the highly discriminative loci MIRU 26, 27 we obtained 28 different types ($h = 0.917$, set no. 3), which we consider to be distinctive enough for initial molecular epidemiological studies.

Discussion

Despite the publication of different typing methods for *M. bovis* strains (IS*6110*, VNTR and spoligotyping) the best methods for the discrimination of *M. bovis* remains to be defined. We did not apply IS*6110* in our study because its discriminatory power for *M. bovis* strains is too low (Serraino et al., 1999 and Kremer et al., 1999; Diguimbaye, unpublished data). With the expectation that 12 MIRU loci and spoligotyping would reasonably differentiate *M. bovis* strains, we applied these two methods in a first step. However, this did not result in a satisfying result (high clustering proportion of 84%). Therefore, the usefulness of 3 ETRs (A, B and C) and the VNTR locus 3232 were further investigated. The 3 ETRs showed to be the most discriminative VNTR loci.

A study on 47 *M. bovis* strains from Northern Ireland (Roring et al., 2004) found six highly ($h > 0.25$) discriminative loci of which in our study 3 (ETR A, B and MIRU 26) and 1 (VNTR 3232) were highly and moderately discriminative, respectively. Two loci (MIRU 24, 40) did not show any polymorphism at all in our study. Aiming at standardization, ETR A, B, MIRU 26 and VNTR 3232 are appropriate to use within both settings when comparing only these two studies.

Analyses of the 12 MIRU loci on *M. tuberculosis* shows that *M. tuberculosis* is more polymorphic than *M. bovis*. Published MIRU data on *M. tuberculosis* from France (Mazars et al., 2001) South Africa (Savine et al., 2002) and USA (Cowan et al., 2002) showed higher allelic diversities in all loci except MIRU 27 and MIRU 24 than in our and the Northern Ireland study. Therefore, typing of *M. tuberculosis* is easier and evaluation of best typing method for *M. bovis* needs to be established independently.

In view of the definition of an agreed set that will be discriminatory enough for *M. bovis* in all geographical settings, we conclude that it is important to carry out more studies with all VNTRs. Ideally, such a set will necessitate few loci (i.e. <10) to ensure a cheap and non-laborious typing of *M. bovis* strains.

For the Chadian strains, we suggest to add the most discriminative MIRU loci 26, 27 to ETR A, B, C for first line typing. In contrast to IS*6110* and spoligotyping, we consider the PCR amplification of these five loci and subsequent agarose gel separation for visualization of the heterogeneity as most appropriate. This approach is also manageable, reproducible and cost-

effective for a laboratory in the South. However, for in-depth studies we recommend not only to use all polymorphic loci (ETR A, B, C; MIRU 4, 16, 20, 26, 27, 31; and VNTR 3232) but also spoligotyping because this ensures the highest power of discrimination.

The results of this study are used within a molecular epidemiology study aiming at identifying risk factors for recent transmission of *M. bovis*. Results will also clarify the trade and trans-border translocations of cattle between Cameroon and Chad. In both countries, *M. bovis* strains lacking DR spacer 30 have been found (Diguimbaye, unpublished data; Njanpop-Lafourcade et al., 2001). Improved discrimination of strains is needed for better tracing back of animals. Furthermore, VNTR typing ensures a good quality control for laboratory cross-contaminations of *M. bovis* cultures especially useful for laboratories with infrastructural restraints.

In conclusion, this work illustrates the necessity of defining the appropriate combination of heterogenic loci for each country and *M. tuberculosis* complex panel studied and therefore advises caution in comparing results of different studies. Furthermore, a first line and in-depth panel of heterogenic loci is suggested for *M. bovis* typing in Chad. Once more studies from other settings are available, a set of most discriminatory VNTRs could be agreed on for *M. bovis* strains.

Acknowledgements

We thank the technicians under the supervision of Dr. F. Baggi of the National Centre for Mycobacteria, Zurich, who have contributed greatly to the project. The NCCR North-South IP-4 is acknowledged for financial support. S.V. Gordon of the Veterinary laboratories agencies, Weybridge, is thanked for complementary analyses and critical review.

Table 1: Determination of heterogeneity at each locus.

No. of copies	Locus no. at MIRU												Locus no. at ETR			Locus no. at 3232[a]
	2[a]	4[b]	10	16	20	23	24	26	27	31[c]	39	40	A	B	C	
0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	-	4	-	-	1	-	-	2	7	-	-	-	-	-	-	-
2	66	1	67	3	66	-	67	1	11	2	67	67	1	-	-	-
3	-	60	-	63	-	-	-	2	49	64	-	-	7	12	6	-
4	-	2	-	1	-	67	-	2	-	1	-	-	48	3	14	-
5	-	-	-	-	-	-	-	55	-	-	-	-	7	25	41	-
6	-	-	-	-	-	-	-	5	-	-	-	-	1	16	5	-
7	-	-	-	-	-	-	-	-	-	-	-	-	3	3	1	3
8	-	-	-	-	-	-	-	-	-	-	-	-	-	7	-	60
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Allelic diversity[d]	0.00	**0.18**	0.00	**0.10**	**0.02**	0.00	0.00	**0.30**	**0.41**	**0.07**	0.00	0.00	**0.45**	**0.74**	**0.55**	**0.16**

a Locus MIRU 2 and 3232 did not amplify in one sample.

MIRU 4 (*b*) and MIRU 31 (*c*) correspond to ETR D and ETR E as defined by other studies.

d Allelic diversity (h) at a locus was calculated as follows: $h = 1 - \Sigma x_i^2[n/(n-1)]$, where xi is the frequency of the ith allele at the locus and n is the number of isolates.

Table 2: Allelic diversities of different loci from *M. bovis* from Northern Ireland and Chad (our study) and *M. tuberculosis* from S. Africa, USA and France.

	Allelic diversity in MIRU locus												Locus no. at ETR			Locus no. at 3232
	2	4[a]	10	16	20	23	24	26	27	31[b]	39	40	A	B	C	
67 *M. bovis* Chad	0.00	**0.18**	0.00	**0.10**	**0.02**	0.00	0.00	**0.30**	**0.41**	**0.07**	0.00	0.00	**0.45**	**0.74**	**0.55**	**0.16**
47 *M. bovis* N. Ireland	0.00	0.00	0.00	**0.02**	0.00	0.00	**0.37**	**0.52**	0.00	0.00	**0.06**	**0.27**	**0.40**	**0.37**	0.00	**0.60**
180 *M.tbc* USA	**0.08**	**0.22**	**0.44**	**0.42**	**0.09**	**0.12**	**0.16**	**0.54**	**0.09**	**0.47**	**0.22**	**0.63**	-	-	-	-
209 *M. tb* S. Africa	**0.14**	**0.26**	**0.70**	**0.28**	**0.06**	**0.54**	0.00	**0.59**	**0.14**	**0.55**	**0.36**	**0.65**	-	-	-	-
72 *M. tb* France	**0.02**	**0.35**	**0.69**	**0.52**	**0.29**	**0.58**	**0.24**	**0.67**	**0.19**	**0.37**	**0.34**	**0.74**	-	-	-	-

MIRU 4 (a) and MIRU 31 (b) correspond to ETR D and ETR E as defined by other studies.

Table 3: Determination of allelic diversities of different heterogeneity loci.

Set No.	VNTR loci combination	Allelic diversity	unique no.	Discrimination profile of *M. bovis* strains (n_{tot} = 67)										
				Cluster no. with size										
				2	3	4	5	6	7	11	12	13	26	30
1	MIRUs	**0.754**	11	3	1	-	-	1	-	1	-	-	-	1
2	ETR A,B,C	**0.906**	9	5	4	-	-	1	1	1	1	-	-	-
3	ETR A,B,C & MIRU 26, 27	**0.917**	15	7	2	1	1	-	-	1	1	-	-	-
4	All VNTRs	**0.922**	22	6	2	1	-	-	-	1	1	-	-	-
5	Spoligotyping	**0.789**	7	4	2	-	-	-	1	-	-	1	1	-
6	All combined	**0.944**	27	5	3	1	-	1	-	1	-	-	-	-

Allelic diversity (h) for each combination was calculated as follows: $h = 1 - \Sigma x_i^2[n/(n-1)]$, where x_i is the frequency of the ith allele for the combination of VNTRs (Set no. 1 to 6) and n is the number of isolates.

References

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

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

Cowan et al., 2002 L.S. Cowan, L. Mosher, L. Diem, J.P. Massey and J.T. Crawford, Variable-number-tandem repeat typing of mycobacterium tuberculosis isolates with low copy numbers of IS6110 by using mycobacterial interspersed repetitive units, *J. Clin. Microbiol.* **40** (2002), pp. 1592–1602.

FMO'Connell, 1998 R. Frothingham and W.A. Meeker-O'Connell, Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats, *Microbiology UK* **144** (1998), pp.

Kamerbeek et al., 1997 J. Kamerbeek, L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal and J. van Embden, Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology, *J. Clin. Microbiol.* **35** (1997), pp. 907–914.

Kremer et al., 1999 K. Kremer, D. van Soolingen, R. Frothingham, W.H. Haas, P.W.M. Hermans, C. Martin, P. Palittapongarnpim, B.B. Plikaytis, L.W. Riley, M.A. Yakrus, J.M. Musser and J.D.A. van Embden, Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility, *J. Clin. Microbiol.* **37** (1999), pp. 2607–2618.

Mazars et al., 2001 E. Mazars, S. Lesjean, A.L. Banuls, M. Gilbert, V. Vincent, B. Gicquel, M. Tibayrenc, C. Locht and P. Supply, High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology, *Proc. Natl. Acad. Sci. U.S.A.* **98** (2001), pp. 1901–1906.

Njanpop-Lafourcade et al., 2001 B.M. Njanpop-Lafourcade, J. Inwald, A. Ostyn, B. Durand, S. Hughes, M.F. Thorel, G. Hewinson and N. Haddad, Molecular typing of *Mycobacterium bovis* isolates from Cameroon, *J. Clin. Microbiol.* **39** (2001), pp. 222–227.

O'Reilly and Daborn, 1995 L.M. O'Reilly and C.J. Daborn, The epidemiology of *Mycobacterium bovis* infections in animals and man: a review, *Tuber. Lung Dis.* **76** (1995) (Suppl 1), pp. 1–46.

Pritchard, 1988 D.G. Pritchard, A century of bovine tuberculosis 1888–1988: conquest and controversy, *J. Comp. Pathol.* **99** (1988), pp. 357–399.

Roring et al., 2002 S. Roring, A. Scott, D. Brittain, I. Walker, G. Hewinson, S. Neill and R. Skuce, Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping, *J. Clin. Microbiol.* **40** (2002), pp. 2126–2133.

Roring et al., 2004 S. Roring, A.N. Scott, H.R. Glyn, S.D. Neill and R.A. Skuce, Evaluation of variable number tandem repeat (VNTR) loci in molecular typing of *Mycobacterium bovis* isolates from Ireland, *Vet. Microbiol.* **101** (2004), pp. 65–73.

Savine et al., 2002 E. Savine, R.M. Warren, G.D. van der Spuy, N. Beyers, P.D. van Helden, C. Locht and P. Supply, Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 loci in serial isolates of *Mycobacterium tuberculosis*, *J. Clin. Microbiol.* **40** (2002), pp. 4561–4566.

Selander et al., 1986 R.K. Selander, D.A. Caugant, H. Ochman, J.M. Musser, M.N. Gilmour and T.S. Whittam, Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics, *Appl. Environ. Microbiol.* **51** (1986), pp. 873–884.

Serraino et al., 1999 A. Serraino, G. Marchetti, V. Sanguinetti, M.C. Rossi, R.G. Zanoni, L. Catozzi, A. Bandera, W. Dini, W. Mignone, F. Franzetti and A. Gori, Monitoring of transmission of tuberculosis between wild boars and cattle: genotypical analysis of strains by molecular epidemiology techniques, *J. Clin. Microbiol.* **37** (1999), pp. 2766–2771.

Skuce et al., 2002 R.A. Skuce, T.P. McCorry, J.F. McCarroll, S.M.M. Roring, A.N. Scott, D. Brittain, S.L. Hughes, R.G. Hewinson and S.D. Neill, Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR–PCR targets, *Microbiology-Sgm* **148** (2002), pp. 519–528.

Supply et al., 2000 P. Supply, E. Mazars, S. Lesjean, V. Vincent, B. Gicquel and C. Locht, Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome, *Mol. Microbiol.* **36** (2000), pp. 762–771.

van Soolingen, 2001 D. van Soolingen, Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements, *J. Intern. Med.* **249** (2001), pp. 1–26.

Chapter VI: Population structure of *Mycobacterium bovis* from a high incidence country: Implications for molecular epidemiology and design of diagnostic candidates

Markus Hilty,[1] Stephen V. Gordon[3], Carmenchu Garcia-Pelayo[3], Javier Nunez-Garcia[3], Colette Diguimbaye, [2] Simeon Cadmus,[4] Esther Schelling,[1] R. Glyn Hewinson[3], and Jakob Zinsstag[1]

Swiss Tropical Institute[1], 4002 Basel, Switzerland,
Laboratoire de Recherches Vétérinaires et Zootechniques[2] de Farcha, N'Djaména, Chad, and
Veterinary Laboratories Agency[3], Weybridge, New Haw, Surrey, England
University of Ibadan[4], Ibadan, Nigeria

Draft paper

Abstract

By using microarray-based comparative genomics, large genomic deletions define 63 and 4 *M. bovis* isolates from Chad as 2 different clones, respectively. The implications for the molecular epidemiology and the choice of antigens used in a diagnostic mix for *M. tuberculosis* complex are discussed.

Microarry based comparative analysis of members of the *Mycobacterium tuberculosis* complex has defined a set of chromosomal deletions which mark all descendants of an ancestral strain (1,8,9,12,14,19). This has allowed 'diagnostic' deletions to be identified that allow an isolate to be unequivocally placed in a strain family. The value of genomic deletions for finding large family groups has been found to be superior to all other genotyping methods (e.g. Spoligotyping) (6). Additionally, knowledge of the genome organization across *M. tuberculosis* strains allows us to inform the choice of antigens used in a diagnostic mix.

Based on previous molecular typing data that allowed us to define phylogenetic extremes (3), the genomic DNA of 4 *M. bovis* strains from Chad with the most divergent spoligotypes (Table 1) were applied to an *M. tuberculosis* amplicon-array and fluorescence scanned with an Affymetrix 428 scanner as described (5). Data were analysed using GeneSpring 5.0 (Silicon Genetics, Redwood City,CA) and Mathematica (Wolfram Research). A cut-off for the normalised test/control ratio of <2.0 was used and results were compared to the control to create gene deletion lists.

Clone 1

For two *M. bovis* strains with spoligotypes 1 and 6 (Table 1), lacking spacer 30, microarray analysis revealed one large genomic deletion, which is described for the first time in this study. Subsequently, primers for amplification of the flanking regions of the deletion were designed (664100 5' actggaccggcaacgacctgg and 669951-5'cgggtgaccgtgaactgcgac). By performing PCR reactions as described (1) for all 67 *M. bovis* strains from Chad and 15 strains from Nigeria (2) and following gel electrophoresis, the size of the amplicons could be estimated by comparison with Size Marker VIII (Roche). PCR products of the two strains analysed by microarray were sequenced with an ABI Prism 310 Genetic Analysis System.

Sequence characterization revealed a deletion of 5320 bp (664281-669600 bp), removing Rv572c-574c and parts of Rv571c and Rv575c. The flanking regions of the deletion showed no similarity to insertion sequences and occurred in a non variable region. The sequences of the junction regions were exactly the same for the two strains analyzed.

63/67 and all the Nigerian strains lacked this deletion while this region wasn't deleted for 4/67 *M. bovis* strains from Chad which lacked spacer 22-24. From this analysis and from the fact that the population structure of *M. bovis* is strictly clonal (1), we conclude that most (63/67) of the Chadian and all of the Nigerian *M. bovis* strains belong to the same clone and therefore must have derived from a single, common cell in the past (18). Additionally, we assume that also the *M. bovis* strains from Cameroon belong to this family as they also all lack spacer 30 although we didn't check these strains for the deletion. The reasons why there

is so little heterogeneity within strains of these countries can be many. For *M. tuberculosis* the finding that one strain is causing most of the disease cases in a population would confirm the suspicion of an outbreak and imply a failure to stop transmission (10). Indeed control programs e.g. vaccination campaigns of test and slaughter policies to stop recent transmission are basically inexistent in Chad, Cameroon and Nigeria. However, the existence of homogeneity of these strains can also mean that there is little competition of other strain families or that this particular strain family is especially good adapted to host and/or the environment. In a recent study was shown, that Mbororo are more susceptible to the Chadian strains than the Arabe breed (3).

The fact that most of the Chadian and Nigerian strains belong to one cluster raises important implications for molecular epidemiological studies. Standard molecular typing tools may not be discriminative enough to distinguish between epidemiologically unlinked strains. In a recent study, a minimum of 5 VNTR loci was defined resulting in a sufficient discriminatory power (7). However, spoligotyping of strains of Nigeria, Cameroon and Chad showed the same genotypes for some of the strains. Although we know that the pastoralists in this area migrate long distances we think that it is more likely that most of these strains have no direct epidemiological link. If we exclude convergent evolution, we think a clonal expansion scenario of some subclones as shown in a recent study from the UK (18), is most likely. However, we cannot draw any conclusion which country is the origin and which is the direction of expansion.

Clone 2

While it is known for *M. bovis* to have lost the ESAT-6 family proteins *esxOP* (RD 5) and *esxVW* (RD 8) (Table 1) 2/4 of our *M. bovis* strains analyzed by comparative genomics had additionally the deletions of Rv3019c/Rv3020c (*esxSR*) and Rv3890/3891 (*esxCD*). We confirmed this finding by DNA sequencing and revealed deletions of 2439 bp (*esxSR*) and 8077 bp (*esxCD*) compared to H37Rv. Subsequent PCR reactions on all 67 strains isolated in Chad showed the presence of these deletions in 4 strains.

ESAT-6 family proteins are encoded by 23 genes (*esxA-W*) and are promising targets for new diagnostics and novel vaccine candidates. The hallmark members of this family, *esxA* (ESAT-6) and *esxB* (CFP-10) are absent from *M. bovis* BCG (due to the RD1 deletion) and have therefore been extensively studied for their role in virulence and for their diagnostic utility (6,16). Additionally, proteins encoded by *esxH* (TB10.4) and its homologues *esxR* (TB10.3) and *esxQ* (TB12.9) are strongly recognized T-cell antigens in humans and animals (17).

However, when choosing vaccine or diagnostic candidates it is important to survey clinical isolates for variation in these targets. Deletion of *esxSR* has been previously described in *M. tuberculosis* strains from France (EMBL accession AJ583832) (11) and the UK (15) and in *M. microti* (EMBL accession AJ550619) (5). To our surprise, using the primers as described (5), sequencing revealed that the deletion of *esxSR* in our *M. bovis* strains from Chad had occurred at exactly the same position to that previously reported for *M. tuberculosis* and *M. microti*, suggesting that this locus is prone to deletion events due to the highly repetitive PE/PPE gene sequences that flank the region. However, we also uncovered a deletion of *esxCD* which isn't the first time found to be deleted too. However, the position of the deletion characterized in our study doesn't equal the one found (13). Adding these new deletions to previous findings (Table 1) shows the high variability of ESAT-6 family members in the *M. tuberculosis* complex.

In the study of Gao *et al* (4), transcriptome analysis across 10 clinical isolates of *M. tuberculosis* revealed that *esx* genes vary in their expression levels between strains. The deletion and variable expression of genes encoding ESAT members suggests that they may be under strong selective pressures linked to immune escape. Hence while ESAT family members are potent antigens, genome analysis has revealed that their encoding genes are frequently deleted, suggesting caution in their use as stand-alone diagnostic reagents. Knowledge of the genome organization across *M. tuberculosis* strains therefore allows us to inform the choice of antigens used in a diagnostic mix.

Acknowledgments

We thank the technicians of the Swiss National Centre for Mycobacteria, the Swiss Tropical Institute, and the "Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha" who have contributed to the project. NCCR North-South IP-4 is acknowledged for financial support.

Table 1: Spoligotypes of chosen *M. bovis* strains for microarray analysis

SP	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
1	26	■	■	□	■	■	■	■	■	□	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	□	□	□	□	□
6	2	■	■	□	■	■	■	■	■	□	■	■	■	□	□	□	□	□	□	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	□	□	□	□	□
15	2	■	■	□	■	■	■	■	■	□	■	■	■	■	■	■	□	■	■	■	□	□	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	□	□	□	□
16	2	■	■	□	■	■	■	■	■	□	■	■	■	■	■	■	□	■	■	■	□	□	□	■	■	■	■	■	■	■	□	□	■	■	■	■	■	■	■	□	□	□	□	□

Table 2: ESAT-6 family variation in MTC members

	Gene name	mRNA expression (*M. tuberculosis*)	Deletion detected	MTC species occurrence of variation (Reference)
Rv0287	*esxG* (TB 9.8)	consistently	ND	
Rv0288	esxH (CFP-7, TB 10.4)	variable	ND	
Rv1037c	*esxI*	variable	ND	
Rv1038c	*esxJ*	variable	ND	
Rv1197	*esxK*	variable	ND	
RV1198	*esxL*	unknown	ND	
Rv1792	*esxM*	consistently	ND	
Rv1793	*esxN*	variable	ND	
Rv2346c	*esxO*	variable	truncation (RD5)	*M. bovis, M. microti (vole)*
Rv2347c	*esxP*	variable	deletion (RD5)	*M. bovis, M. microti (vole)*
Rv3017c	*esxQ* (TB 12.9)	unexpressed	ND	
Rv3019c	*esxR* (TB 10.3)	low	deletion (MiD4)	*M. microti* (5) ***M. bovis*** **(this study)** *M. tuberculosis* (11,15)
Rv3020c	*esxS*	low	deletion (MiD4)	*M. microti* (5) ***M. bovis*** **(this study)** *M. tuberculosis* (11,15)
Rv3444c	*esxT*	unexpressed	ND	
Rv3445	*esxU*	unknown	ND	
Rv3619c	*esxV*	consistently	deletion (RD8)	*M. microti, M. bovis* (1)
Rv3620c	*esxW*	variable	deletion (RD8)	*M. microti, M. bovis* (1)
Rv3874	*esxB* (CFP-10)	variable	deletion (RD1mic) deletion (RD1)	*M. microti* (5) *M. bovis BCG* (1)
Rv3875	*esxA* (ESAT-6)	variable	deletion (RD1mic) deletion (RD1)	*M. microti* (5) *M. bovis BCG* (1)
Rv3890c	*esxC*	low	deletion deletion truncation	***M. bovis (13)*** ***M. bovis*** **(this study)** *M. tuberculosis* (19)
Rv3891c	*esxD*	variable	deletion	***M. bovis (13)*** ***M. bovis*** **(this study)**
Rv3904c	*esxE*	unknown	ND	
Rv3905c	*esxF*	low	ND	

ND: None detected at sensitivity of array used

Reference List

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

2. **Cadmus, S., S. Palmer, M. Okker, J. Dale, K. Gover, N. Smith, K. Jahans, R. G. Hewinson, and S. V. Gordon**. 2006. Molecular Analysis of Human and Bovine Tubercle Bacilli from a Local Setting in Nigeria. Journal of Clinical Microbiology **44**:29-34.

3. **Diguimbaye-Djaibé C., Hilty M., Ngandolo R, Mahamat HH., Pfyffer G. E., Baggi F., Hewinson G., Tanner M., Zinsstag J. and Schelling E.** *Mycobacterium bovis* Isolates from Tuberculous Lesions in Chadian Zebu Carcasses. 2006. Emerg.Infect.Dis. **12**(5):769-771

4. **Gao, Q., K. E. Kripke, A. J. Saldanha, W. Yan, S. Holmes, and P. M. Small**. 2005. Gene expression diversity among Mycobacterium tuberculosis clinical isolates. Microbiology **151**:5-14.

5. **Garcia-Pelayo, M. C., K. C. Caimi, J. K. Inwald, J. Hinds, F. Bigi, M. I. Romano, D. van Soolingen, R. G. Hewinson, A. Cataldi, and S. V. Gordon**. 2004. Microarray analysis of Mycobacterium microti reveals deletion of genes encoding PE-PPE proteins and ESAT-6 family antigens. Tuberculosis.(Edinb.) **84**:159-166.

6. **Hill, P. C., D. Jackson-Sillah, A. Fox, K. L. Franken, M. D. Lugos, D. J. Jeffries, S. A. Donkor, A. S. Hammond, R. A. Adegbola, T. H. Ottenhoff, M. R. Klein, and R. H. Brookes**. 2005. ESAT-6/CFP-10 fusion protein and peptides for optimal diagnosis of mycobacterium tuberculosis infection by ex vivo enzyme-linked immunospot assay in the Gambia. J.Clin.Microbiol. **43**:2070-2074.

7. **Hilty, M., C. Diguimbaye, E. Schelling, F. Baggi, M. Tanner, and J. Zinsstag**. 2005. Evaluation of the discriminatory power of variable number tandem repeat (VNTR) typing of Mycobacterium bovis strains. Vet.Microbiol. **109**:217-222.

8. **Hirsh, A. E., A. G. Tsolaki, K. DeRiemer, M. W. Feldman, and P. M. Small**. 2004. Stable association between strains of Mycobacterium tuberculosis and their human host populations. Proc.Natl.Acad.Sci.U.S.A **101**:4871-4876.

9. **Kato-Maeda, M., J. T. Rhee, T. R. Gingeras, H. Salamon, J. Drenkow, N. Smittipat, and P. M. Small**. 2001. Comparing genomes within the species Mycobacterium tuberculosis. Genome Res. **11**:547-554.

10. **Malik, A. N. and P. Godfrey-Faussett**. 2005. Effects of genetic variability of Mycobacterium tuberculosis strains on the presentation of disease. Lancet Infect.Dis. **5**:174-183.

11. **Marmiesse, M., P. Brodin, C. Buchrieser, C. Gutierrez, N. Simoes, V. Vincent, P. Glaser, S. T. Cole, and R. Brosch**. 2004. Macro-array and bioinformatic analyses reveal mycobacterial 'core' genes, variation in the ESAT-6 gene family and new phylogenetic markers for the Mycobacterium tuberculosis complex. Microbiology **150**:483-496.

12. **Mostowy, S., D. Cousins, J. Brinkman, A. Aranaz, and M. A. Behr**. 2002. Genomic deletions suggest a phylogeny for the Mycobacterium tuberculosis complex. J.Infect.Dis. **186**:74-80.

13. **Mostowy, S., J. Inwald, S. Gordon, C. Martin, R. Warren, K. Kremer, D. Cousins, and M. A. Behr**. 2005. Revisiting the evolution of Mycobacterium bovis. J.Bacteriol. **187**:6386-6395.

14. **Nguyen, D., P. Brassard, D. Menzies, L. Thibert, R. Warren, S. Mostowy, and M. Behr**. 2004. Genomic characterization of an endemic Mycobacterium tuberculosis strain: evolutionary and epidemiologic implications. J.Clin.Microbiol. **42**:2573-2580.

15. **Rajakumar, K., J. Shafi, R. J. Smith, R. A. Stabler, P. W. Andrew, D. Modha, G. Bryant, P. Monk, J. Hinds, P. D. Butcher, and M. R. Barer**. 2004. Use of genome level-informed PCR as a new investigational approach for analysis of outbreak-associated Mycobacterium tuberculosis isolates. J.Clin.Microbiol. **42**:1890-1896.

16. **Ravn, P., M. E. Munk, A. B. Andersen, B. Lundgren, J. D. Lundgren, L. N. Nielsen, A. Kok-Jensen, P. Andersen, and K. Weldingh**. 2005. Prospective evaluation of a whole-blood test using Mycobacterium tuberculosis-specific antigens ESAT-6 and CFP-10 for diagnosis of active tuberculosis. Clin.Diagn.Lab Immunol. **12**:491-496.

17. **Skjot, R. L., I. Brock, S. M. Arend, M. E. Munk, M. Theisen, T. H. Ottenhoff, and P. Andersen**. 2002. Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. Infect.Immun. **70**:5446-5453.

18. **Smith, N. H., J. Dale, J. Inwald, S. Palmer, S. V. Gordon, R. G. Hewinson, and J. M. Smith**. 2003. The population structure of Mycobacterium bovis in Great Britain: clonal expansion. Proc.Natl.Acad.Sci.U.S.A **100**:15271-15275.

19. **Tsolaki, A. G., A. E. Hirsh, K. DeRiemer, J. A. Enciso, M. Z. Wong, M. Hannan, Goguet de la Salmoniere YO, K. Aman, M. Kato-Maeda, and P. M. Small**. 2004. Functional and evolutionary genomics of Mycobacterium tuberculosis: insights from genomic deletions in 100 strains. Proc.Natl.Acad.Sci.U.S.A **101**:4865-4870.

Chapter VII: Genetic diversity in *Mycobacterium ulcerans* isolates from Ghana revealed by a newly identified locus containing a variable number of tandem repeats

Markus Hilty,[1*] Dorothy Yeboah-Manu,[1,2*] Daniel Boakye,[2] Ernestina-Mensah-Quainoo,[3] Simona Rondini,[1] Esther Schelling,[1] David Ofori-Adjei,[2] Françoise Portaels[4], Jakob Zinsstag[1] and Gerd Pluschke[1]

Swiss Tropical Institute[1], 4002 Basel, Noguchi Memorial Institute for Medical Research[2], Legon, Ghana, Tema Municipal Health Directorate, Tema, Ghana[3], and Institute of Tropical Medicine[4], 2000 Antwerp, Belgium

Published in *Journal of Bacteriology* 2006 Feb;188(4):1462-5

[*]Contributed equally

Abstract

Molecular typing methods applied so far for *Mycobacterium ulcerans* isolates have not been able to identify genetic differences among isolates from Africa. This apparent lack of genetic diversity among *M. ulcerans* isolates is indicative for a clonal population structure. We analysed the genetic diversity of 71 African isolates, including 57 strains from Ghana, by variable number of tandem repeats (VNTR) typing based on a newly identified polymorphic locus designated ST1 and the previously described locus, MIRU 1. Three different genotypes were found in Ghana, demonstrating for the first time genetic diversity of *M. ulcerans* in an African country. While the ST1/MIRU 1 allele combination BD/BAA seems to dominate in Africa, it was only rarely found in isolates from Ghana, where the combination BD/B was dominating and observed in all districts analysed. A third variant genotype (C/BAA) was found only in the Amansie-West district. Results are indicative for the emergence and spreading of new genetic variants of *M. ulcerans* within Ghana and support the potential of VNTR-based typing for genotyping of *M. ulcerans*.

Introduction

Mycobacterium ulcerans, the causative agent of Buruli ulcer (BU) is an emerging pathogen particularly in Sub-Saharan African countries, and is also found in tropical and sub-tropical regions of Asia, the Western Pacific and Latin America (4). BU is characterized by chronic, necrotic lesions of subcutaneous tissues. Due to the lack of an established effective antimicrobial therapy, surgical excision and skin grafting is currently the recommended treatment (27).

While it is known that proximity to slow flowing or stagnant water bodies is a risk factor for *M. ulcerans* infection, the exact mode of transmission has remained an enigma (4). This is partly because no molecular typing method is available that has sufficiently high resolution for micro-epidemiological analyses. The apparent lack of genetic diversity of *M. ulcerans* within individual geographical regions (7, 8, 16, 19-21) is indicative for a clonal population structure. Genetic analyses suggest the recent divergence of *M. ulcerans* from *M. marinum* (5, 26), which is well known as fish pathogen and can cause limited granulomatous skin infections in humans (10, 11, 13). One of the hallmarks of the emergence of *M. ulcerans* as a more severe pathogen is the acquisition of a 174-kb plasmid bearing a cluster of genes necessary for the synthesis of the macrolide toxin mycolactone responsible for the massive tissue destruction seen in BU (22).

Variable number of tandem repeat (VNTR) typing is a PCR-based technique identifying alleles of defined regions of DNA that contain a variable number of copies of short sequence stretches. Resolution of the method is cumulative and can be increased by inclusion of additional loci. Tandem repeats are easily identified from genome sequence data, measurement of PCR fragment sizes is relatively straightforward and VNTR typing data can be digitalized and compared between different laboratories. Availability of complete genomic sequences has facilitated identification of repetitive genetic elements of *M. tuberculosis* (12, 15, 24, 25), *M. bovis* (14, 18) and *M. avium* (6), including short tandem repeats designated exact tandem repeats (ETRs) and mycobacterial interspersed repetitive units (MIRUs). Strain typing with these sets of polymorphic loci is developing into an important tool in the epidemiological analysis of tuberculosis (9) and ordinary agarose gel electrophoretic separation of PCR products is usually sufficient to estimate the number of repeat units in an allele. In a study of *M. bovis* strains from Chad, VNTR-typing of a distinct number of loci is most discriminative for strains of the same clone (14).

More recently, MIRUs and other VNTRs have also been described for *M. ulcerans* and *M. marinum* (3, 23) typing. Most of the described sequences are orthologues of the *M.*

tuberculosis genome database and their resolution seems to be comparable to that of the currently most discriminatory methods, the 2426 PCR analysis (19) and IS2404-Mtb2 PCR (2) which discriminate among isolates from different geographical origin, but not among strains from different endemic regions of Africa.

We describe in this report a new *M. ulcerans* specific VNTR locus (ST1) which together with the previously described MIRU 1 (23) differentiated clinical isolates from Ghana into three VNTR allele combinations.

Methods

Identification of the VNTR locus ST1: A tandem repeat finder software (http://www.c3.biomath.mssm.edu/trf.thml) was used to screen the *M. marinum* sequence data bank (www.sanger.ac.uk/projects/M_marinum) and identified a tandem repeat containing locus which was designated ST1. This locus is present both in *M. marinum* and in *M. ulcerans* (http://genopole.pasteur.fr/Mulc/BuruList.html), but not in *M. tuberculosis* (http://www.sanger.ac.uk/Projects/M_tuberculosis). A forward (ctgaggggatttcacgaccag) and a reverse primer (cgccacccgcggacacagtcg) located in the sequences flanking the identified locus and yielding a PCR product of 423 bp was designed. Genomic sequences corresponding to the primers were 100 % identical for *M. marinum* and *M. ulcerans*.

Bacterial strains: A panel of 11 *M. ulcerans* clinical isolates of human origin from diverse geographical origin and of 6 *M. marinum* clinical isolates of human origin from Switzerland was used to assess the polymorphism of ST1 (Table 1). The *M. marinum* isolates were from patients living in the agglomeration of Zurich, except for *M. marinum* N119 that was isolated from a patient living in Biel. The year of isolation was 1995 for strains 853 and 894, 1997 for strains 8972 and 946 and 1998 for strains N119 and 3023. In order to analyse the diversity of African *M. ulcerans* strains, 66 additional clinical isolates (12 from Benin and 54 from Ghana) were included in this study. The Ghanaian strains were isolated (28) between 2001 and 2003 from patients being treated at the Amasaman Health Centre in the Greater Accra Region of Ghana (48 isolates) or the Saint Martin Hospital Agroyesum in the Ashanti Region of Ghana (6 isolates). The residential origin of the isolates is as indicated in the supporting table.

DNA Extraction: DNA was extracted as described (17). Briefly, small bacterial pellets were heated for 1 h at 95°C in 500 µl of an extraction mixture (50 mM Tris-HCl, 25 mM EDTA, and 5% monosodium glutamate). One hundred microliters of a 50-mg/ml lysozyme solution was then added and incubated for two hours at 37°C. 70 µl of proteinase K-10x buffer (100 mM Tris-HCl, 50 mM EDTA, 5% sodium dodecyl sulphate [pH 7.8]) and 10 µl of a 20-

mg/ml proteinase K solution was added. After incubation at 45°C overnight, 300 µl of 0.1-mm-diameter zirconia beads (BioSpec Products) were added to each sample and vortexed at full speed for 4 min. Beads and large debris were removed by brief centrifugation, and the supernatants were transferred to fresh tubes for phenol-chloroform (Fluka) extraction. The DNA contained in the upper phase was precipitated with ethanol and re-suspended in 100 µl of water.

PCR analysis and sequencing of PCR products: PCR reaction mixtures contained 1x *Taq* PCR buffer, deoxynucleoside triphosphates (0.2 mM each), 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), a 0.5 µM concentration of the primer pair and mycobacterial DNA in a final volume of 20 µl. Addition of 5 % DMSO to the reaction mix improved the yield of PCR products. The reaction was carried out using a Perkin-Elmer 9600 cycler starting with a denaturing step of 10 min at 95°C. After denaturation, the PCR was performed for 40 cycles of 0.5 min at 94°C, 0.5 min at 65°C and 1 min at 72°C. The reactions were terminated by an incubation of 10 min at 72°C. PCR fragments were analysed by agarose gel electrophoresis using 2% NuSieve agarose. The size of the amplicons was estimated by comparison with Size Marker VIII (Roche). PCR products were directly sequenced with an ABI Prism 310 Genetic Analysis System. PCR products for ST1 of 423 bp and 369 bp corresponded to a copy number of 2 and 1, respectively. For MIRU 1, copy numbers were assigned as described (23).

Results

Identification and characterization of a new VNTR locus found in *M. ulcerans* and *M. marinum*

A new VNTR locus designated ST1, was identified by screening of the *M. marinum* sequence data bank with a tandem repeat finder software. Orthologues of ST1 were found in *M. ulcerans*, but not in *M. tuberculosis.* Its repeat length of 54 bp is suitable for size analysis by standard agarose gel electrophoresis. In contrast to MIRUs (23), ST1 is not intergenic, but part of a pseudogene and therefore of no known functional interest (personal communication). When eleven *M. ulcerans* isolates of geographically diverse origin were typed by agarose gel electrophoresis of PCR products, two different alleles of ST1 were identified (Table 1). While eight strains had two repeats, two isolates, one from French Guyana and one of the two analysed isolates from Ghana had only one repeat. Of seven *M. marinum* clinical isolates tested, all five strains from patients in the agglomeration of Zurich (strains 8972, 946, 3023, 853 and 894) had three repeats, whereas strain N119 isolated from a different part of

Switzerland and the reference strain used for the *M. marinum* genome sequencing project, both had two repeats. Sequence analysis of PCR products reconfirmed the size differences observed by agarose gel electrophoresis and identified six sequence variants of the repeat unit (designated A – F; Fig 1). Unlike many other VNTRs (1), ST1 showed no micro-deletions, but only single nucleotide polymorphisms (SNPs) within the sequence variants. The *M. ulcerans* strains from China and Japan turned out to have a different allele (CF; Table 1) than the other *M. ulcerans* strains with two repeats (BD). A third allele with two repeats (AC) and an allele with three repeats (ACE) was found in *M. marinum* (Table 1). Thus sequencing of ST1 improved the discrimination power of *M. ulcerans* and *M. marinum* strains compared to gel electrophoresis analysis alone and revealed distinctive genotypes for *M. marinum* compared to *M. ulcerans*.

Diversity of *M. ulcerans* isolates from Ghana

Evidence for diversity of the ST1 locus in African isolates (Table 1) prompted us to analyse additional collections of 12 disease isolates from Benin and 54 isolates from Ghana (Table 2). All strains from Benin and the majority of Ghanaian strains had an ST1 allele (BD) with two repeats. However, in most strains from the Amansie West district (including ITM-970359; Table 1), a second allele with only one repeat (C) was identified (Table 2). When the Ghanaian isolates were tested also for diversity in the loci MIRU 1 (23), VNTR 8, 9 and 19, previously described as polymorphic within *M. ulcerans* strains of different geographical origin (3), diversity was also found in locus MIRU 1. Sequence analysis of PCR products of selected strains reconfirmed the size differences observed by agarose gel electrophoresis and identified two sequence variants of the MIRU 1 repeat unit (designated A and B; Fig 1).

Altogether three VNTR allele combinations were found among the clinical isolates from Ghana (Table 2, Fig. 2 and 3). While all isolates from the Ga, Akwapim South, Ahafo-Ano North and Akim Abuakwa districts had the ST1/MIRU 1 allele combination 1 (BD/B), two allele combinations, i.e. 1 and 2 (C/BAA) were found among the five isolates from the Amansie-West district. A third allele combination (BD/BAA) was found in two strains (Agy99 from the Ga district and ITM 97-0359 from the Ashanti region) isolated before 2000 in Ghana and in all other African isolates.

Discussion

Molecular typing methods such as multi-locus sequence typing, 16S rRNA sequencing, restriction fragment length polymorphism and variable number of tandem repeats typing have revealed a remarkable lack of genetic diversity of *M. ulcerans* and a clonal population structure within given geographical regions. The discriminatory power of all these methods is particularly insufficient to differentiate between African isolates. Innovative molecular genetic fingerprinting methods are therefore required for local epidemiological studies aiming to reveal transmission pathways and environmental reservoirs of *M. ulcerans*. First attempts to use VNTR typing for *M. ulcerans* (3, 23) have identified variable loci suitable for discrimination of disease isolates at continental level. In this study we used a newly identified (ST1) and four previously described VNTRs to analyse genetic diversity within a collection of 71 *M. ulcerans* strains from Africa, including 57 isolates from Ghana. Three of the previously described VNTRs i.e. VNTR 8, 9 and 19 (3) were not able to discriminate among the African strains. Yet MIRU 1 (23) and the newly identified locus (ST1) defined three subgroups within the Ghanaian strains.

The fact that two allele combinations (BD/B and C/BAA) differing from the common African combination (BD/BAA) were found within a recent (2001-2003) collection of Ghanaian isolates is indicative for an ongoing microevolution of *M. ulcerans* and for the spreading of new variants within Ghana. It is tempting to hypothesize that allele combination 3 (BD/BAA) represents an ancestral like genotype and that the others evolved by reduction in the repeat unit numbers in the ST1 locus (from BD to C) or in the MIRU 1 locus (from BAA to B), respectively. While conversion of the MIRU 1 locus from BAA to B could be explained by deletion of the two A repeat units, conversion of the ST1 locus from BD to C by a deletional mechanism would require that a central sequence stretch of the BD repeat region comprising portions of both the B and the D repeat unit would have been lost, yielding the hybrid repeat unit C.

While allele combination 3 seems to be the most common in Africa, most of the *M. ulcerans* strains from Ghana analysed, had the allele combination 1. This genotype was found in all Ghanaian districts included in this study. Allele combination 2 dominated in the Amansie West district, but was found exclusively there. Follow up of the temporal and spatial patterns of emergence and spreading of genotypes may contribute in future to our understanding of the transmission and epidemiology of Buruli ulcer. From the present data, we cannot draw any conclusions why certain variant appear to be the more successful than others. The fact, that we were not able to sub-group the 47 isolates from the Ga district by VNTR (with the only

exception of the 'older' isolate Agy99) reconfirms that *M. ulcerans* has a clonal population structure associated with a low rate of genomic drift. Availability of the fully assembled and annotated genome sequence of *M. ulcerans* in the near future will facilitate identification of further polymorphic VNTR loci potentially contributing to further refinement of genetic fingerprinting of *M. ulcerans* isolates.

Acknowledgements

We acknowledge Dr. Edwin Ampadu, of the Ghana National Buruli Ulcer Control program for his assistances in clinical sample collection. NCCR North-South IP-4 is acknowledged for financial support and Anthony Ablordey for critical review. Many thanks also to Franca Baggi of the Swiss Centre of Mycobacteria for providing *M. marinum* strains and the heads of the *M. ulcerans* and *M. marinum* genome sequencing projects for the permission to use the sequence data.

Table 1: ST1 alleles of *M. ulcerans* and *M. marinum* disease isolates

Species	Isolate	Country of origin	Number of repeats	Arrangement of the variant repeat DNA sequences
M. ulcerans	ITM 8756	Japan	2	**C F**
	ITM 980912	China	2	**C F**
	ITM 941328	Malaysia	2	**B D**
	ITM 884	Australia	2	**B D**
	ITM 9357	PNG	2	**B D**
	ITM 7922	French Guyana	1	**C**
	ITM 970359+	Ghana	1	**C**
	ITM 970321	Ghana	2	**B D**
	ITM 940886	Benin	2	**B D**
	ITM 940662	Côte d'Ivoire	2	**B D**
	ITM 960658	Angola	2	**B D**
M. marinum	894/1995	Switzerland	3	**A C E**
	853/1995	Switzerland	3	**NOT DONE**
	8972/1997	Switzerland	3	**NOT DONE**
	946/1997	Switzerland	3	**NOT DONE**
	3023/1998	Switzerland	3	**NOT DONE**
	N119/1998	Switzerland	2	**NOT DONE**
	*M. marinum**	unknown	2	**A C**

*isolate used for the *M. marinum* genome sequencing project

+isolated in 1997 from a patient living in the Amansie West district

Table 2: ST1 and MIRU 1 allele combinations of *M. ulcerans* strains from Africa

Country of origin	District	Number of strains	ST1 allele	MIRU 1 allele	Allele combination
Ghana	Ga district	47	2 (BD)	1 (B)	1
	Ga district	1	2 (BD)	3 (BAA)	3
	Akwapim South	1	2 (BD)	1 (B)	1
	Akim Abuakwa	1	2 (BD)	1 (B)	1
	Ahafo-Ano	1	2 (BD)	1 (B)	1
	Amansie West	1	2 (BD)	1 (B)	1
	Amansie West	4	1 (C)	3 (BAA)	2
	unknown°	1	2 (BD)	3 (BAA)	3
Benin		13	2 (BD) +	3* (BAA)+	3
Côte d'Ivoire		1	2 (BD)	3*	3
Angola		1	2 (BD)	3*	3

VNTR copy numbers for ST1 and MIRU 1 were determined and allele combinations assigned (1-3). Sequence profiles of *M. ulcerans* strains are shown in brackets. °isolated at the Saint Martin's hospital in the Ashanti region; * MIRU 1 copy numbers as previously described (23); +only one strain (ITM 94-0886) was analysed by sequencing (23).

Allele	ST1 repeat unit sequences
A	CCGGTTCTGTTTCGTCCGGTGCGACCGCTGGCACTGTCTCGACCGGTGCGACGA
B	....G....C.......
C	....G...........G
D	.......G...G.C...........................G...........G
E	-T.....G...G.C...........................G...........G
F	.......G...G.C....A......................G...........G

Allele	MIRU 1 repeat unit sequences
A*	ATGAGCCAGCCGGCGACGATGCAGAGCGAAGCGATGAGGAGGAGCGGCGCCAG
B	G...A..C..T..

Figure 1: Sequence variation of ST1 and MIRU 1 tandem repeat units

(-): Base deletions; (.): identical sequence positions.

*Allele A of MIRU 1 corresponds to variant A2 (23).

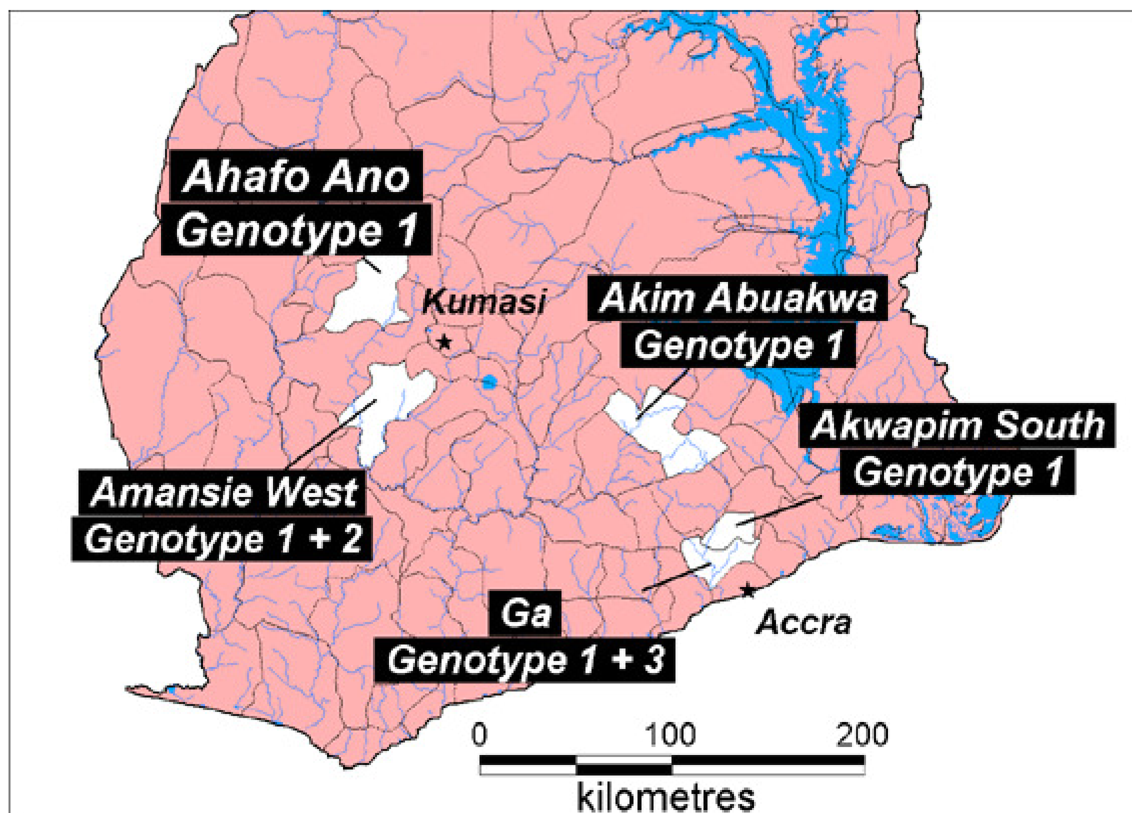

Figure 2: Map of southern Ghana showing the residential districts of patients from whom the Ghanaian isolates analysed in this study were obtained

ST1/MIRU 1 allele combinations are genotype 1: BD/B, genotype 2: C/BAA and genotype 3: BD/BAA.

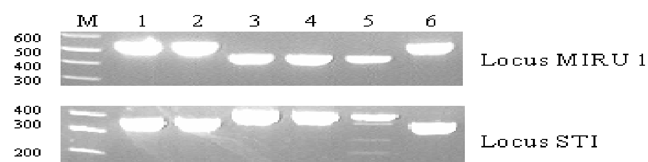

Figure 3: Agarose gel electrophoretic analysis of PCR products from amplifications with MIRU 1 primers (upper Panel) and STI primers (lower panel)

Results with selected isolates are shown. 1: strain (Amansie-West district); 2: strain (Amansie-West district); 3: strain (Ga district); 4: strain (Ga district); 5: strain (Ga district); 6: strain (Amansie-West district);

Reference List

1. **Ablordey, A., M. Hilty, P. Stragier, J. Swings, and F. Portaels**. 2005. Comparative Nucleotide Sequence Analysis of Polymorphic Variable-Number Tandem-Repeat Loci in Mycobacterium ulcerans. J.Clin.Microbiol. **43**:5281-5284.

2. **Ablordey, A., R. Kotlowski, J. Swings, and F. Portaels**. 2005. PCR amplification with primers based on IS2404 and GC-rich repeated sequence reveals polymorphism in Mycobacterium ulcerans. J.Clin.Microbiol. **43**:448-451.

3. **Ablordey, A., J. Swings, C. Hubans, K. Chemlal, C. Locht, F. Portaels, and P. Supply**. 2005. Multilocus variable-number tandem repeat typing of Mycobacterium ulcerans. J.Clin.Microbiol. **43**:1546-1551.

4. **Asiedu, K., R. Scherpbier, and M. Raviglione**. 2000. Buruli ulcer - *Mycobacterium ulcerans* infection. WHO document WHO/CDS/CPE/GBUI/2000.1.

5. **Boddinghaus, B., T. Rogall, T. Flohr, H. Blocker, and E. C. Bottger**. 1990. Detection and Identification of Mycobacteria by Amplification of Ribosomal-Rna. Journal of Clinical Microbiology **28**:1751-1759.

6. **Bull, T. J., K. Sidi-Boumedine, E. J. Mcminn, K. Stevenson, R. Pickup, and J. Hermon-Taylor**. 2003. Mycobacterial interspersed repetitive units (MIRU) differentiate Mycobacterium avium subspecies paratuberculosis from other species of the Mycobacterium avium complex. Molecular and Cellular Probes **17**:157-164.

7. **Chemlal, K., K. De Ridder, P. A. Fonteyne, W. M. Meyers, J. Swings, and E. Portaels**. 2001. The use of IS2404 restriction fragment length polymorphisms suggests the diversity of Mycobacterium ulcerans from different geographical areas. American Journal of Tropical Medicine and Hygiene **64**:270-273.

8. **Chemlal, K., G. Huys, P. A. Fonteyne, V. Vincent, A. G. Lopez, L. Rigouts, J. Swings, W. M. Meyers, and F. Portaels**. 2001. Evaluation of PCR-restriction profile analysis and IS2404 restriction fragment length polymorphism and amplified fragment length polymorphism fingerprinting for identification and typing of Mycobacterium ulcerans and M-marinum. Journal of Clinical Microbiology **39**:3272-3278.

9. **Crawford, J. T.** 2003. Genotyping in contact investigations: a CDC perspective. International Journal of Tuberculosis and Lung Disease **7**:S453-S457.

10. **Dailloux, M., C. Laurain, R. Weber, and P. Hartemann**. 1999. Water and nontuberculous mycobacteria. Water Research **33**:2219-2228.

11. **Dobos, K. M., F. D. Quinn, D. A. Ashford, C. R. Horsburgh, and C. H. King**. 1999. Emergence of a unique group of necrotizing mycobacterial diseases. Emerging Infectious Diseases **5**:367-378.

12. **Frothingham, R. and W. A. Meeker-O'Connell**. 1998. Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats. Microbiology-Uk **144**:1189-1196.

13. **Gluckman, S. J.** 1995. Mycobacterium-Marinum. Clinics in Dermatology **13**:273-276.

14. **Hilty, M., C. Diguimbaye, E. Schelling, F. Baggi, M. Tanner, and J. Zinsstag**. 2005. Evaluation of the discriminatory power of variable number tandem repeat (VNTR) typing of Mycobacterium bovis strains. Vet.Microbiol. **109**:217-222.

15. **Mazars, E., S. Lesjean, A. L. Banuls, M. Gilbert, V. Vincent, B. Gicquel, M. Tibayrenc, C. Locht, and P. Supply**. 2001. High-resolution minisatellite-based typing as a portable approach to global

analysis of Mycobacterium tuberculosis molecular epidemiology. Proceedings of the National Academy of Sciences of the United States of America **98**:1901-1906.

16. **Portaels, F., P. A. Fonteyne, H. DeBeenhouwer, P. DeRijk, A. Guedenon, J. Hayman, and W. M. Meyers**. 1996. Variability in 3' end of 16S rRNA sequence of Mycobacterium ulcerans is related to geographic origin of isolates. Journal of Clinical Microbiology **34**:962-965.

17. **Rondini, S., E. Mensah-Quainoo, H. Troll, T. Bodmer, and G. Pluschke**. 2003. Development and application of real-time PCR assay for quantification of Mycobacterium ulcerans DNA. Journal of Clinical Microbiology **41**:4231-4237.

18. **Skuce, R. A., T. P. McCorry, J. F. McCarroll, S. M. M. Roring, A. N. Scott, D. Brittain, S. L. Hughes, R. G. Hewinson, and S. D. Neill**. 2002. Discrimination of Mycobacterium tuberculosis complex bacteria using novel VNTR-PCR targets. Microbiology-Sgm **148**:519-528.

19. **Stinear, T., J. K. Davies, G. A. Jenkin, F. Portaels, B. C. Ross, F. Oppedisano, M. Purcell, J. A. Hayman, and P. D. R. Johnson**. 2000. A simple PCR method for rapid genotype analysis of Mycobacterium ulcerans. Journal of Clinical Microbiology **38**:1482-1487.

20. **Stinear, T., B. C. Ross, J. K. Davies, L. Marino, R. M. Robins-Browne, F. Oppedisano, A. Sievers, and P. D. R. Johnson**. 1999. Identification and characterization of IS2404 and IS2606: Two distinct repeated sequences for detection of Mycobacterium ulcerans by PCR. Journal of Clinical Microbiology **37**:1018-1023.

21. **Stinear, T. P., G. A. Jenkin, P. D. R. Johnson, and J. K. Davies**. 2000. Comparative genetic analysis of Mycobacterium ulcerans and Mycobacterium marinum reveals evidence of recent divergence. Journal of Bacteriology **182**:6322-6330.

22. **Stinear, T. P., A. Mve-Obiang, P. L. C. Small, W. Frigui, M. J. Pryor, R. Brosch, G. A. Jenkin, P. D. R. Johnson, J. K. Davies, R. E. Lee, S. Adusumilli, T. Garnier, S. F. Haydock, P. F. Leadlay, and S. T. Cole**. 2004. Giant plasmid-encoded polyketide synthases produce the macrolide toxin of Mycobacterium ulcerans. Proceedings of the National Academy of Sciences of the United States of America **101**:1345-1349.

23. **Stragier, P., A. Ablordey, W. M. Meyers, and F. Portaels**. 2005. Genotyping Mycobacterium ulcerans and Mycobacterium marinum by using mycobacterial interspersed repetitive units. J.Bacteriol. **187**:1639-1647.

24. **Supply, P., J. Magdalena, S. Himpens, and C. Locht**. 1997. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Molecular Microbiology **26**:991-1003.

25. **Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht**. 2000. Variable human minisatellite-like regions in the Mycobacterium tuberculosis genome. Molecular Microbiology **36**:762-771.

26. **Tonjum, T., D. B. Welty, E. Jantzen, and P. L. Small**. 1998. Differentiation of Mycobacterium ulcerans, M-marinum, and M-haemophilum: Mapping of their relationships to M-tuberculosis by fatty acid profile analysis, DNA-DNA hybridization, and 16S rRNA gene sequence analysis. Journal of Clinical Microbiology **36**:918-925.

27. **van der Werf, T. S., T. Stinear, Y. Stienstra, W. T. A. van der Graaf, and P. L. Small**. 2003. Mycolactones and Mycobacterium ulcerans disease. Lancet **362**:1062-1064.

28. **Yeboah-Manu, D., T. Bodmer, E. Mensah-Quainoo, S. Owusu, D. Ofori-Adjei, and G. Pluschke**. 2004. Evaluation of decontamination methods and growth media for primary isolation of Mycobacterium ulcerans from surgical specimens. J.Clin.Microbiol. **42**:5875-5876.

Chapter VIII: Comparative Nucleotide Sequence Analysis of Polymorphic Variable-Number Tandem-Repeat Loci in *Mycobacterium ulcerans*

Anthony Ablordey,[1] Markus Hilty,[2] Pieter Stragier,[1] Jean Swings,[3,4] and Françoise Portaels[1]

Mycobacteriology Unit, Institute of Tropical Medicine, B-2000 Antwerp, Belgium,[1]
Swiss Tropical Institute, 4002, Basel, Switzerland,[2]
Laboratory of Microbiology,[3] BCCM/LMG Culture Collection, University Ghent, B-9000 Ghent, Belgium[4]

Published in *Journal of Clinical Microbiology* 2005 Oct;43(10):5281-4

Abstract

We analyzed a set of variable-number tandem-repeat (VNTR) loci to assess their nucleotide sequence diversity in isolates of three *Mycobacterium ulcerans* genotypes. Sequence variants in two loci resulted in intraspecies resolution of Southeast Asian and Asian genotypes in contrast to a homogenous sequence composition among African isolates. Nucleotide sequence polymorphism in repeat units can enhance discrimination of VNTR loci.

Mycobacterium ulcerans causes Buruli ulcer, a necrotizing skin disease in tropical and subtropical regions (4, 10). The epidemiology of Buruli ulcer is poorly understood, due in part to the highly restricted genetic diversity in *M. ulcerans*, especially among isolates with common geographic origins (1, 2, 5, 6, 11, 13, 14), and also to the difficulty in obtaining cultures from environmental specimens (1, 10).

Tandem-repeat (TR) loci have enormous potential as highly evolving genomic regions suitable for typing species with low genetic diversity. Their use in molecular epidemiology studies have contributed significantly to the identification of sources of infection, a better understanding of disease transmission, and strain-trait correlations (8, 9, 12).

To investigate the potential of TRs in providing highly discriminatory markers for studying molecular diversity in *M. ulcerans*, we demonstrated allele-length polymorphism associated with nine variable-number tandem-repeat (VNTR) loci. This allowed inter- and intraspecies differentiation in a representative collection of *Mycobacterium marinum* and *M. ulcerans* (2). Intraspecies discrimination in *M. ulcerans* was, however, limited among isolates within the same geographic region (2). Different isolates from Africa, Southeast Asia, or Asia could not be distinguished by allele-length analysis, after PCR amplification of nine VNTR loci. Such isolates were also not distinguished by multilocus sequence typing (15), mycobacterial interspersed repetitive unit-VNTR typing (16), and IS*2404*-restriction fragment length polymorphism typing (5).

In this study, we carried out a comparative sequence analysis of the VNTR loci to further assess the contribution of nucleotide sequence polymorphism to allelic diversity in isolates belonging to the African, Southeast Asian and Asian *M. ulcerans* genotypes. The investigation involved sequence analysis of nine VNTR loci in three isolates (including sequence strain) belonging to the African genotype, and four loci (8, 9, 18, and 19) in isolates of the Asian and Southeast Asian type (Table 1). The African isolates were of Angolan, Beninese, and Ghanaian (sequence strain) origins. The Southeast Asian genotype comprises isolates of Australian, Papua New Guinean, and Malaysian origins, while isolates from Japan and China formed the Asian genotype. All *M. ulcerans* isolates were subcultured (from frozen stocks of the collection of the Institute of Tropical Medicine, Antwerp [ITM]) onto Löwenstein-Jensen medium and incubated at 32°C for 4 weeks. Isolates were further characterized phenotypically and tested for the presence of IS*2404* and IS*2606* insertion sequences as previously described (13, 18).

TR loci were bioinformatically identified by applying the TR Finder algorithm on *M. marinum* genome sequences (available *M. ulcerans* genome sequences not accessible for TR

Finder analysis), which also generated a consensus pattern for each locus. Details of TR discovery, DNA extraction, PCR primers, and amplification conditions have been previously described (2). Purified PCR products were sequenced by using the ABI 310 genetic analysis system.

For each locus, TR sequences of the different isolates were aligned and compared with the consensus pattern. All nine loci were found to consist of heterogeneous arrays of repeat units (variants) with deletions and/or nucleotide substitutions (Table 2). Locus 8 was the most conserved in both species, with no nucleotide deletion and two substitutions in sequence variants among all *M. ulcerans* isolates.

For each locus, the individual repeat variants were assigned designations (Table 2). While some repeat variants were found exclusively either in *M. ulcerans* (e.g., G_{19}, H_{19}, or D_{18}) or in *M. marinum* (e.g., A_{18} or B_{19}), others variant occurred in both species (e.g., A_8 or A_9).

Sequence profiles were generated at each locus for the isolates by combining these designations. Comparison of the sequence profiles (which defines an allele at a given locus) facilitates the identification of sequence types (Table 3).

Among the African isolates, corresponding loci featured 100% TR sequence identity; consequently, intraspecies differentiation within this genotype was not possible. Among Southeast Asian isolates, nucleotide sequence homology was complete in all except for loci 9 and 18, for which point mutations resulted in different allelic states. In locus 9, a single-nucleotide deletion in a repeat variant in the Malaysian isolate (ITM 94-1328, with profile $A_9A_9C_9$) differentiated it from the Australian isolate (ITM 94-1324) and Papua New Guinean isolate (ITM 94-1331), both with the $A_9A_9D_9$ sequence profile. Each of the isolates, however, harboured a unique sequence variant at locus 18 (D_{18}, C_{18}, and E_{18}, respectively, for isolates ITM 94-1328, ITM 94-1324, and ITM 94-1331), permitting the complete resolution of the Southeast Asia genotype. Locus 18 also resolved the Asian type into China and Japan genotypes (Table 3).

Although polymorphism at TR loci can occur either as a result of variation in the number of repeat units (length polymorphism) or as a result of nucleotide sequence changes between individual repeat units (sequence polymorphism) (12), the practical ease and lower cost of analyzing length polymorphism (by agarose gel electrophoresis) over sequencing have promoted the use of the former approach for routine typing purposes. Few studies on sequence polymorphism in TR loci have yielded mixed results. While some studies have indicated incremental gain in strain discrimination when length polymorphism data were complemented with sequence analysis (3, 7), this has not been realized in others (9, 17).

In this study, we showed the occurrence of sequence polymorphism in two TR loci, which exhibit no length polymorphism among isolates of two *M. ulcerans* genotypes. A general trend of TR sequence conservation in isolates from the same geographic region was noticed. This was most pronounced among the African isolates, which displayed complete sequence homology across the nine VNTR loci. Consistent with previous data (1, 2, 5, 6, 11, 14-16), the lack of sequence variants in this investigation further emphasizes the clonal homogeneity and recent evolutionary origin and distribution of the African genotype (15).

In contrast, sequence analysis revealed three Southeast Asian alleles and two alleles within the Asian genotype. Notably, the discrimination of these genotypes corroborates the data from IS*2404*-Mtb2 PCR (which differentiates between the isolates from China and Japan and also among the three Southeast Asian isolates) (1) and 2426 PCR (14), which discriminates among the Southeast Asian but not between the Asian isolates. Isolates of these two genotypes show limited differences in their repetitive-sequence-based PCR profiles. Differences in their VNTR sequence profiles therefore are significant in further highlighting differences among these isolates. A combination of the sequence and length polymorphism data results in a total of 11 *M. ulcerans* alleles compared to 8 indexed by length polymorphism analysis alone and 10 alleles by IS*2404*-Mtb2 PCR on the same set of isolates. The conservation of TR loci in the two *Mycobacterium* species and with much sequence degeneration in *M. ulcerans* is consistent with the proposed origin of *M. ulcerans* from *M. marinum* through a reductive genome evolution (15).

Sequence polymorphisms among *M. ulcerans* isolates involved single-nucleotide substitutions and microdeletions. For clonal organisms, and also across VNTR loci, such point mutations are often not considered major sources of genetic variation among isolates. However, data accruing from whole-genome sequence analyses of a number of organisms and also from sequence analysis of several genetic markers indicate that even in highly clonal species like *Mycobacterium tuberculosis*, *Bacillus anthracis*, and *Yersinia pestis*, many thousands of point mutations can be discovered when large portions of genomes are investigated (8). This theme is thus further reinforced by sequence data from this investigation. Complementation of sequence and length polymorphism data should potentially increase the discriminatory power of the VNTR-typing method. It is envisaged that this approach would be more useful for genotyping *M. ulcerans* and other highly monomorphic species.

Acknowledgments

This work was partly supported by grants from the Fund for Scientific Research, Flanders, Belgium (F.W.O.-Vlaanderen, contract no. G.0301.01 and G.0471.03N). A.A. was supported by a grant from the Damien Foundation (Brussels, Belgium).

We thank Pim de Rijk, Krista Fissette, and Cécile Uwizeye for the excellent technical work.

Table 1: VNTR profiles of *M. ulcerans* and *M. marinum*

Species	Isolates[a]	Origin	VNTR Allelic Profile (by locus no.)									VNTR/MLST/IS2404 RFLP[b] Type	MIRU[c]-VNTR Type
			1	4	6	8	9	14	15	18	19		
M. ulcerans	ITM 94-1324	Australia	1	2	1	3	3	1	1	1	2	South East Asian	Asian
	ITM 94-1328	Malaysia	1	2	1	3	3	1	1	1	2	South East Asian	Asian
	ITM 94-1331	Papua N.Guinea	1	2	1	3	3	1	1	1	2	South East Asian	Asian
	ITM 98-912	China	1	2	2	3	4	3	1	2	4	Asian	Asian
	ITM 8756	Japan	1	2	2	3	4	3	1	2	4	Asian	Asian
	ITM 97-658	Angola	1	1	1	3	2	1	1	1	2	African	African
	ITM 97-104	Benin	1	1	1	3	2	1	1	1	2	African	African
	Seq.strain	Ghana	1	1	1	3	2	1	1	1	2	African	African
	ITM 842	Surinam	2	1	1	1	2	2	2	1	3		
M.marinum	Seq. strain		5	4	5	2	3	4	3	2	9		

[a] The profile of the Surinam type was included to indicate polymorphism, at loci 1, 8, and 15. ITM, Institute of Tropical Medicine.

[b] MLST, multilocus sequence typing (15); RFLP, restriction fragment length polymorphism (5).

[c] MIRU, mycobacterial interspersed repetitive unit (16).

Table 2: Multiple sequence alignment of repeat unit[a]

Locus	Sequence	Variant	Species Occurring
1	ATCGCCCGACTCCTCCTCCGGCCTCACCGGCCGGTATCGTCGCCGCGCACCACCCCA	A1	MM
	..................................C.............---------	B1	MU
	..................................C......................	C1	MM
	..........................T..............................	D1	MM
	..........................T.....................---------	E1	MM
4	GGTCGCCTCGCTCCCATCACTCGCCAAGCTCGCTCTGCTCGCTCGGCTCCCAAACCCAACA	A4	MM
	..........................C..................................	B4	MM
	....GG.....--	C4	MU
	....GG.......	D4	MM
	....---------	E4	MM
6	GTGGTGGTCGCGAAACCGGCGAAGCCGGGCGAAGCGGGCCACCACCGACAAGCCCC	A6	MM
	....----------------	B6	MM
	..........T.............................----------------	C6	MU
	----------------.......................................	D6	MM
8	AGTGGTGACCGCCAGCGCGGCGGGGAGCCGGGCGCAGCGGGTCGCCACCATCAAATCC	A8	MM/MU
	................A...	B8	MU
	......................A...................................	C8	MU
9	GTGGCGATCGCAAGCGCGGCCCAGCCGGGGGCAGCGGGTCGCCACCAAGGTGGCGGC	A9	MM/MU
	....---------------	B9	MM
	..........T....T.............---------.T....-------------	C9	MU
	..........T....T.............---------.T....-------------	D9	MU
	..........G.....T..	E9	MU
	.G........T....T.............---------......-------------	F9	MU
14	GCCCTCGGTCGCGACCCGCCGCGCCCGGCTCCGCCGCGCTCGCGATCGCTCCAC	A14	MM
	...................................A..................	B24	MM
	...........................---------------------------	C14	MM
	.A.........................---------------------------	D14	MU
15	AGCCGGCTCCGCTCAGCCGGCTCCGGCTCAATTCGCCGACTTCGCTCGCCGGCC	A15	MM
	......................--------------------------------	B15	MM
	..A...................--------------------------------	C15	MU
18	CCGGTTCCCCCGGTATCACCAGTACCGCTCCCCGTACCACCCGTATCACCGGTACCGCCGCTC	A18	MM
	...T.G...C.........	B18	MU
	..TT.G............CGGCACC......................TGGC..C.........	C18	MU
	.GT.AC...........CGGCAC........................TGGC.ATGGTGGTG..	D18	MU
	.GTT.G...TGGC.ATGGT-G....	E18	MU
	.G..GA.......GG..C.GG..A........GTG.......C....CTG...CTG.TG.TGA	F18	MU
	.G..GA...........C.GG..C.G.....GGTG.......C....CTG...C.G.TG....	G18	MU
	...TAG....C.TGG.G.TA..GG...A.....A....GAA.CGG.G.....G..G.---..T	H18	MU
	...T.G....A.TGG.G.T...G....A.....A....GAA.CGG.G.....G..G.---..T	I18	MU
19	GGGGATCGCAAGCCCGGCGACGCCGGGCGCCGCGGGTCACCACCAACAATTCCCGC	A19	MM
	....G.......	B19	MM
	....T	C19	MM
	....T...C......	D19	MM
	......................................G......GA..-------	E19	MM
	...............................A......G......T...C......	F19	MU
	---------------------------------.....G......T...C......	G19	MU
	---------------------------------.....G......-----------	H19	MU
	.........GC....................A.................G......	I19	MU
	....C......	J19	MU
	......................................G......-----------	K19	MU

[a] -, base deletion; ., identical nucleotide position; MM, *M. marinum*; MU, *M. ulcerans*.

Table 3: Sequence profiles of *M. ulcerans* isolates and the *M. marinum* sequence strain

Locus 1	Sequence profile	Locus 4	Sequence profile	Locus 6	Sequence profile
M. marinum(Seq. Strain)	$A_1 C_1 C_1 D_1 E_1$	*M. marinum*(Seq. Strain)	$A_4 B_4 D_4 E_4$	*M. marinum*(Seq. Strain)	$A_6 A_6 A_6 B_6 D_6$
M. ulcerans (Seq. Strain)	B_1	*M. ulcerans* (Seq. Strain)	C_4	*M. ulcerans* (Seq. Strain)	C_6
ITM 96-658	B_1	ITM 96-658	C_4	ITM 96-658	C_6
ITM 97-104	B_1	ITM 97-104	C_4	ITM 97-104	C_6
Locus 8		**Locus 9**		**Locus 14**	
M. marinum(Seq. Strain)	$A_8 A_8$	*M. marinum*(Seq. Strain)	$A_9 A_9 B_9$	*M. marinum*(Seq. Strain)	$A_{14} A_{14} B_{14} C_{14}$
M. ulcerans (Seq. Strain)	$A_8 A_8 B_8$	*M. ulcerans* (Seq. Strain)	$A_9 C_9$	*M. ulcerans* (Seq. Strain)	D_{14}
ITM 96-658	$A_8 A_8 B_8$	ITM 96-658	$A_9 C_9$	ITM 96-658	D_{14}
ITM 97-104	$A_8 A_8 B_8$	ITM 97-104	$A_9 C_9$	ITM 97-104	D_{14}
ITM 1324	$A_8 A_8 B_8$	ITM 1324	$A_9 A_9 D_9$		
ITM 1328	$A_8 A_8 B_8$	ITM 1328	$A_9 A_9 C_9$		
ITM 1331	$A_8 A_8 B_8$	ITM 1331	$A_9 A_9 D_9$		
ITM 8756	$A_8 C_8 C_8$	ITM 8756	$A_9 A_9 E_9 F_9$		
ITM 98-912	$A_8 C_8 C_8$	ITM 98-912	$A_9 A_9 E_9 F_9$		
Locus 15		**Locus 18**		**Locus 19**	
M. marinum(Seq. Strain)	$A_{15} A_{15} B_{15}$	*M. marinum*(Seq. Strain)	$A_{18} A_{18}$	*M. marinum*(Seq. Strain)	$A_{19} B_{19} B_{19} B_{19} C_{19} C_{19} D_{19} E_{19}$
M. ulcerans (Seq. Strain)	C_{15}	*M. ulcerans* (Seq. Strain)	B_{18}	*M. ulcerans* (Seq. Strain)	$F_{19} G_{19} H_{19}$
ITM 96-658	C_{15}	ITM 96-658	B_{18}	ITM 96-658	$F_{19} G_{19} H_{19}$
ITM 97-104	C_{15}	ITM 97-104	B_{18}	ITM 97-104	$F_{19} G_{19} H_{19}$
		ITM 1324	C_{18}	ITM 1324	$F_{19} H_{19}$
		ITM 1328	D_{18}	ITM 1328	$F_{19} H_{19}$
		ITM 1331	E_{18}	ITM 1331	$F_{19} H_{19}$
		ITM 8756	$F_{18} H_{18}$	ITM 8756	$F_{19} I_{19} J_{19} K_{19}$
		ITM 98-912	$G_{18} I_{18}$	ITM 98-912	$F_{19} I_{19} J_{19} K_{19}$

References

1. **Ablordey, A., R. Kotlowski, J. Swings, and F. Portaels.** 2005. PCR amplification with primers based on IS*2404* and GC-rich repeated sequence reveals polymorphism in *Mycobacterium ulcerans*. J. Clin. Microbiol. **43:**448-450.[Abstract/Free Full Text]

2. **Ablordey, A., J. Swings, C. Hubans, K. Chemlal, C. Locht, F. Portaels, and P. Supply.** 2005. Multilocus variable-number tandem repeats typing of *Mycobacterium ulcerans*. J. Clin. Microbiol. **43:**1546-1551.[Abstract/Free Full Text]

3. **Amonsin. A., L. L. Li, Q. Zhang, J. P. Bannantine, A. S. Motiwala, S. Sreevatsan, and V. Kapur.** 2004. Multilocus short sequence repeat sequencing approach for differentiating among *Mycobacteriun avium* subsp *paratuberculosis* strains. J. Clin. Microbiol. **42:**1694-1702.[Abstract/Free Full Text]

4. **Asiedu, K., R. Scherpbier, and M. Raviglione.** 2000. Executive summary, p. 1-4. *In* W.H.O./CDS/CPE/GBUI/2000.1. Buruli ulcer infection: *Mycobacterium ulcerans* infection. World Health Organization, Geneva, Switzerland.

5. **Chemlal, K., K. de Ridder, P. A. Fonteyne, W. M. Meyers, J. Swings, and F. Portaels.** 2001. The use of *IS2404* restriction fragment length polymorphisms suggests the diversity of *Mycobacterium ulcerans* from different geographic areas. Am. J. Trop. Med. Hyg. **64:**270-273.[Abstract/Free Full Text]

6. **Chemlal, K., G. Huys, P. A. Fonteyne, V. Vincent, A. G. Lopez, L. Rigouts, J. Swings, W. M. Meyers, and F. Portaels.** 2001. Evaluation of PCR-restriction profile analysis and *IS2404* restriction fragment length polymorphism and amplified fragment length polymorphism fingerprinting for identification and typing of *Mycobacterium ulcerans* and *Mycobacterium marinum*. J. Clin. Microbiol. **39:**3272-3278.[Abstract/Free Full Text]

7. **Frothingham, R.** 1995. Differentiation of strains in *Mycobacterium tuberculosis* complex by DNA sequence polymorphisms, including rapid identification *of M. bovis* BCG. J. Clin. Microbiol. **33:**840-844.[Abstract]

8. **Keim, P., M. N. van Ert, T. Pearson, A. J. Vogler, L. Y. Huynh, and D. M. Wagner.** 2004. Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales. Infect. Genet. Evol. **4:**205-213.[CrossRef][Medline]

9. **Overduin, P., L. Schouls, P. Roholl, A. van der Zanden, N. Mahmmod, A. Herrewegh, and D. van Soolingen.** 2004. Use of multilocus variable-number tandem repeat analysis for typing *Mycobacterium avium* subsp. *paratuberculosis*. J. Clin. Microbiol. **42:**5022-5028.[Abstract/Free Full Text]

10. **Portaels, F.** 1995. Epidemiology of mycobacterial diseases. Clin. Dermatol. **13:**207-222.[CrossRef][Medline]

11. **Portaels, F., P. A. Fonteyne, H. de Beenhouwer, P. de Rijk, A. Guédénon, J. Hayman, and W. M. Meyers.** 1996. Variability in the 3' end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to the geographic origin of isolates. J. Clin. Microbiol. **34:**962-965.[Abstract]

12. **Roring, S., A. Scott, D. Brittain, I. Walker, G. Hewinson, S. Neill, and R. Skuce.** 2002. Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing tandem repeats and spoligotyping. J. Clin. Microbiol. **40:**2126-2132.[Abstract/Free Full Text]

13. **Stinear, T. P., J. K. Davis, L. Marino, R. M. Robin-Browne, F. Oppedisano, A. Sievers, and P. D. R. Johnson.** 1999. Identification and characterization of IS*2404* and IS*2606*: two distinct repeated sequences for detection of *Mycobacterium ulcerans* by PCR. J. Clin. Microbiol. **37:**1018-1023.[Abstract/Free Full Text]

14. **Stinear, T. P., J. K. Davis, G. A. Jenkins, F. Portaels, B. C. Ross, F. Oppedisano, M. Purcell, J Hayman, and P. D. R. Johnson.** 2000. A simple PCR method for rapid genotype analysis of *Mycobacterium ulcerans*. J. Clin. Microbiol. **38:**1482-1487.[Abstract/Free Full Text]

15. **Stinear, T. P., G. A. Jenkins, P. D. R J. Johnson, and J. K. Davis.** 2000. Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. J. Bacteriol. **182:**6322-6330.[Abstract/Free Full Text]

16. **Stragier, P., A. Ablordey, W. M. Meyers, and F. Portaels.** 2005. Genotyping *Mycobacterium ulcerans* and *Mycobacterium marinum* by using mycobacterial interspersed repetitive units. J. Bacteriol. **187:**1639-1647.[Abstract/Free Full Text]

17. **Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht.** 2000. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol. Microbiol. **39:**3563-3571.

18. **Vincent Levi-Frebault, V., and F. Portaels.** 1992. Proposed minimal standards for the genus mycobacterium and description of new slowly growing mycobacteria species. Int. J. Syst. Bacteriol. **42:**315-323.[Abstract]

Chapter IX: General discussion and conclusions

9.1 Abstract

The aim of this section is to review the studies whose evaluations of the discriminatory power of the three popular typing methods for *M. tuberculosis* complex (MTC) strains reach the conclusions that IS*6110* RFLP has a higher discriminatory power than MIRU-VNTR which itself is more discriminative than spoligotyping. In contrast, we additionally show that spoligotyping can be more discriminative than MIRU-VNTR typing, depending on the geographical location and genotype family chosen. Furthermore, we review that these genotyping tools are stable enough over time thus justifying their usage although possible convergent evolution and little heterogeneity may require the use of several different and appropriate genotyping tools to exclude biases. This is particularly recommended in high incidence countries where heterogeneity within strains is low and convergent evolution more probable.

We also review the importance of factors such as the awareness of reinfection versus relapse and mixed infections versus microevolution of a single strain to human tuberculosis control, especially in high incidence, African countries. Furthermore, knowledge about the degree and risk factors of ongoing tuberculosis transmission could reveal new target groups and result in suggesting an improved Direct Observed Treatment Short course (DOTS). However, adapting DOTS to distinct population groups cannot be suggested without knowing the variability of locally perceived tuberculosis. Therefore the linking of social science with molecular epidemiological studies is suggested.

The same molecular epidemiological methods prove additionally useful in investigating MTC transmission within or between different animal species. In the case of *M. bovis*, some animals were reviewed to act as maintenance and spillover hosts, respectively. The knowledge of the natural reservoirs of *M. bovis* is important as it has potential to be transmitted to humans. However, so far, no *M. bovis* has been found in human samples of Chad and there are several possible explanations for this discussed.

We conclude this chapter with a short overview of the genotyping of a different mycobacterium, *M. ulcerans.* In contrast to tuberculosis much less is known about the transmission of Buruli Ulcer, the disease caused by *M. ulcerans*, but a new VNTR typing may be very promising for micro epidemiological studies in the future.

9.2 Features of molecular epidemiological typing tools

9.2.1 Discriminatory power of IS6110 RFLP, spoligotyping and MIRU-VNTR

Today, a large number of genotyping tools for the fingerprinting of MTC exists, of which IS*6110*-RFLP, spoligotyping and MIRU-VNTR are very commonly used. Recently, the discriminatory power of these tools has been evaluated on representative strain collections from different geographical regions. In a test panel of 90 *M. tuberculosis* complex strains from 38 countries, the discriminatory power of the IS6110-RFLP was shown to be the most discriminatory tool compared to spoligo- and MIRU-VNTR typing (25). A different study showed MIRU-VNTR to be more discriminative than spoligotyping in a strain collection containing 90 *M. tuberculosis* complex and 10 non-*M. tuberculosis* complex strains, as well as 31 duplicated DNA samples (24).

However, although such evaluation of the discriminatory power on representative strain collections is valid, results can vary if evaluations focus on strains from only one geographical area or on a specific member of the MCT. When analyzing the Beijing strain of *M. tuberculosis*, spoligotyping has a lower discriminatory power compared to the other typing tools, because spoligospacers 1-34 are naturally deleted (14). However, for the Cameroon family strains from Chad, MIRU-VNTR typing is slightly less discriminative than spoligotyping (9). Strains of the Cameroon family have mainly been isolated in Cameroon, Chad and Nigeria. Characteristic chromosomal deletions are described and strains furthermore unequally lack spacers 23-25 in their spoligotyping patterns.

Evaluation of the discriminatory power of *Mycobacterium bovis* of Ireland revealed a higher allelic diversity of MIRUs compared to spoligotyping (0.69 vs. 0.74) (33). In contrast, allelic diversity for *M. bovis* strains from our study from Chad were 0.75 and 0.79, for MIRUs and spoligotyping, respectively (18).

These results show that the standardization of a molecular genotyping approach, which is valid for all MTC members, is difficult. Therefore evaluating of the discriminatory powers within strains of the same geographical area or within the same strain family before performing molecular epidemiological studies is recommended.

9.2.2 Molecular clock

Besides the ability to discriminate between strains, typing tools also have to be robust enough to show which strains belong to the same cluster. This is also dependent on the molecular clock of the typing tools and should be taken into consideration when using a typing tool. The

faster a molecular clock the more discriminative it is if horizontal gene transfer is excluded. In various recent studies, molecular clocks have been evaluated.

- IS6110 RFLP vs. spoligotyping

An analysis of 165 serial *M. tuberculosis* isolates obtained from 56 patients revealed that 5 (9%) were infected with isolates with changes in their IS*6110* fingerprint patterns but no changes were observed for spoligotyping. A statistically significant correlation could be found between changes in insertion sequence (IS) patterns and the increased time intervals over which the isolates were obtained (29). In a different study, based on serial isolates spanning for the most part <3 months, the half-life for a change in IS6110-RFLP was extrapolated to be 3.2 years (95% confidence interval, 2.1–5.0) (8). In *M. tuberculosis* strains from South Africa evolutionary changes were observed in 4% of the strains, and a half-life ($t_{1/2}$) of 8.74 years was calculated, assuming a constant rate of change over time. This rate may be composed of a high rate of change seen during the early disease phase (t $(_{1/2})$ 0.57 years) and a low rate of change seen in the late disease phase (t $(_{1/2})$ 10.69 years). The early rate probably reflects change occurring during active growth prior to therapy, while the low late rate may reflect change occurring during or after treatment (42).

- MIRU-VNTR vs. IS6110

To assess the temporal stability of MIRUs, 123 serial isolates belonging to a variety of distinct IS*6110* restriction fragment length polymorphism (RFLP) families were genotyped and separated by up to 6 years. All 12 MIRU VNTR loci were completely identical within the groups of serial isolates in 55 of 56 groups (98.2%), although 11 pairs of isolates from the same patients with conserved MIRU VNTRs displayed slightly different IS*6110* RFLP profiles. These results indicate that MIRU VNTRs are relatively stable over time (34).
These studies show that the present genotyping tools are stable enough over time and their use therefore justified.

- Large genomic deletions received by micro array analysis

Recently, large genomic deletions of *M. tuberculosis* (5, 20, 38) and their use for genome level informed PCR (GLIP) (28, 31) and deligotyping (16) have been presented. The deletions are supposed to happen unidirectional and are researched with the microarray technique (22, 27, 28). Three different types of large genomic deletions were distinguished (5). The first type describes the deletion of mobile genetic elements (prophages and insertion sequences) and the second and third the *IS6110* and non repetitive mediated deletions, respectively. Research into the presence of large sequence deletions resulted in the definition

of epidemiologically important clones (22, 28, 31). Furthermore, the deletions often hamper or delete potential ORF and might therefore affect the feature of the pathogen.
The non repetitive mediated deletions (Type 3) are also called diagnostic deletions and allow an isolate to be unequivocally placed in a strain family. The value of genomic deletions for finding large family groups is considered to be superior as the turnaround time is slower than for all other genotyping methods. Based on the assumption that all stains of a strain family represent a cluster, the further use of genotyping methods leads to more refinement and eventual subclustering.

9.2.3 Low heterogeneity and Convergence: the need for higher discriminatory power

- Adding of VNTRs

Comparison of our data from Chad with Ireland (18, 33) showed a low discriminatory power of MIRUs compared to similar studies on *M. tuberculosis*. However, the VNTR typing was shown to become more discriminative when adding further VNTRs e.g. exact tandem repeats (ETRs) to the 12 MIRUs. Adding ETR A, B, and C resulted in higher discrimination than the use of all 12 MIRUs combined. Therefore a combination of different types of VNTRs is suggested (18, 33).

- Convergence in VNTR typing because highly polymorphic loci lower sensitivity of clustering

VNTR typing of MTC strains from different settings have shown different allelic diversities at the VNTR loci. However, MIRU 40 and 26 seem to be very polymorphic for *M. tuberculosis*. MIRU 26 is also polymorphic for *M. bovis* with, in contrast, ETR A and B much more diverse than MIRU 40 (18).
The higher turnaround time of certain VNTR loci can result in a convergent evolution of epidemiologically unlinked strains and therefore lower the sensitivity of clustering which means that certain strains found in Nigeria (6) have the same VNTR type as strains from Chad and convergence cannot be excluded. However, this problem can best be addressed by the inclusion of a second genotyping method, e.g. spoligotyping and a sensitivity of clustering of mostly 100% is common.

- Convergence Spoligotyping

Possible convergent evolution of spoligotyping has also been shown. Homologous recombination between adjacent IS*6110* elements leads to extensive deletion in the DR region, again demonstrating a dependent evolutionary mechanism. Different isolates from the

same strain family and isolates from different strain families were observed to converge to the same spoligotype pattern (41).

9.3 Practical usage of molecular epidemiological results with special consideration of Africa

In recent years, molecular typing of the *M. tuberculosis* complex has greatly increased our knowledge about the mode of disease transmission. Molecular typing can help in suggesting adapted control strategies and potentially tackles and provides links to many important topics related to the disease.

9.3.1 Reinfection versus relapse or mixed infection versus micro evolution: the 'correct' diagnosis

A study from South Africa showed that 19% of all patients were simultaneously infected with Beijing and non-Beijing strains, and 57% of patients infected with a Beijing strain were also infected with a non-Beijing strain. These results suggest that multiple infections are frequent, implying high reinfection rates and an absence of efficient protective immunity conferred by the initial infection (43). Because Cameroon family strains are so predominant in our study (9) this raises the question, whether these particular *M. tuberculosis* strains play a similar role in Chad, Cameroon and Nigeria. Multiple infections with Cameroon and non-Cameroon family strains could be investigated in a similar manner by a 2 strain specific PCR (9).

A different study of clinical data, also using samples from South Africa, suggests that first-line therapy can select for a resistant subpopulation, whereas poor adherence or second-line therapy resulted in the re-emergence of the drug-susceptible subpopulations (40). This is important for treatment control.

However, care must be taken not to confuse mixed infection with microevolution of a single strain. While a classic mixed infection consists of 2 or more completely different strains (different strain families) a heterogeneous mycobacterium population structure can also evolve because of a mutation of one infectious agent in the reproduction process within the same host.

In a recent study investigating mixed infections, the MIRU-VNTR technique was applied to search for cases infected by more than one clone. Clonal variants within the same host were detected in 3 out of 115 cases (2.6%), including cases with clones which were indistinguishable by restriction fragment length polymorphism or spoligotyping. In one case, coinfecting clonal variants differed in antibiotic susceptibilities (15).

In another study, infections with different bacterial subpopulations were detected in samples from eight patients (8.2%), with the frequency of detectable mixed infections in the study population estimated to be 2.1%. Genotypic variations were found to be independent of drug susceptibility, and the various molecular markers evolved independently in most cases (35).
In conclusion, we suggest that MIRU-VNTR typing is a potentially valuable tool to investigate reinfection, mixed infection, microevolution or the relapse of tuberculosis. These results may prove especially important for adapted human tuberculosis control in high incidence countries such as Chad

9.3.2 Degree of ongoing transmission, global mycobacterial population structure and outbreak investigations

While the basic principles of tuberculosis transmission are well understood, the advent and use of molecular methods in epidemiological studies have shown that traditional contact tracing may not always be accurate, leading to identification of previously unrecognized source cases. The assessment of recent transmission and/or reactivation (44) remains of particular interest in resource poor settings where scarce resources for control need to be directed to transmission hotspots. In Chad, the overall heterogeneity using spoligotyping and MIRU/ETR typing was high in 40 *M. tuberculosis* samples analyzed, however we also found that a substantial proportion of strains (33 %) are part of the Cameroon family. Within this family, strains evolved differently and therefore have slightly different VNTR and/or spoligotypes (9). We propose that evolved strains, derived from a common ancestor should be considered together with clustered strains to represent chains of ongoing transmission. In a recent study, a data set which identifies newly evolved strains has been generated. Inclusion of these evolved strains into various molecular epidemiological calculations significantly increases the ability to estimate ongoing transmission in a particular high incidence study setting (39).
In our study the Cameroon family was the most predominant group of *M. tuberculosis* strains which is also most common for Nigeria, Cameroon and Chad (9). In the future, investigation into the reasons why some strains are predominant may clarify which bacterial factors contribute to disease. This knowledge has the potential to influence control and prevention strategies for tuberculosis (26). Predominant strains should also be identified in developing countries to study the differential pathogenesis between strains (12) and also to reveal the true extent of genetic diversity of the pathogen (4).

Additionally, genotyping studies could also enhance the investigation of outbreaks. In a study of a nosocomial outbreak of multidrug-resistant tuberculosis caused by Mycobacterium bovis in 31 patients, 30 of whom were also infected with human immunodeficiency virus, all 31 died of progressive tuberculosis. All *M. bovis* strains had identical spoligotyping patterns and showed resistance to 12 antituberculosis drugs. Reinfection was suggested in 11 cases and confirmed in 4 by molecular typing methods (32). In 2001 the largest recognized outbreak of tuberculosis in a United Kingdom school was detected in Leicester (31). The index patient was a 14-year-old student who had been complaining of a chronic cough for 9 months prior to being diagnosed with sputum smear-positive cavitary pulmonary tuberculosis (13).
In high incidence countries tuberculosis outbreaks are rarely investigated because of a lack of genotyping facilities, but it is important as it could contribute to innovative control strategies.

9.3.3 Linking epidemiological and social science studies

Within the framework of NCCR North-South, our molecular epidemiological data is linked to a social science study on the perception of tuberculosis by Moustapha Ould Taleb. He found that biomedical tuberculosis symptoms of nomads in Mauritania and Chad correspond to various perceptions of illness categories. For example the terms "Kouha" (cough) and "Soualla" (cough) correspond to perceived hereditary tuberculosis and "Lebroud or Legtoua" to tuberculosis acquired through cold temperatures, nutrition (e.g. powdered sugar is particularly incriminated as a cause in Chad) or hard work (understood here as the hardship of pastoralist work). The modern Arabic medical terminology "Soul" for tuberculosis is unknown to nomads of Mauritania who continue to use names which either correspond to single symptoms such as the cough ("kouha"), fatigue ("Azer") or to stigmatizing images such "Sahat elmoumnin",(the illness of the believers) "kouha Elkahla" (serious cough) etc.
The aims behind joint collaboration are as follows:

a) To evaluate the variability of local perceptions of TB. As local perceptions of illness determine treatment seeking behaviour this is crucial in adapting DOTS to mobile populations.
b) To compare perceptions of TB with prevalence of the disease. Although people associate many terms with suffering from tuberculosis not all are related to clinical diagnosed TB.
c) To investigate the type of perception compared with the clustering of molecular characterized TB strains. This would reveal if certain perceptions are risk factors for clustering and therefore for recent transmission.

d) To evaluate whether certain strains exclusively match certain types of tuberculosis e.g. "Kouha" and "Soualla". If different tuberculosis strains or strain families show slightly different clinical symptoms, this could have far reaching implications for our understanding of the tuberculosis disease.
e) To research whether perceived inherited tuberculosis indicates high ongoing transmission at the household level. As "Kouha" and "Soualla" are considered to be inherited tuberculosis, this probably indicates that tuberculosis transmission often takes place at the household level (M. Ould Taleb personal. communication). Were this to be proven by molecular epidemiology, it would suggest the need for enforced tuberculosis control at the household level.

9.3.4 Inter animal species transmission

Molecular typing methods can clarify the sources of infection and the major routes of the transmission and spread of bovine tuberculosis (TB) and their risk factors (36). They are also necessary for eco-systemic analyses of transmission chains between wildlife and livestock (17). For control polices of eradicating *M. bovis* in cattle, it is important to know whether a wildlife species act as a maintenance or spillover host. A recent study in the UK showed that the culling of badgers reduces cattle TB incidence in the areas where culling takes place, but increases incidence in adjoining areas (11). Additionally, studies have revealed that deer (7, 17), wild boars (17) and brushtail possum (7) also act as maintenance hosts. Other animal species rather act as spillover hosts, for example goats, sheep, rabbits and pigs (7). Today it is assumed that spillover hosts are mainly infected by host adapted *M. bovis* substrains rather than by classical *M. bovis* strains e.g. goats which are infected with *M. bovis* sups. caprae. Therefore, these animal species are considered to contribute to the transmission pathways of the classic *M. bovis* strains to a far lesser extent. In high incidence countries like Chad, the natural reservoir and spillover hosts of the classical *M. bovis* are largely unknown. Future research on different hosts, particularly camels in the Chad setting, is therefore highly recommended.

Future studies may even show *M. bovis* strain preference within different cattle breeds. Preliminary results show that the *M. bovis* population structure of the more tuberculosis susceptible mbororo is slightly more homogenic than of the arabe breed from Chad. This seems to indicate that the breed mbororo is more likely associated with recent transmissions which raises the question if *M. bovis* is better adapted towards this breed (10).

9.3.5 Zoonotic transmission

Last but not least, molecular epidemiology can also investigate the transmission of tuberculosis between animals and humans. *M. bovis* is considered to have zoonotic potential and it is certainly very important to know the percentage of human tuberculosis caused by *M. bovis* especially in high incidence countries (3). In Nigeria the rate of *M. bovis* from humans was 4 *M. bovis* strains from 102 sputum samples (21) and 3 *M. bovis* strains cultured from 55 sputum samples (6). However, spoligo- and VNTR-typing in the latter study revealed that the three *M. bovis* strains isolated from humans did not match the 15 *M. bovis* strains isolated from cattle. This was despite the fact that they all had spoligotyping patterns similar to the dominant pattern found in cattle. This surprising result may be due to the small number of strains analyzed from cattle but it also raises questions about direct animal to human transmission. Similarly, the spoligotypes of 4/5 *M. bovis* strains isolated from humans from Tanzania did not match the types of 31 *M. bovis* strains from Tanzanian cattle (23). In Cameroon, only a single *M. bovis* strain was isolated from 455 human specimens suggesting the low importance of *M. bovis* in the overall burden of human tuberculosis in this country (30).

To prove zoonotic transmission was one of the objectives of this thesis. It was thought that the combination of high prevalence of *M. bovis* in cattle and the habit of drinking unpasteurized milk and eating raw meat presented significant risks for transmission. However, no *M. bovis* has so far been found in human samples of Chad. Here are several possible explanations:

a) <u>Insufficient numbers and incorrect study populations</u>

The numbers of isolated MTC strains from humans and animals from Chad are very few but are the very first ones isolated in this country (9, 10). As the first objective of the thesis of C. Diguimbaye was to establish a mycobacterial laboratory in Chad, attention has not been given to a high number or a careful selection of isolates. Only pulmonary and a few urine samples of human patients from the main hospital of N'Djaména were included (9).

An ongoing study currently additionally analyzes extra pulmonary tuberculosis in children from N'Djaména. However, preliminary results show that *M. bovis* is not present in these samples. In future, the inclusion of extra pulmonary samples of adults is also planned. Additionally, we plan to evaluate the burden of *M. bovis* infection within nomadic pastoralists who have close contact with cattle and are therefore at high risk.

b) <u>Reduced risk of transmission in semi-arid climates and in extensive livestock systems</u>

The sampling of tuberculosis specimens from Chad took place in N'Djaména where the climate is dry and semi-arid. There are indications that zoonotic transmission is more probable in humid areas and therefore inclusion of a more southerly region should be taken into consideration. Furthermore, the extensive livestock systems used by the pastoralists does not involve the use of stables. This may also reduce the risk of zoonotic transmission.

c) Less pathogenic strains

It is known that humans are not natural reservoirs for *M. bovis*, but can act as spillover hosts. However, it is not known whether certain *M. bovis* strains are more pathogenic for humans than others. As no *M. bovis* in humans has been found, this may mean that the Chadian strains are less pathogenic for humans.

9.4 Genotyping in *M. ulcerans*

While there are many studies evaluating genotyping methods for MTC, the situation for *M. ulcerans* is so far not as developed. The discriminatory power of genotyping methods is still insufficient for performing micro epidemiological studies. Therefore new genotyping methods have to be developed and evaluated.

As for MTC, VNTR typing of MIRUs (37) and other VNTRs (2) have been implemented for *M. ulcerans*. Evaluations of the discriminatory power showed the ability to separate isolates on a continental level but not within the contents. A study evaluating the sequences of VNTR loci showed that sequencing does not enhance the discriminatory power (1). It is therefore of special importance that a new VNTR-locus (designated STI) and a previously presented MIRU locus have recently been shown to discriminate between *M. ulcerans* strain within Ghana, and therefore within Africa, for the first time (19).

9.5 Ten key messages and recommendations of this thesis

1. MIRU/VNTR typing for *M. tuberculosis* strains from Chad is as discriminative as spoligotyping. Recommendation: MIRU-VNTR typing is a very promising tool for investigating mixed infection which might occur in high percentages in high incidence countries like Chad (Chapter III).

2. Using microarry analysis, the Cameroon family was identified as a highly prevalent clone and characterized 33 % of *M. tuberculosis* strains of Chad. Fortunately, this clone is not currently associated with drug resistance. Recommendation: Evaluation of risk factors for recent transmissions of Cameroon family members could lead to recommendations on how to

interrupt the transmission of this clone and contribute to alternative control strategies (Chapter III).

3. Although the risk of infection with *M. bovis* is high for humans in Chad, analysis of 40 pulmonary and urine samples of clinical suspected tuberculosis cases revealed that none of the patients were infected by *M. bovis* (Chapter III). Recommendation: Evaluation of extra pulmonary samples from risk population groups (e.g. pastoralists). It is also recommended to look at *M. bovis* as a possible subpopulation of mixed infections in humans.

4. Drug resistance testing of 40 *M. tuberculosis* cases from Chad revealed a high percentage of Isoniazid resistance (33 %) (Chapter III). Recommendation: Carry out molecular epidemiological studies analyzing the risk factors of transmission of drug resistant strains. Implementation of a method permitting faster detection of resistance is also desirable.

5. A study carried out in a slaughter house in Chad revealed that the mbororo breed is more susceptible to *M. bovis* than the arabe breed. Recommendation: Investigate the immunological and genetic consequences (Chapter IV).

6. Spoligo- and MIRU-VNTR typing revealed a remarkably homogenetic population structure within *M. bovis* strains. However, the use of 2 MIRUs and 3 ETRs allows reasonably high discrimination to take place. Recommendation: Use these 5 loci to investigate and prove possible animal to human transmission and other molecular epidemiological objectives in the future (Chapter V).

7. A high proportion of Chadian (63/67), Nigerian and Cameroon *M. bovis* strains are members of the same clone defined by a large genomic deletion. There are indications that subclones found in the different study sites are due to clonal expansion. Recommendation: Investigate the possible spread of *M. bovis* strains from one country to the other (Chapter VI). However, we also recommend looking at different hosts (camels, goats) in order to show if other animal reservoirs (maintenance hosts) exist for this particular *M. bovis* clone.

8. A high proportion of Chadian strains showed deletion affecting ESAT-6 family members, which are promising targets for new diagnostics and novel vaccine candidates. Recommendation: Investigate genome organization across *M. tuberculosis* strains to inform the choice of antigens used in a diagnostic mix (Chapter VI).

9. A new and a previously described VNTR locus revealed genetic diversity within African *M. ulcerans* strains for the first time. Recommendation: Use these two VNTRs for extended analysis on various panels of *M. ulcerans*. However, development of new genotyping tools has to continue as the discriminatory power is still low. This could allow for micro epidemiological studies in the future (Chapter VII).

10. Sequencing of various VNTRs revealed that the discriminatory power of sequencing is only slightly higher than that of Agarose Gel electrophoresis. Recommendation: Given that sequencing does not significantly enhance the discriminatory power, and is more laborious and expensive, it is not recommended for use, especially not within laboratories with infrastructural constraints (Chapter VIII).

9.6 References of conclusion

1. **Ablordey, A., M. Hilty, P. Stragier, J. Swings, and F. Portaels**. 2005. Comparative Nucleotide Sequence Analysis of Polymorphic Variable-Number Tandem-Repeat Loci in Mycobacterium ulcerans. J.Clin.Microbiol. **43**:5281-5284.

2. **Ablordey, A., J. Swings, C. Hubans, K. Chemlal, C. Locht, F. Portaels, and P. Supply**. 2005. Multilocus variable-number tandem repeat typing of Mycobacterium ulcerans. J.Clin.Microbiol. **43**:1546-1551.

3. **Ayele, W. Y., S. D. Neill, J. Zinsstag, M. G. Weiss, and I. Pavlik**. 2004. Bovine tuberculosis: an old disease but a new threat to Africa. Int.J.Tuberc.Lung Dis. **8**:924-937.

4. **Borsuk, S., M. M. Dellagostin, S. G. Madeira, C. Lima, M. Boffo, I. Mattos, P. E. Almeida da Silva, and O. A. Dellagostin**. 2005. Molecular characterization of Mycobacterium tuberculosis isolates in a region of Brazil with a high incidence of tuberculosis. Microbes.Infect. **7**:1338-1344.

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

6. **Cadmus, S., S. Palmer, M. Okker, J. Dale, K. Gover, N. Smith, K. Jahans, R. G. Hewinson, and S. V. Gordon**. 2006. Molecular Analysis of Human and Bovine Tubercle Bacilli from a Local Setting in Nigeria. Journal of Clinical Microbiology **44**:29-34.

7. **Corner, L. A.** 2005. The role of wild animal populations in the epidemiology of tuberculosis in domestic animals: How to assess the risk. Vet.Microbiol.

8. **de Boer, A. S., M. W. Borgdorff, P. E. de Haas, N. J. Nagelkerke, J. D. van Embden, and D. van Soolingen**. 1999. Analysis of rate of change of IS6110 RFLP patterns of Mycobacterium tuberculosis based on serial patient isolates. J.Infect.Dis. **180**:1238-1244.

9. **Diguimbaye C., Hilty M. Ngandolo R**., *et al.* 2006. Molecular characterization and drug resistance testing of *Mycobacterium tuberculosis* isolates from Chad. J.Clin.Microbio. Apr;**44**(4):1575-7

10. **Diguimbaye-Djaibé C., Hilty M., Ngandolo R, Mahamat HH., Pfyffer G. E., Baggi F., Hewinson G., Tanner M., Zinsstag J. and Schelling E.** *Mycobacterium bovis* Isolates from Tuberculous Lesions in Chadian Zebu Carcasses. 2006. Emerg.Infect.Dis. **12**(5):769-771

11. **Donnelly, C. A., R. Woodroffe, D. R. Cox, F. J. Bourne, C. L. Cheeseman, R. S. Clifton-Hadley, G. Wei, G. Gettinby, P. Gilks, H. Jenkins, W. T. Johnston, A. M. Le Fevre, J. P. McInerney, and W. I. Morrison**. 2005. Positive and negative effects of widespread badger culling on tuberculosis in cattle. Nature .

12. **Easterbrook, P. J., A. Gibson, S. Murad, D. Lamprecht, N. Ives, A. Ferguson, O. Lowe, P. Mason, A. Ndudzo, A. Taziwa, R. Makombe, L. Mbengeranwa, C. Sola, N. Rastogi, and F. Drobniewski**.

2004. High rates of clustering of strains causing tuberculosis in Harare, Zimbabwe: a molecular epidemiological study. J.Clin.Microbiol. **42**:4536-4544.

13. **Ewer, K., J. Deeks, L. Alvarez, G. Bryant, S. Waller, P. Andersen, P. Monk, and A. Lalvani**. 2003. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of Mycobacterium tuberculosis infection in a school tuberculosis outbreak. Lancet **361**:1168-1173.

14. **Ferdinand, S., G. Valetudie, C. Sola, and N. Rastogi**. 2004. Data mining of Mycobacterium tuberculosis complex genotyping results using mycobacterial interspersed repetitive units validates the clonal structure of spoligotyping-defined families. Res.Microbiol. **155**:647-654.

15. **Garcia, d., V, R. N. Alonso, S. Andres, M. J. Ruiz Serrano, and E. Bouza**. 2005. Characterization of clonal complexity in tuberculosis by mycobacterial interspersed repetitive unit-variable-number tandem repeat typing. J.Clin.Microbiol. **43**:5660-5664.

16. **Goguet de la Salmoniere YO, C. C. Kim, A. G. Tsolaki, A. S. Pym, M. S. Siegrist, and P. M. Small**. 2004. High-throughput method for detecting genomic-deletion polymorphisms. J.Clin.Microbiol. **42**:2913-2918.

17. **Hermoso, d. M., A. Parra, A. Tato, J. M. Alonso, J. M. Rey, J. Pena, A. Garcia-Sanchez, J. Larrasa, J. Teixido, G. Manzano, R. Cerrato, G. Pereira, P. Fernandez-Llario, and d. M. Hermoso**. 2005. Bovine tuberculosis in wild boar (Sus scrofa), red deer (Cervus elaphus) and cattle (Bos taurus) in a Mediterranean ecosystem (1992-2004). Prev.Vet.Med.

18. **Hilty, M., C. Diguimbaye, E. Schelling, F. Baggi, M. Tanner, and J. Zinsstag**. 2005. Evaluation of the discriminatory power of variable number tandem repeat (VNTR) typing of Mycobacterium bovis strains. Vet.Microbiol. **109**:217-222.

19. **Hilty, M., D. Yeboah-Manu, D. Boakye, E. Mensah-Quainoo, S. Rondini, E. Schelling, D. Ofori-Adjei, F. Portaels, J. Zinsstag and G. Pluschke.** 2006. A new VNTR locus revealing genetic diversity in *Mycobacterium ulcerans* isolates from Ghana. J.Bacteriol. Feb;**188**(4):1462-5

20. **Hirsh, A. E., A. G. Tsolaki, K. DeRiemer, M. W. Feldman, and P. M. Small**. 2004. Stable association between strains of Mycobacterium tuberculosis and their human host populations. Proc.Natl.Acad.Sci.U.S.A **101**:4871-4876.

21. **Idigbe, E. O., C. E. Anyiwo, and D. I. Onwujekwe**. 1986. Human pulmonary infections with bovine and atypical mycobacteria in Lagos, Nigeria. J.Trop.Med.Hyg. **89**:143-148.

22. **Kato-Maeda, M., J. T. Rhee, T. R. Gingeras, H. Salamon, J. Drenkow, N. Smittipat, and P. M. Small**. 2001. Comparing genomes within the species Mycobacterium tuberculosis. Genome Res. **11**:547-554.

23. **Kazwala, R. R., L. J. Kusiluka, K. Sinclair, J. M. Sharp, and C. J. Daborn**. 2005. The molecular epidemiology of Mycobacterium bovis infections in Tanzania. Vet.Microbiol.

24. **Kremer, K., C. Arnold, A. Cataldi, M. C. Gutierrez, W. H. Haas, S. Panaiotov, R. A. Skuce, P. Supply, A. G. van der Zanden, and D. van Soolingen**. 2005. Discriminatory Power and Reproducibility of Novel DNA Typing Methods for Mycobacterium tuberculosis Complex Strains. J.Clin.Microbiol. **43**:5628-5638.

25. **Kremer, K., D. van Soolingen, R. Frothingham, W. H. Haas, P. W. M. Hermans, C. Martin, P. Palittapongarnpim, B. B. Plikaytis, L. W. Riley, M. A. Yakrus, J. M. Musser, and J. D. A. van Embden**. 1999. Comparison of methods based on different molecular epidemiological markers for typing of Mycobacterium tuberculosis complex strains: Interlaboratory study of discriminatory power and reproducibility. Journal of Clinical Microbiology **37**:2607-2618.

26. **Malik, A. N. and P. Godfrey-Faussett**. 2005. Effects of genetic variability of Mycobacterium tuberculosis strains on the presentation of disease. Lancet Infect.Dis. **5**:174-183.

27. **Mostowy, S., D. Cousins, J. Brinkman, A. Aranaz, and M. A. Behr**. 2002. Genomic deletions suggest a phylogeny for the Mycobacterium tuberculosis complex. J.Infect.Dis. **186**:74-80.

28. **Nguyen, D., P. Brassard, D. Menzies, L. Thibert, R. Warren, S. Mostowy, and M. Behr**. 2004. Genomic characterization of an endemic Mycobacterium tuberculosis strain: evolutionary and epidemiologic implications. J.Clin.Microbiol. **42**:2573-2580.

29. **Niemann, S., E. Richter, and S. Rusch-Gerdes**. 1999. Stability of Mycobacterium tuberculosis IS6110 restriction fragment length polymorphism patterns and spoligotypes determined by analyzing serial isolates from patients with drug-resistant tuberculosis. J.Clin.Microbiol. **37**:409-412.

30. **Niobe-Eyangoh, S. N., C. Kuaban, P. Sorlin, P. Cunin, J. Thonnon, C. Sola, N. Rastogi, V. Vincent, and M. C. Gutierrez**. 2003. Genetic biodiversity of Mycobacterium tuberculosis complex strains from patients with pulmonary tuberculosis in Cameroon. J.Clin.Microbiol. **41**:2547-2553.

31. **Rajakumar, K., J. Shafi, R. J. Smith, R. A. Stabler, P. W. Andrew, D. Modha, G. Bryant, P. Monk, J. Hinds, P. D. Butcher, and M. R. Barer**. 2004. Use of genome level-informed PCR as a new investigational approach for analysis of outbreak-associated Mycobacterium tuberculosis isolates. J.Clin.Microbiol. **42**:1890-1896.

32. **Rivero, A., M. Marquez, J. Santos, A. Pinedo, M. A. Sanchez, A. Esteve, S. Samper, and C. Martin**. 2001. High rate of tuberculosis reinfection during a nosocomial outbreak of multidrug-resistant tuberculosis caused by Mycobacterium bovis strain B. Clin.Infect.Dis. **32**:159-161.

33. **Roring, S., A. N. Scott, H. R. Glyn, S. D. Neill, and R. A. Skuce**. 2004. Evaluation of variable number tandem repeat (VNTR) loci in molecular typing of Mycobacterium bovis isolates from Ireland. Vet.Microbiol. **101**:65-73.

34. **Savine, E., R. M. Warren, G. D. van der Spuy, N. Beyers, P. D. van Helden, C. Locht, and P. Supply**. 2002. Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 loci in serial isolates of Mycobacterium tuberculosis. Journal of Clinical Microbiology **40**:4561-4566.

35. **Shamputa, I. C., A. L. Rigouts, and F. Portaels**. 2004. Molecular genetic methods for diagnosis and antibiotic resistance detection of mycobacteria from clinical specimens. APMIS **112**:728-752.

36. **Skuce, R. A., S. W. McDowell, T. R. Mallon, B. Luke, E. L. Breadon, P. L. Lagan, C. M. McCormick, S. H. McBride, and J. M. Pollock**. 2005. Discrimination of isolates of Mycobacterium bovis in Northern Ireland on the basis of variable numbers of tandem repeats (VNTRs). Vet.Rec. **157**:501-504.

37. **Stragier, P., A. Ablordey, W. M. Meyers, and F. Portaels**. 2005. Genotyping Mycobacterium ulcerans and Mycobacterium marinum by using mycobacterial interspersed repetitive units. J.Bacteriol. **187**:1639-1647.

38. **Tsolaki, A. G., A. E. Hirsh, K. DeRiemer, J. A. Enciso, M. Z. Wong, M. Hannan, Goguet de la Salmoniere YO, K. Aman, M. Kato-Maeda, and P. M. Small**. 2004. Functional and evolutionary genomics of Mycobacterium tuberculosis: insights from genomic deletions in 100 strains. Proc.Natl.Acad.Sci.U.S.A **101**:4865-4870.

39. **van der Spuy, G. D., R. M. Warren, M. Richardson, N. Beyers, M. A. Behr, and P. D. van Helden**. 2003. Use of genetic distance as a measure of ongoing transmission of Mycobacterium tuberculosis. J.Clin.Microbiol. **41**:5640-5644.

40. **Van Rie, A., T. C. Victor, M. Richardson, R. Johnson, G. D. van der Spuy, E. J. Murray, N. Beyers, N. C. van Pittius, P. D. van Helden, and R. M. Warren**. 2005. Reinfection and mixed infection cause changing Mycobacterium tuberculosis drug-resistance patterns. Am.J.Respir.Crit Care Med. **172**:636-642.

41. **Warren, R. M., E. M. Streicher, S. L. Sampson, G. D. van der Spuy, M. Richardson, D. Nguyen, M. A. Behr, T. C. Victor, and P. D. van Helden**. 2002. Microevolution of the direct repeat region of Mycobacterium tuberculosis: implications for interpretation of spoligotyping data. J.Clin.Microbiol. **40**:4457-4465.

42. **Warren, R. M., G. D. van der Spuy, M. Richardson, N. Beyers, M. W. Borgdorff, M. A. Behr, and P. D. van Helden**. 2002. Calculation of the stability of the IS6110 banding pattern in patients with persistent Mycobacterium tuberculosis disease. J.Clin.Microbiol. **40**:1705-1708.

43. **Warren, R. M., T. C. Victor, E. M. Streicher, M. Richardson, N. Beyers, N. C. Gey van Pittius, and P. D. van Helden**. 2004. Patients with active tuberculosis often have different strains in the same sputum specimen. Am.J.Respir.Crit Care Med. **169**:610-614.

44. **Wootton, S. H., B. E. Gonzalez, R. Pawlak, L. D. Teeter, K. C. Smith, J. M. Musser, J. R. Starke, and E. A. Graviss**. 2005. Epidemiology of pediatric tuberculosis using traditional and molecular techniques: Houston, Texas. Pediatrics **116**:1141-1147.

Appendix 1: Variable host-pathogen compatibility in *Mycobacterium tuberculosis*

Sebastien Gagneux[*†‡], Kathryn DeRiemer[†§], Tran Van[†], Midori Kato-Maeda[†¶], Bouke C. de Jong[†‖], Sujatha Narayanan[**], Mark Nicol[††], Stefan Niemann[‡‡], Kristin Kremer[§§], M. Cristina Gutierrez[¶¶], Markus Hilty[‖‖], Philip C. Hopewell[¶], Peter M. Small[*†††]

[*]Institute for Systems Biology, Seattle, WA, USA, [†]Division of Infectious Diseases and Geographic Medicine, Stanford University Medical Centre, Stanford, CA, USA, [§]University of California, Davis, USA, [¶]Division of Pulmonary and Critical Care Medicine, San Francisco General Hospital and the University of California, San Francisco, CA, USA, [‖]MRC Laboratories, Fajara, The Gambia, [**]Department of Immunology, Tuberculosis Research Centre, Chennai, India, [††]Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa, [‡‡]Forschungszentrum Borstel, National Reference Centre for Mycobacteria, Borstel, Germany, [§§]Mycobacteria Reference Unit, National Institute of Public Health and the Environment, Bilthoven, The Netherlands, [¶¶]Centre National de Reference des Mycobacteries, Institut Pasteur, Paris, France, [‖‖]Swiss Tropical Institute, Basel, Switzerland, [†††]Bill and Melinda Gates Foundation, Seattle, WA, USA

Published in

Proceedings of the National Academy of Sciences of U S A 2006 Feb 21;103(8):2869-73

Abstract

Mycobacterium tuberculosis remains a major cause of morbidity and mortality worldwide. Studies have reported human pathogens to have geographically structured population genetics, some of which have been linked to ancient human migrations. However, no study has addressed the potential evolutionary consequences of such longstanding human-pathogen associations. Here we demonstrate that the global population structure of *M. tuberculosis* is defined by six phylogeographical lineages, each associated with specific, sympatric human populations. In an urban cosmopolitan environment, mycobacterial lineages were much more likely to spread in sympatric than in allopatric patient populations. Tuberculosis cases that did occur in allopatric hosts disproportionately involved high-risk individuals with impaired host resistance. These observations suggest that mycobacterial lineages are adapted to particular human populations. If confirmed, our findings have important implications for tuberculosis control and vaccine development.

Introduction

Several studies have reported geographically structured populations in human pathogens (1-4). Recently, the genetic population structure of *Helicobacter pylori* and *Mycobacterium leprae* have been linked to ancient human migrations (1, 4, 5). Such long-standing host-pathogen associations could lead to adaptive genetic changes between interacting host and pathogen populations. Studies in invertebrate model systems have shown that pathogens can adapt to specific host species (6). However, no example of host-specific pathogen adaptation has yet been documented in pathogens affecting different human populations. The observation of geographically structured populations of human pathogens implies that particular strains and their corresponding patient populations can be classified as sympatric or allopatric (6). Compatibility, defined as the ability of a given pathogen to infect a given host, often differs in sympatric versus allopatric host-pathogen combinations with sympatric combinations usually displaying a greater compatibility (6).

M. tuberculosis occurs world-wide and is still killing 2-3 million people each year (7). New tools for tuberculosis control are urgently needed, including a more effective vaccine (8). A series of genotyping tools for *M. tuberculosis* have been developed (9). Most of these make use of mobile genetic elements or repetitive DNA. Even though these tools have been invaluable for detecting ongoing tuberculosis transmission, the markers upon which they are based change relatively rapidly, making it difficult to define deep phylogenetic relationships (4). In contrast, large sequence polymorphisms (LSPs) represent unique event polymorphisms that can be used to construct robust phylogenies for *M. tuberculosis* (10). An additional advantage is that once LSPs have been identified (e.g. by comparative whole-genome hybridization), simple PCR can be used to screen large numbers of strains in a high-throughput fashion.

In this study, we used comparative genomic and molecular epidemiological tools to define the global population structure of *M. tuberculosis* and to investigate its influence on the transmission dynamics of *M. tuberculosis* in San Francisco during an eleven-year period.

Materials and Methods

Molecular epidemiology in San Francisco. In an ongoing population-based molecular epidemiological study in San Francisco, California (11), 2807 tuberculosis patients were enrolled between January 1991 and December 2001. Of these patients, 2382 (84.9%) had *M. tuberculosis* isolated in culture. Demographic and epidemiological data, including place of birth and self-defined ethnicity, were recorded for each patient and IS*6110* restriction fragment length polymorphism (RFLP) genotyping was performed on 2141 (89.9%) of the bacterial isolates following standardized methods (11). Isolates with matching (clustered) RFLP patterns were considered part of a chain of tuberculosis transmission. The protocols and the procedures for the protection of human subjects were approved by Stanford University and the University of California, San Francisco.

Global sample of *M. tuberculosis*. Fifty of the strains included in the global sample had unique RFLP patterns and were isolated from US-and foreign-born patients from San Francisco. We previously reported that these patients represented cases of reactivation of infections acquired in their respective country of origin and that the genomic deletion profiles of these strains were associated with the respective patient's place of birth (10). Therefore, the unique foreign-born cases from San Francisco could be used to sample the diversity of *M. tuberculosis*. We validated this approach in 108 reference strains obtained from several additional strain collections representative of specific geographic areas (Supplementary Table 1). These reference strains were selected because they represented the most common genotypes in the corresponding geographic areas based on our previous molecular epidemiological studies (9, 12) (B.D., S.Na., M.N., S.Ni., and M.H. unpublished). We then screened an additional 709 unique strains isolated from US-and foreign-born patients from San Francisco. Eight strains from different patient clusters comprising only US-born individuals were also included.

Identification of large sequence polymorphisms. Comparative whole-genome hybridization was performed using an Affymetrix DNA chip (Santa Clara, CA, USA) following procedures described previously (13). Genomic regions putatively deleted in the test strains compared to the sequenced reference strain H37Rv were identified using the DelScan software (AbaSci, San Pablo, CA, USA). Putative deletions were confirmed by PCR and sequencing (13).

Lineage determination by PCR and multiplex real-time PCR. We used the phylogenetically informative LSPs to screen by PCR and/or TaqMan multiplex real-time PCR (Applied Biosystems, Foster City, CA, USA) an additional 679 unique strains, as well as one isolate representative of each of the 184 (97.7% of all) patient clusters that occurred in San Francisco between 1991 and 2001. The screening results from the clustered isolates were extrapolated to the remaining isolates of the respective clusters. The primer and probe sequences used in this study are shown in Supplementary Tables 2 and 3. The Euro-American lineage was defined based on a characteristic seven base pair deletion in pks15/1 (ref. (14)) or the ctg to cgg substitution at codon 463 of katG (ref. (15)), which are known to be equivalent markers (14, 16).

Lineage-specific transmission in San Francisco. Of 2141 patients with available RFLP data, 1849 (86.4%) were born in the US, China, The Philippines, Central America including Mexico, and Vietnam. This set of 1849 patients represented our sampling frame. We classified these patients as follows: all clustered patients, all cases with drug-resistance, all patients born in Vietnam or in Central America for which DNA was available, and a random selection of strains with unique RFLP patterns recovered from patients born in the US, China or The Philippines (the three largest patient populations in San Francisco). Overall, 71.4% of eligible patients (1321 patients) and their isolates were included in this part of the study, comprising 66.6% (493/740) patients born in the US, 67.3% (301/447) patients born in China, 69.5% (251/361) patients born in The Philippines, 89.7% (140/156) patients born in Central America, and 93.8% (136/145) patients born in Vietnam.

Statistical analysis. The number of secondary cases in each lineage was determined by subtracting the number of RFLP clusters from the total number of clustered cases (17). Because prevalent bacteria have a greater opportunity to transmit we translated the number of secondary cases in each lineage into lineage-specific secondary case rates by dividing the number of secondary cases in a lineage by the sum of all index cases (the number of clusters plus all the unique cases) belonging to the same lineage. To compare transmission rates between lineages, we then transformed the lineage-specific secondary case rates into secondary case rate ratios by dividing the secondary case rate of the lineage of interest by the secondary case rate of the other two lineages combined. To calculate the host population-specific secondary case rate we made the simplifying assumption that any index case in San Francisco, regardless of which host population he or she belonged, could have infected the

secondary case in question. Thus, we used as the denominator the sum of the number of clusters plus unique cases for the whole of San Francisco. A total of 188 patient clusters with 604 secondary cases occurred in San Francisco between 1991 and 2001. For the analysis, there were 184 clusters (97.9%), with their corresponding 596 (98.7% of all) secondary cases, with at least one isolate with DNA available for screening. Overall, 1349 tuberculosis cases with a unique RFLP pattern occurred during the same time period, 754 (55.9%) of which had lineage information available (including 213 cases who were not part of the five main patient populations). To account for the number of unique cases which were not screened for lineage-defining markers, we weighted the denominator of the secondary case rate by multiplying the number of unique cases in each lineage by 1.79 (1349 total unique cases/754 screened unique cases).

In order to identify the risk determinants of transmission of allopatric strains in the US-born population, we sought associations between the three lineages and patient characteristics using univariate analyses with a 3 x 2 χ^2 test of proportions with two degrees of freedom. Variables with a p-value < 0.20 in the 3 x 2 comparison were further tested by individual 2 x 2 comparisons using the regular χ^2 test of proportions or the Fisher's two-tailed exact test. All variables with a p-value < 0.20 in the 2 x 2 univariate analysis and biological plausibility were considered for the multivariate logistic regression model. We performed forward stepwise model construction and compared the log likelihood ratios of successive models until the final, most parsimonious model was identified. We used the Hosmer-Lemeshow goodness-of-fit test to validate the final models (18). Statistical analyses were performed with Stata (version 7E; Stata Corporation, College Station, Texas, USA).

Results and Discussion

In order to define the global population structure of *M. tuberculosis*, we performed genomic deletion analysis on a global sample of 875 strains originating from 80 countries (Table 1 and Supplementary Table 1). This sample included strains isolated from foreign-born tuberculosis patients in San Francisco who contracted the infection in their country of origin, and was complemented with geographically representative strains from other reference collections. We analyzed a subset of 111 strains by comparative whole-genome hybridization (13). The results of 74 of these experiments were published elsewhere (13, 19, 20), and 37 are reported here. We identified 19 phylogenetically informative and lineage-specific LSPs (Fig. 1a and Supplementary Table 2). These LSPs were confirmed by sequencing, validated by PCR and sequencing in 72 additional strains, and used to screen the remaining 692 strains by PCR or multiplex real-time PCR (Supplementary Tables 2 and 3). We used as additional phylogenetic markers the previously reported regions of difference (RD) TbD1 and RD9 (ref. (21)), the seven base pair deletion in the *pks15/1* locus (14), and the *katG*463 ctg to cgg substitution (15).

The analysis of our global sample of 875 strains revealed six main lineages and 15 sub-lineages of *M. tuberculosis* (Fig. 1a, Table 1, Supplementary Tables 1 and 4). Some of these lineages correspond to strain groupings that have previously been reported. For example, the Indo-Oceanic lineage includes a group of strains that have been referred to as 'ancestral' due to the fact that they conserve the TbD1 genomic region, which is deleted in 'modern' strains of *M. tuberculosis* (21). The East-Asian lineage includes, but is not limited to, the Beijing family of strains (20). The West-African lineages 1 and 2 correspond to strains that have traditionally been named *M. africanum* (19), and the Euro-American lineage regroups strains that have generally been described as principal genetic group 2 and 3 (ref. (14-16)).

Besides confirming some of the mycobacterial groupings that have been described previously, our analysis of an extended global strain collection revealed that the population genetics of *M. tuberculosis* is highly geographically structured. Each of the six main lineages was associated with particular geographical areas, and the lineage names reflect these geographical associations (Fig. 1b and Table 1). For example, the East-Asian lineage is dominant in many countries of the Far East, and the Indo-Oceanic lineage occurs all around the Indian Ocean. The Euro-American lineage is clearly the most frequent lineage in Europe and the Americas, but specific sublineages within the Euro-American lineage predominate

also in different regions of Africa and the Middle East (Fig. 1a and b). While we did observe such geographical substructuring within the Euro-American lineage, no other sub-lineage was associated with any specific geographical area (results not shown).

Though most other areas were associated with only one or two lineages, all six main lineages were represented in Africa (Fig. 1b). These included the two West-African lineages that did not occur elsewhere, as well as the Indo-Oceanic lineage, the most ancestral of the six lineages, which was associated with East Africa. A recent study suggests that ancestral mycobacteria may have already affected early hominids in East Africa around 3 million years ago (22). Taken together, these findings are consistent with a scenario for the origin and evolution of human tuberculosis, in which *M. tuberculosis* expanded and diversified during its spread out of East Africa. This speculative scenario suggests that *M. tuberculosis* might be significantly older than previously estimated (15). As a consequence, different *M. tuberculosis* lineages may have adapted to different human host populations.

Taking advantage of the cosmopolitan setting of San Francisco, with its diverse tuberculosis patient and bacterial populations, we investigated the effects of host-pathogen mixing on the occurrence of secondary cases of tuberculosis. We used multiplex real-time PCR to screen for the main lineages of *M. tuberculosis* in a stratified random sample of 1321 isolates, corresponding to 71% of all tuberculosis cases reported in San Francisco between 1991 and 2001 who were born in the United States (US), China, The Philippines, Vietnam, or Central America. These patients represent the five largest tuberculosis patient populations in San Francisco. This sample included all the RFLP clustered cases belonging to any of these five populations as well as a random sample of unique cases. The clustered cases were considered part of relatively recent chains of tuberculosis transmission in San Francisco and the unique cases were considered to have developed tuberculosis as a consequence of reactivation of latent infection (11).

Our results showed that 99.6% of all isolates in San Francisco belonged to three of the six main lineages. Twenty-six percent of the 1321 isolates belonged to the Indo-Oceanic lineage, 26% to the East-Asian lineage, and 48% to the Euro-American lineage. When we stratified the bacterial lineage data by the five patient populations, a strong association was evident (Fig. 2a; Pearson χ^2_8=1295, p<0.0001). In four of the five patient populations, one specific lineage accounted for at least 72% of all tuberculosis cases.

We explored whether the association between lineage of *M. tuberculosis* and human population reflects host-specific differential transmission of mycobacterial lineages, using RFLP clustering as a proxy for transmission (17). We hypothesized that lineages that are rare

in a specific human population are not adapted to transmit and cause secondary cases in this specific human population. We first calculated the secondary case rate ratios of the three *M. tuberculosis* lineages irrespective of the patient's place of birth. All three lineages had statistically different secondary case rate ratios (Supplementary Table 5). In San Francisco, patients infected with the Euro-American lineage were three times more likely to generate a secondary case during the eleven-year study period than patients infected with any other strain. The Indo-Oceanic lineage had a significantly lower secondary case rate ratio and the East-Asian lineage the lowest. When we calculated the lineage-specific secondary case rate ratios stratified by human population, we found that in every instance, the secondary case rate ratios of sympatric lineages was significantly greater in comparison to that of allopatric lineages in the same population (Fig. 2b and Supplementary Table 5). Taken together, these observations suggest that particular lineages of *M. tuberculosis* might be adapted to specific human populations and mal-adapted to others.

Given that some tuberculosis cases were caused by allopatric lineages, we investigated the characteristics of patients with disease caused by allopatric lineages. We chose to look at the US-born population because it represents the largest patient population in San Francisco (Fig. 2a) and that sociological determinants of transmission are well documented. The characteristics of the US-born patients, stratified by the three lineages of *M. tuberculosis*, are presented in Supplementary Table 6. Significant variables were selected for multivariate logistic regression modelling. The multivariate analysis revealed that US-born patients of self-defined Chinese and Filipino ethnicity tend to harbour the same strains as patients born in China and the Philippines, respectively (Table 2). Because self-defined ethnicity is a good predictor of human genetic ancestry (23) these findings provide significant additional support for the importance of this host-pathogen association.

Another study has reported similar associations between *M. tuberculosis* strain families and human populations (16). Such host-pathogen associations, while indicative, do not by themselves provide proof that specific host-pathogen adaptations occur. They can also be explained by sociological and epidemiological factors (6). For example, social mixing is non-random among ethnic groups in San Francisco, which certainly impacts transmission of *M. tuberculosis* between US-and foreign-born individuals in the short term (24, 25). However, some foreign strains, for example those associated with Chinese immigrants, must have been introduced into San Francisco repeatedly since the beginning of the Gold Rush in the 1800s (26). We propose that over such large time frames, there has been ample opportunity for the spread of foreign strains into the US-born population.

We cannot exclude the possibility that social factors contribute or even drive our observation of lineage-specific association with particular human populations. However, further results from our multivariate analysis support biological causality for the observed host-pathogen association. US-born tuberculosis patients of non-Chinese and non-Filipino ethnicity infected with allopatric strains (i.e. belonging to the Indo-Oceanic or East-Asian lineages) were more likely to be HIV positive or homeless (Table 2). This suggests that although these lineages are less adapted to transmit and cause disease in fully competent members of allopatric human populations, they can do so in the context of impaired host resistance. Such differences in host-pathogen compatibility or local adaptation have been associated with host-specific pathogen adaptation and demonstrated in several invertebrate host-pathogen model systems (6).

Overall, our findings demonstrate a global genetic population structure for *M. tuberculosis*, and support the notion that this pathogen has adapted to specific human populations. These results have implications for tuberculosis control efforts, especially for the development of new vaccines. The importance of strain genetic variation for vaccine escape has been documented in several bacterial species (27-29). In Bacille Calmette-Guerin (BCG), the currently available tuberculosis vaccine, significant geographical variation in protective efficacy has been observed (8). Environmental factors and differences in vaccine strain have been invoked (8, 30-32), but our findings suggest that regional differences in host-pathogen interactions could be partially responsible. Although recent progress has been made in the development of new tuberculosis vaccines (33), the global population structure of *M. tuberculosis* and host-specific pathogen adaptation may need to be considered when engineering and evaluating new vaccine candidates.

Acknowledgements

We thank Bruce Levin and James H. Jones for their valuable comments on the manuscript, and Colette Diguimbaye and Erik Post for providing strains. This research was supported by the Swiss National Science Foundation and the Novartis Foundation (S.G.), the NIH (K.D., P.H.), and the Wellcome Trust (P.S.).

Table 1. Assignment of 875 strains of *M. tuberculosis* from 80 countries to six main phylogenetic lineages in eleven geographic regions

Geographic region (number of countries)	Total strains	Indo-Oceanic lineage	East-Asian lineage	East-African-Indian lineage	Euro-American lineage	West-African lineage 1	West-African lineage 2
Americas (18)	207	6	7		194		
Europe (14)	35	1	4		30		
North Africa/Middle East (6)	10				10		
West Africa (8)	28				12	9	7
Central Africa (5)	15			1	14		
South Africa (1)	5		2		3		
East Africa (6)	20	3		8	9		
Indian Subcontinent (4)	17	3	1	10	3		
Southeast Asia (9)	272	169	73	1	29		
East Asia (7)	262	17	190		55		
Pacific Islands (2)	4				4		
Total (80)	875	199	277	20	363	9	7

Table 2. Risk factors independently associated with one of three *M. tuberculosis* lineages in 490 US-born patients from San Francisco

M. tuberculosis lineage	Risk factor	Adjusted odds ratio	(95% CI)	P-value
East-Asian	Chinese ethnicity	19.8	(4.6-84.2)	<0.001
	Homelessness	3.0	(1.4-6.2)	0.004
Indo-Oceanic	Filipino ethnicity	43.2	(5.6-335)	<0.001
	Age ≥ 45 years	3.9	(1.5-10.1)	0.004
	HIV positive	3.4	(1.3-8.7)	0.01
Euro-American	Chinese ethnicity	0.18	(0.06-0.6)	0.004

Note: CI=confidence interval

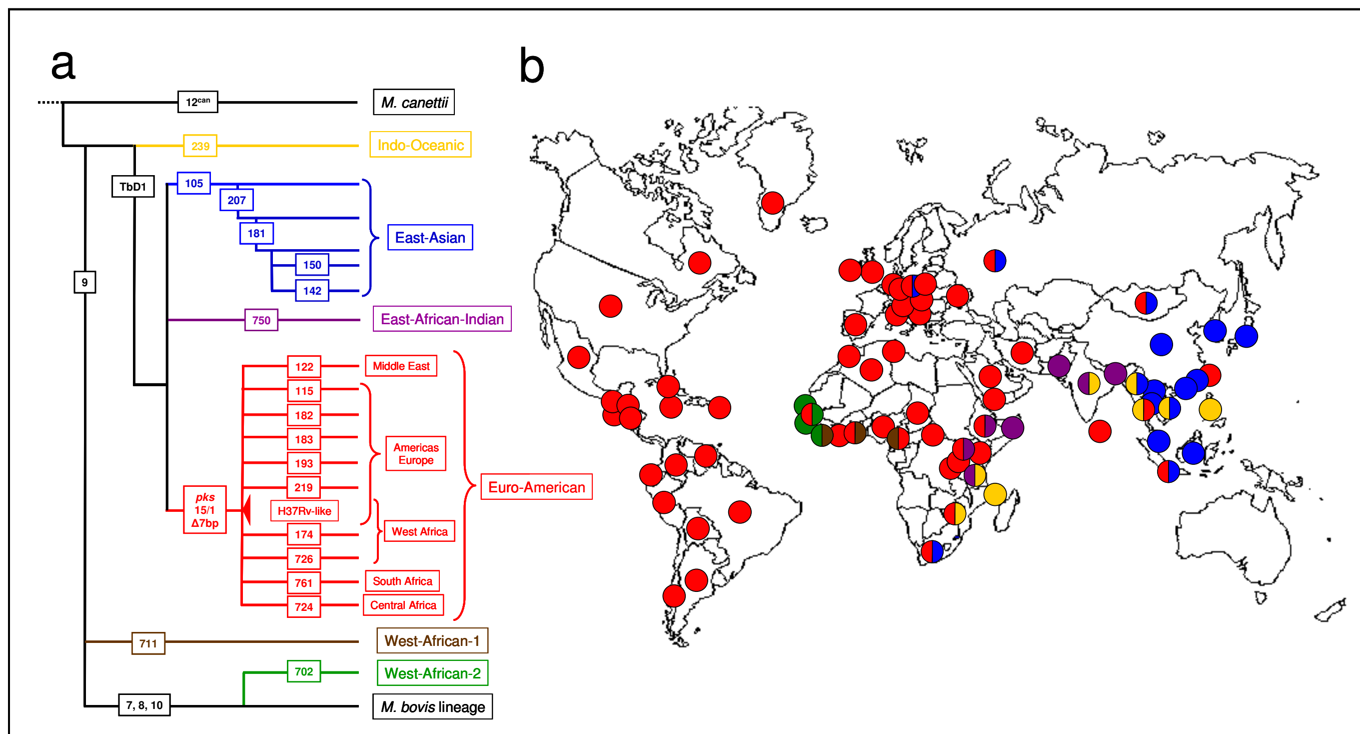

Fig. 1. The global population structure and geographical distribution of *M. tuberculosis*. **(a)** Large sequence polymorphisms (LSPs) define a global phylogeny for *M. tuberculosis*. The name of the lineage-defining LSPs or regions of difference (RDs) are shown in rectangles. The geographic regions associated with specific lineages are indicated. **(b)** The six main lineages of *M. tuberculosis* are geographically structured. Each dot corresponds to one of 80 countries represented in the global strain collection. The colours of the dots relate to the six main lineages defined in Fig. 1a and indicate the dominant lineage(s) in the respective countries.

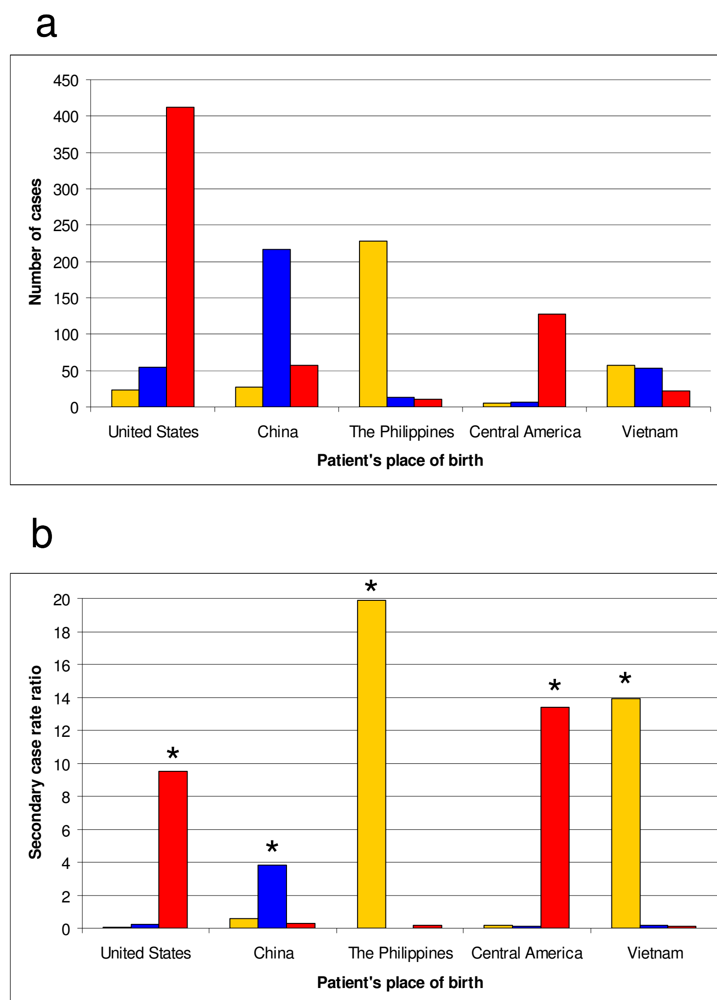

Figure 2. Lineage-specific prevalence and transmission of *M. tuberculosis* in San Francisco (1991 to 2001). **(a)** Prevalent strains in San Francisco are strongly associated with sympatric patient populations. The sums of both unique and clustered cases are shown. **(b)** The propensity of specific mycobacterial lineages to transmit is significantly higher in sympatric compared to allopatric patient populations (asterisk: comparison to the other two lineages combined: p <0.0001; yellow=Indo-Oceanic lineage, blue=East-Asian lineage, red=Euro-American lineage).

References

1. Falush, D., Wirth, T., Linz, B., Pritchard, J. K., Stephens, M., Kidd, M., Blaser, M. J., Graham, D. Y., Vacher, S., Perez-Perez, G. I., Yamaoka, Y., Megraud, F., Otto, K., Reichard, U., Katzowitsch, E., Wang, X., Achtman, M. & Suerbaum, S. (2003) *Science* **299,** 1582-5.
2. Musser, J. M., Kroll, J. S., Granoff, D. M., Moxon, E. R., Brodeur, B. R., Campos, J., Dabernat, H., Frederiksen, W., Hamel, J. & Hammond, G. (1990) *Rev. Infect. Dis.* **12,** 75-111.
3. Agostini, H. T., Yanagihara, R., Davis, V., Ryschkewitsch, C. F. & Stoner, G. L. (1997) *Proc. Natl. Acad. Sci. U S A* **94,** 14542-6.
4. Monot, M., Honore, N., Garnier, T., Araoz, R., Coppee, J. Y., Lacroix, C., Sow, S., Spencer, J. S., Truman, R. W., Williams, D. L., Gelber, R., Virmond, M., Flageul, B., Cho, S. N., Ji, B., Paniz-Mondolfi, A., Convit, J., Young, S., Fine, P. E., Rasolofo, V., Brennan, P. J. & Cole, S. T. (2005) *Science* **308,** 1040-2.
5. Wirth, T., Wang, X., Linz, B., Novick, R. P., Lum, J. K., Blaser, M., Morelli, G., Falush, D. & Achtman, M. (2004) *Proc. Natl. Acad. Sci. U S A* **101,** 4746-51.
6. Woolhouse, M. E., Webster, J. P., Domingo, E., Charlesworth, B. & Levin, B. R. (2002) *Nat. Genet.* **32,** 569-77.
7. World Health Organization (2005) (WHO, Geneva).
8. Andersen, P. & Doherty, T. M. (2005) *Nat. Rev. Microbiol.*
9. Kremer, K., van Soolingen, D., Frothingham, R., Haas, W. H., Hermans, P. W., Martin, C., Palittapongarnpim, P., Plikaytis, B. B., Riley, L. W., Yakrus, M. A., Musser, J. M. & van Embden, J. D. (1999) *J. Clin. Microbiol.* **37,** 2607-18.
10. Hirsh, A. E., Tsolaki, A. G., DeRiemer, K., Feldman, M. W. & Small, P. M. (2004) *Proc. Natl. Acad. Sci. U S A* **101,** 4871-6.
11. Jasmer, R. M., Hahn, J. A., Small, P. M., Daley, C. L., Behr, M. A., Moss, A. R., Creasman, J. M., Schecter, G. F., Paz, E. A. & Hopewell, P. C. (1999) *Ann. Intern. Med.* **130,** 971-978.
12. Niobe-Eyangoh, S. N., Kuaban, C., Sorlin, P., Cunin, P., Thonnon, J., Sola, C., Rastogi, N., Vincent, V. & Gutierrez, M. C. (2003) *J. Clin. Microbiol.* **41,** 2547-53.
13. Tsolaki, A. G., Hirsh, A. E., DeRiemer, K., Enciso, J. A., Wong, M. Z., Hannan, M., Goguet de la Salmoniere, Y. O., Aman, K., Kato-Maeda, M. & Small, P. M. (2004) *Proc. Natl. Acad. Sci. U S A* **101,** 4865-70.
14. Marmiesse, M., Brodin, P., Buchrieser, C., Gutierrez, C., Simoes, N., Vincent, V., Glaser, P., Cole, S. T. & Brosch, R. (2004) *Microbiology* **150,** 483-96.
15. Sreevatsan, S., Pan, X., Stockbauer, K. E., Connell, N. D., Kreiswirth, B. N., Whittam, T. S. & Musser, J. M. (1997) *Proc. Natl. Acad. Sci. U S A* **94,** 9869-74.
16. Baker, L., Brown, T., Maiden, M. C. & Drobniewski, F. (2004) *Emerg. Infect. Dis.* **10,** 1568-77.
17. Burgos, M., DeRiemer, K., Small, P. M., Hopewell, P. C. & Daley, C. L. (2003) *J. Infect. Dis.* **188,** 1878-84.
18. Selvin, S. (1998) in *Monographs in Epidemiology and Biostatistics* (Oxford University Press, New York), Vol. 28, pp. 385-391.
19. Mostowy, S., Onipede, A., Gagneux, S., Niemann, S., Kremer, K., Desmond, E. P., Kato-Maeda, M. & Behr, M. (2004) *J. Clin. Microbiol.* **42,** 3594-9.
20. Tsolaki, A. G., Gagneux, S., Pym, A. S., Goguet de la Salmoniere, Y. O., Kreiswirth, B. N., Van Soolingen, D. & Small, P. M. (2005) *J. Clin. Microbiol.* **43,** 3185-91.
21. 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) *Proc. Natl. Acad. Sci. U S A* **99,** 3684-9.
22. Gutierrez, C., Brisse, S., Brosch, R., Fabre, M., Omais, B., Marmiesse, M., Supply, P. & Vincent, V. (2005) *PLoS Pathogens* **1,** 1-7.
23. Rosenberg, N. A., Pritchard, J. K., Weber, J. L., Cann, H. M., Kidd, K. K., Zhivotovsky, L. A. & Feldman, M. W. (2002) *Science* **298,** 2381-5.
24. Chin, D. P., DeRiemer, K., Small, P. M., de Leon, A. P., Steinhart, R., Schecter, G. F., Daley, C. L., Moss, A. R., Paz, E. A., Jasmer, R. M., Agasino, C. B. & Hopewell, P. C. (1998) *Am. J. Respir. Crit. Care Med.* **158,** 1797-1803.
25. Jasmer, R. M., Ponce, d. L., Hopewell, P. C., Alarcon, R. G., Moss, A. R., Paz, E. A., Schecter, G. F. & Small, P. M. (1997) *Int. J. Tuberc. Lung Dis.* **1,** 536-541.
26. http://en.wikipedia.org/wiki/Chinatown%2C_San_Francisco.
27. Gagneux, S. P., Hodgson, A., Smith, T. A., Wirth, T., Ehrhard, I., Morelli, G., Genton, B., Binka, F. N., Achtman, M. & Pluschke, G. (2002) *J. Infect. Dis.* **185,** 618-26.
28. Van Loo, I. H. & Mooi, F. R. (2002) *Microbiology* **148,** 2011-8.

29. Mbelle, N., Huebner, R. E., Wasas, A. D., Kimura, A., Chang, I. & Klugman, K. P. (1999) *J. Infect. Dis.* **180,** 1171-6.
30. Behr, M. A., Wilson, M. A., Gill, W. P., Salamon, H., Schoolnik, G. K., Rane, S. & Small, P. M. (1999) *Science* **284,** 1520-1523.
31. Rook, G. A., Dheda, K. & Zumla, A. (2005) *Nat. Rev. Immunol.* **5,** 661-7.
32. Fine, P. E. (1995) *Lancet* **346,** 1339-45.
33. McShane, H., Pathan, A. A., Sander, C. R., Keating, S. M., Gilbert, S. C., Huygen, K., Fletcher, H. A. & Hill, A. V. (2004) *Nat. Med.* **10,** 1240-4.

Appendix 2: Species identification of non-tuberculous mycobacteria from humans and cattle of Chad

C. Diguimbaye-Djaibé[1], V. Vincent[2], E. Schelling[3], M. Hilty[3], R. Ngandolo[1], HH. Mahamat[1], G. Pfyffer[4], F. Baggi[5], M. Tanner[3], J. Zinsstag[3]

[1]Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha, N'Djaména, Chad, [2.]Centre de Référence de Mycobactéries, Institut Pasteur, Paris, France and [3]Swiss Tropical Institute, Basel, [4] Department of Medical Microbiology, Kantonsspital Luzern, Luzern, [5] National Centre for Mycobacteria, University of Zurich, Switzerland

Published in *Schweizerisches Archiv für Tierheilkunde* 2006;148(5):251-6

Summary

In Chad, during a study on tuberculosis in humans and cattle, 52 non-tuberculous mycobacteria (NTM) strains were isolated. By means of INNO-LiPA, PRA-*hsp65* amplification and sequencing of 16S rDNA, NTM species of 25/52 isolates were identified. *M. fortuitum* complex (8) was the most frequent species, followed by *M. nonchromogenicum* (4) and *M. avium* complex (4). PRA method could identify *M. fortuitum* 3rd variant among isolates derived from cattle specimens. This finding could confirm the existence of farcy in the Chadian cattle population as *M. fortuitum* 3rd variant and putitative pathogen *M. farcinogenes* can't be distinguished by the methods used in this study. Half of the NTM isolates could not be specified and we considered them as contaminants from the environment.

Keywords: non –tuberculous mycobacteria, Chad, molecular methods, INNO-LiPA assay, PRA amplification, sequencing of the 16S gene

Zusammenfassung

Während einer Studie im Tschad, welche die Tuberkulose bei Menschen und Rindern untersucht, wurden 52 nicht-Tuberkulse Mykobakterien (NTM) isoliert. Mit Hilfe von INNO-LiPA tests, PRA-*hsp65* Amplifizierung und Sequenzierung der 16S rDNS, konnten 25/52 Isolaten identifiziert werden. *M. fortuitum* complex war die häufigste Species, gefolgt von *M. nonchromogenicum* (4) und *M. avium* complex (4). Die PRA Methode konnte bei Isolaten, die von Kühen stammen, die dritte Variante von *M. fortuitum* identifizieren. Dieser Befund könnte die Existenz von Farcy in der tschadischen Kuhpopulation bedeuten, weil die dritte Variante von *M. fortuitum* vom vermuteten Farcy Erreger *M. farcinogenes* nicht mit den in dieser Studie angewendeten Methoden unterschieden werden kann. Die Hälfte der NTM Isolate konnten nicht identifiziert werden und wir betrachten diese als Kontaminationen aus der Umwelt.

Schlüsselwörter: nicht-Tuberkulse Mykobakterien (NTM), Tschad, molekulare Methoden, INNO-LiPA, PRA Amplifizierung, Sequenzierung des 16S Gens

Résumé

Au Tchad, lors d'une étude de la tuberculose humaine et animale, 52 souches de mycobactéries non tuberculeuses (MNT) ont été isolées. La caractérisation génétique des isolats a été réalisée au moyen des tests INNO-LIPA, PRA-hsp65 et le séquençage du 16 rDNA. 25/52 isolats ont pu être identifié. *M. fortuitum* le complexe (8) était l'espèce la plus fréquente, suivie par *M. nonchromogenicum* (4) et *M. avium* le complexe (4). La méthode PRA a pu spécifier *M. fortuitum* variante 3 chez le bétail. Cette découverte peut apporter une preuve supplémentaire sur l'existence du farcin dans le cheptel tchadien, sachant que *M. fortuitum* variante 3 et *M. farcinogenes* ne peuvent pas être distingués par les méthodes utilisées dans cette étude. L'autre moitié des MNT n'ont pas pu être spécifié et nous les avons considéré comme étant des polluants environnementaux.

Mots clés : mycobactérie non tuberculeuse, Tchad, méthodes moléculaires, INNO-LiPA, PRA-*hsp65,* séquençage de 16S ADN

Introduction

With the increase in human tuberculosis cases and the advent of HIV/AIDS, there has been resurgence in interest in diseases caused by non-tuberculous mycobacteria (NTM). NTM are subdivided into rapid and slow growers. Their ecologic niche is the environment, as they have been found in soil, plants, house dust and water. In contrast, animals are not considered as an important reservoir for NTM (Saiman, 2004). However, they can cause infections in humans and animals (Phillips and von Reyn, 2001; Hamid et al., 1991; Alander-Damsten et al., 2003; Valheim et al., 2001). Mycobacteria cause a variety of illnesses, which have profound individual and public health implications. The clinical symptomatology of these diseases is not different from classical tuberculosis (Dvorska et al., 2001), but their therapy is problematic due to the high resistance to antituberculous drugs seen for most ubiquitous mycobacteria (Schutt-Gerowitt, 1995).

Reports on NTM infections in humans and animals in Africa are scarce. Most published studies are from South Africa, and specifically on investigations in the South African gold mines where *Mycobacterium kansasii* and *M. scrofulaceum* were the main causes of mycobacterial diseases (Churchyard et al., 1999; Corbett et al., 1999) and the first case of infection with *M. marinum* since 1987 was reported (Mousdicas and Saxe, 1987). For the others part of Africa, information can be found in studies on AIDS patients. In Burkina Faso, Ledru et al. (1996) found that 6.5% of mycobacterial isolations from AIDS patients were NTM without further specification, and in Nigeria, Idigbe et al. (1994) identified 20% *M. avium* and 10% *M. kansasii* among their isolates. In livestock, the serological investigation detected antibodies to *M. paratuberculosis* in camels and goats in Kenya (Paling et al., 1988). *M. farcinogenes* was described as main causal agent of bovine farcy in Sudan (Hamid et al., 2002).

In Chad, during a study of two years on tuberculosis in humans and animals, numerous *Mycobacterium tuberculosis* complex (MTC) and NTM isolates were obtained. The purpose of the present article is to report the different NTM species found among mycobacterial strains from Chad.

Materials and Methods

Isolates and study sites

1) Specimens collected in 5 Chadian health centres (sputum and urine) and in one slaughterhouse (tubercles from lymph nodes, lung, spleen, liver and pleural cavity of condemned cattle's carcass) in N'Djaména, were subjected to decontamination and cultivation. Obtained mycobacterial isolates were identified by biochemical testing (Kent and Kubica, 1985). On the basis of biochemical tests results, the isolates were categorised in *M. tuberculosis* complex (MTC) and non-tuberculous mycobacteria (NTM). These preliminary studies were performed at the "Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha (LRVZ/F)" in Chad.

2) Thirty six NTM had been sent to the Institut Pasteur (IP) in Paris for identification NTM species by molecular method.

3) Sixteen NTM strains were characterized at the National Centre of Mycobacteria (NCM) Zurich.

Molecular methods

1) The INNO-LiPA assay was carried out according to manufacturer's instructions and using the reagents provided with the LiPA kit (Versant® INNO-LiPA HCV II). The protocol consisted of PCR amplification, hybridization of the PCR products to the strips, detection and interpretation of the results (Suffys et al., 2001)

2) PRA amplification was performed according to the procedure described by Telenti et al. (1993). This method amplified a 439-bp fragment of the *hsp65* gene.

3) Real-time PCR

DNA extraction and subsequently amplification and identification were carried out according to the procedure described by Kraus et al. (2001). This method allowed the classification of NTM and MTC strains which were all previously categorised as MTC by biochemistry.

4) Sequencing of the 16S gene

The obtained PCR products were used to perform the sequencing of the 16S gene. The sequence processing was done with computer software from ABI PRISM™ 310 (Applied Biosystems). Alignments of mycobacterial 16S rDNA sequences were done with the Model 310 (version 3.4.1.) alignment tool. All probe sequences were subsequently matched with sequences in the GenBank by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) to detect sequence similarity. A similarity of 98 to 99% suggests that the obtained sequence likely

derives from this species (Turenne et al., 2001). The search was performed at the National Centre of Mycobacteria in Zurich.

Results

At the LRVZ of N-Djaména, biochemical testing revealed a total of 52 NTM isolates, which were further characterized by three different molecular methods (INNO-LiPA, PRA-*hsp65* and 16S (rDNA). We analyzed 36 isolates by INNO-LiPA and PRA-*hsp65* at the NTM and 16 isolates by only sequencing of the 16S (rDNA) at the NCM. 25 of 52 isolates resulted in the identification of NTM isolates by at least one of these tools (Table 1).

M. fortuitum complex was identified for eight isolates from seven cattle and one human origins and was found the most. Six of them were classified as *M. fortuitum* supsp. perigrum (with INNO-LiPA) of which three were further characterized as *M. fortuitum* 3rd variant by PRA-*hsp65. Mycobacterium avium* complex was found for four isolates of which one and one of human (626 UR) and cattle origin (502 GG; Table 1) respectively, were classified as *M. intracellulare*. We received also four *M. nonchromogenicum* of cattle origin of which two and two were classified as subsp. mucogenicum and type I, respectively. The three remaining isolates of cattle origin were identified as *Mycobacterium* IWGMT.90093, *M. smiae* and *M. Szulgai/trivialé/brumae.* Further human isolates were *M. mariokaense* (two), *M. celatum* (one), *M. chelonae*, *Mycobacterium* sp.N120 and also *M. smiae* (one) (Table 1).

Discussion and conclusion

M. fortuitum complex was the most frequent NTM species (8/25) in this study and among them, 3 isolates from cattle were identified as the 3rd variant by PRA. Two studies have demonstrated the exact identity of 16S rDNA of *M. senegalense, M. farcinogenes* and *M. fortuitum* 3rd variant (Kirschner et al., 1992; Turenne et al., 2001). This finding is interesting because it confirms the existence of bovine farcy in the Chadian cattle population which has not been done since 1963 (Perpézat et al., 1963). However, we can't draw any conclusion if the responsible pathogen for farcy is *M. farcinogenes* (like in Sudan) or *M. senegalense* (like a case found in Chad), because of the lack of discrimination of the methods used (Hamid et al., 2002).

Only four isolates were identified as *M. avium* complex and none was *M. kansasii*, while usually these two mycobacteria are the most common NTM in clinical specimens and biopsies (Shih et al., 1997; Marras and Daley, 2002; Thorel, 1980; Pate et al., 2004). Concerning the remaining mycobacteria found in our study, they are rarely isolated but most of them were described as potential pathogens. *Mycobacterium simiae* is commonly found in the environment and was rarely associated with human disease. However, some cases of disease caused by *M. simiae* in AIDS and non-AIDS patients have been reported (Vandercam et al., 1996; Huminer et al., 1993; Bell et al., 1983; Lavy and Yoshpe-Purer, 1982). *M. celatum* was described as cause of fatal pulmonary infection in an old woman (Bux-Gewehr et al., 1998) and of disseminated infection in domestic ferret (Valheim et al., 2001). *M. chelonae* was frequently isolated from patients with cystic fibrosis (Hjelt et al., 1994; Tomashefski et al., 1996; Fauroux et al., 1997). *M. terrae* complex is composed of *M. nonchromogenicum, M. terrae*, and *M. triviale*. They are uncommon colonizers of human epithelia and generally regarded as non-pathogenic (Lee et al., 2004). However, *M. nonchromogenicum* may occasionally cause human disease such as pulmonary infection and tenosynovitis (Peters and Morice, 1991).

We obtained many NTM usually found in the environment by sequencing because this method is not focused on pathogen NTM strains. However, we have not confirmed our sequencing results by another molecular method as suggested (Hafner et al. 2004). Generally, NTM isolates should be further characterised because NTM infections can cause disease and first line antituberculosis drug treatment may be not efficacious. In another hand, it will be interesting for veterinarians to know about the importance of NTM in cattle, particularly in interpretation of tuberculin test.

Acknowledgements

We thank the technicians of the "Centre de Reference des Mycobactéries" of Institut Pasteur, the National Center for Mycobacteria, the Swiss Tropical Institute, and the "Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha" who have contributed to the project. The Swiss National Science Foundation is acknowledged for financial support.

Table 1 Results of NTM species identification of human and cattle origin from Chad with the three methods INNO-LiPA, PRA-*hsp65* and16S (rRNA) sequencing.

N° of strain	Origin of specimen	INNO-LiPA	PRA-*hsp65*	16S rDNA (% identity)
407CR/G	human	*M. fortuitum* subsp. *peregrinum*	*M. peregrinum/ M. porcinum*	*N/D*
219GG	Cattle (Mbororo)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum* subsp. *peregrinum*	*N/D*
446GG	Cattle (Mbororo)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum*	*N/D*
455GG	Cattle (Mbororo)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum* 3rd variant	*N/D*
454GG	Cattle (Arabe)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum* 3rd variant	*N/D*
548PM	Cattle (Arabe)	*M. fortuitum subsp. peregrinum*	*M. fortuitum* 3rd variant	*N/D*
483PM/P	Cattle (Arabe)	*N/D*	*N/D*	*M. fortuitum* (99 %)
490GG/P	Cattle (Arabe)	*N/D*	*N/D*	*M. fortuitum* (99 %)
582PM	Cattle (Arabe)	NI	*M. nonchromogenicum* subsp.*mucogenicum*	*N/D*
441PM	Cattle (Mbororo)	NI	*M. nonchromogenicum* subsp. *mucogenicum*	*N/D*
464FOIE	Cattle (Arabe)	NI	*M. nonchromgenicum* typeI	*N/D*
522PM	Cattle (Mbororo)	NI	*M. nonchromogenicum* type I	*N/D*
663PM	Cattle (Mbororo)	*M. avium* complex	*M. intracellulare/ MAI/ scrofulaceum*	*N/D*
502GG	Cattle (Arabe)	*M. avium* complex	*M. intracellulare*	*N/D*
444GG	Cattle (Arabe)	*M. avium* complex	-	*N/D*
626UR	human	*M. intracellulare*	*M. intracell/ MAI/scrofulaceum*	*N/D*
661GG/G	Cattle (Arabe)	*N/D*	*N/D*	*M. simiae* (99 %)
277CR/G	human	*N/D*	*N/D*	*M. simiae* (100 %)
280CR/P	human	*N/D*	*N/D*	*M. moriokaense* (99 %)
381UR/P	human	*N/D*	*N/D*	*M. moriokaense* (98 %)
637GG/G	Cattle (Arabe)	*N/D*	*N/D*	*Mycobacterium* IWGMT.90093 (99 %)
269CR/P	human	*N/D*	*N/D*	*M. celatum* (98 %)
430CR/G	human	*N/D*	*N/D*	*M. chelonae* (99 %)
685CR/P	human	*N/D*	*N/D*	*Mycobacterium* sp.N120 (98 %)
559PM	Cattle (Arabe)	NI	*Szulgai/trivialé/brumae*	N/D

NI: not identified. N/D: not done.

References

Alander-Damsten Y.K., Brander E.E., Paulin L.G.: Panniculitis, due to *Mycobacterium smegmatis,* in two Finnish cats. J.Feline.Med.Surg. 2003, 5:19-26.

Bell, R. C., J. H. Higuchi, W. N. Donovan, I. Krasnow, and W. G. Johanson, Jr.: *Mycobacterium simiae*. Clinical features and follow-up of twenty-four patients. Am.Rev.Respir.Dis. 1983, 127:35-38.

Bux-Gewehr, I., H. P. Hagen, S. Rusch-Gerdes, and G. E. Feurle.: Fatal pulmonary infection with *Mycobacterium celatum* in an apparently immunocompetent patient. J.Clin.Microbiol. 1998, 36:587-588.

Churchyard, G. J., I. Kleinschmidt, E. L. Corbett, D. Mulder, and K. M. De Cock.: Mycobacterial disease in South African gold miners in the era of HIV infection. Int.J.Tuberc.Lung Dis. 1999, 3:791-798.

Corbett, E. L., L. Blumberg, G. J. Churchyard, N. Moloi, K. Mallory, T. Clayton, B. G. Williams, R. E. Chaisson, R. J. Hayes, and K. M. De Cock.: Nontuberculous mycobacteria: defining disease in a prospective cohort of South African miners. Am.J.Respir.Crit Care Med. 1999, 160:15-21.

Dvorska, L., M. Bartos, C. Martin, W. Erler, and I. Pavlik.: Strategies for differentiation, identification and typing of medically important species of Mycobacteria by molecular methods. Vet.Med.-Czech. 2001, 46:309-328.

Fauroux, B., B. Delaisi, A. Clement, C. Saizou, D. Moissenet, C. Truffot-Pernot, G. Tournier, and T. H. Vu.: Mycobacterial lung disease in cystic fibrosis: a prospective study. Pediatr.Infect.Dis.J. 1997, 16:354-358.

Hafner, B., H. Haag, H. K. Geiss, and O. Nolte.: Different molecular methods for the identification of rarely isolated non-tuberculous mycobacteria and description of new hsp65 restriction fragment length polymorphism patterns. Mol.Cell Probes 2004, 18:59-65.

Hamid, M. E., G. E. Mohamed, M. T. Abu-Samra, S. M. el Sanousi, and M. E. Barri.: Bovine farcy: a clinico-pathological study of the disease and its aetiological agent. J.Comp Pathol. 1991, 105:287-301.

Hamid, M. E., A. Roth, O. Landt, R. M. Kroppenstedt, M. Goodfellow, and H. Mauch.: Differentiation between *Mycobacterium farcinogenes* and *Mycobacterium senegalense* strains based on 16S-23S ribosomal DNA internal transcribed spacer sequences. J.Clin.Microbiol. 2002, 40:707-711.

Hjelt, K., N. Hojlyng, P. Howitz, N. Illum, E. Munk, N. H. Valerius, K. Fursted, K. N. Hansen, I. Heltberg, and C. Koch.: The role of Mycobacteria Other Than Tuberculosis (MOTT) in patients with cystic fibrosis. Scand.J.Infect.Dis. 1994, 26:569-576.

Huminer, D., S. Dux, Z. Samra, L. Kaufman, A. Lavy, C. S. Block, and S. D. Pitlik.: *Mycobacterium simiae* infection in Israeli patients with AIDS. Clin.Infect.Dis. 1993, 17:508-509.

Idigbe, E. O., A. Nasidi, C. E. Anyiwo, C. Onubogu, S. Alabi, R. Okoye, O. Ugwu, and E. K. John.: Prevalence of human immunodeficiency virus (HIV) antibodies in tuberculosis patients in Lagos, Nigeria. J.Trop.Med.Hyg. 1994, 97:91-97.

Kent P.T., and G. P. Kubica.: Public health mycobacteriology- a guide for the level III laboratory. U.S. Departement of health and human Services publication, Atlanta, Ga. 1985.

Kirschner, P., M. Kiekenbeck, D. Meissner, J. Wolters, and E. C. Bottger.: Genetic heterogeneity within *Mycobacterium fortuitum* complex species: genotypic criteria for identification. J.Clin.Microbiol. 1992, 30:2772-2775.

Kraus, G., A. Cleary, N. Miller, R. Seivright, A. K. Young, G. Spruill, and H. J. Hnatyszyn.: Rapid and specific detection of the *Mycobacterium tuberculosis* complex using fluorogenic probes and real-time PCR. Mol.Cell.Probes 2001, 15:375-383.

Lavy, A. and Y. Yoshpe-Purer.: Isolation of *Mycobacterium simiae* from clinical specimens in Israel. Tubercle. 1982, 63:279-285.

Ledru, S., B. Cauchoix, M. Yameogo, A. Zoubga, J. Lamande-Chiron, F. Portaels, and J. P. Chiron. : Impact of short-course therapy on tuberculosis drug resistance in South-West Burkina Faso. Tuber.Lung Dis. 1996, 77:429-436.

Lee, C. K., H. M. Gi, Y. Cho, Y. K. Kim, K. N. Lee, K. J. Song, J. W. Song, K. S. Park, E. M. Park, H. Lee, and G. H. Bai.: The genomic heterogeneity among *Mycobacterium terrae* complex displayed by sequencing of 16S rRNA and hsp 65 genes. Microbiol.Immunol. 2004, 48:83-90.

Marras, T. K. and C. L. Daley.: Epidemiology of human pulmonary infection with nontuberculous mycobacteria. Clin.Chest Med. 2002, 23:553-567.

Mousdicas, N. and N. Saxe.: Fish-tank granuloma. The first reported case in South Africa. S.Afr.Med.J. 1987, 71:321-322.

Paling, R. W., S. Waghela, K. J. Macowan, and B. R. Heath.: The occurrence of infectious diseases in mixed farming of domesticated wild herbivores and livestock in Kenya. II. Bacterial diseases. J.Wildl.Dis. 1988, 24:308-316.

Pate, M., I. Zdovc, T. Pirs, B. Krt, and M. Ocepek.: Isolation and characterization of *Mycobacterium avium* and *Rhodococcus equi* from granulomatous lesions of swine lymph nodes in Slovenia. Acta Vet.Hung. 2004, 52:143-150.

Perpézat, A., F. Mariat and M. Thomé.: Importance du farcin chez le Zébu du Tchad. Bull.Soc.Path.Exot. 1963, 56:375-383.

Peters, E. J. and R. Morice.: Miliary pulmonary infection caused by *Mycobacterium terrae* in an autologous bone marrow transplant patient. Chest 1991, 100:1449-1450.

Phillips, M. S. and C. F. von Reyn.: Nosocomial infections due to nontuberculous mycobacteria. Clin.Infect.Dis. 2001, 33:1363-1374.

Saiman, L.: The mycobacteriology of non-tuberculous mycobacteria. Paediatr.Respir.Rev. 2004, 5 Suppl A:S221-S223.

Schutt-Gerowitt, H.: On the development of mycobacterial infections. I. A review concerning the common situation. Zentralbl.Bakteriol. 1995, 283:5-13.

Shih, J. Y., P. R. Hsueh, L. N. Lee, H. C. Wang, P. C. Yang, S. H. Kuo, and K. T. Luh.: Nontuberculous mycobacteria isolates: clinical significance and disease spectrum. J.Formos.Med.Assoc. 1997, 96:621-627.

Suffys, P. N., R. A. da Silva, M. de Oliveira, C. E. Campos, A. M. Barreto, F. Portaels, L. Rigouts, G. Wouters, G. Jannes, G. van Reybroeck, W. Mijs, and B. Vanderborght.: Rapid identification of Mycobacteria to the species level using INNO-LiPA Mycobacteria, a reverse hybridization assay. J.Clin.Microbiol. 2001, 39:4477-4482.

Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Bottger, and T. Bodmer.: Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J.Clin.Microbiol. 1993, 31:175-178.

Thorel, M. F.: [Mycobacteria identified in a centre for veterinary research between 1973 and 1979 (author's transl)]. Ann.Microbiol.(Paris) 1980, 131:61-69.

Tomashefski, J. F., Jr., R. C. Stern, C. A. Demko, and C. F. Doershuk.: Nontuberculous mycobacteria in cystic fibrosis. An autopsy study. Am.J.Respir.Crit Care Med. 1996, 154:523-528.

Turenne, C. Y., L. Tschetter, J. Wolfe, and A. Kabani.: Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous Mycobacterium species. J.Clin.Microbiol. 2001, 39:3637-3648.

Valheim, M., B. Djonne, R. Heiene, and D. A. Caugant.: Disseminated *Mycobacterium celatum* (type 3) infection in a domestic ferret (Mustela putorius furo). Vet.Pathol. 2001, 38:460-463.

Vandercam, B., J. Gala, B. Vandeweghe, J. Degraux, G. Wauters, L. Larsson, A. Bourlond, and F. Portaels.: *Mycobacterium simiae* disseminated infection in a patient with acquired immunodeficiency syndrome. Infection 1996, 24:49-51.

Appendix 3: Methods

1. Ligation mediated PCR

1.1 Preparation of the DNA and linker

a) Extraction of DNA

Place 100 µl of TE in 2 ml screw-cap tubes.
Take sufficient colonies from the culture medium and suspend in the TE
Incubate in the oven for 30 min at 100°C (to ensure that the bacteria are destroyed and DNA is extracted).

b) Digestion of the DNA with *Sal*I

For 1 reaction:

DNA	17 µl (if TE)
Neb 4 buffer	2 µl
*Sal*I	1 µl
H_2O	qsp 20 µl

Incubate for at least 1h at 37°C (dry water bath)

- Check the digestion on a 0.8 % agarose gel. **Warning:** if no or partial digestion occurs, dialyse the samples for ½ h on a filter (0.05 µm pore size) and redigest the extract for 1 h at 37°C with 1 µl of *Sal*I + 2 µl of buffer
- Estimate the amount of DNA to be ligated on 0.8 % agarose gels by loading 2 µl of DNA/ *Sal*I + 2 µl of loading buffer.

c) Ligation of DNA to the linker

MIX	1 Reaction	20 Reactions
Ligase T4 buffer10X	2 µl	40 µl
Linker*	1 µl	20 µl
Ligase T4 (1 U/µl)	1 µl	20 µl
DNA	As estimated above	
H_2O	qsp 20 µl final	

* see appendix ligation mediated PCR for preparation
* always keep the linker on ice

<u>Ligation conditions:</u>

5 min	5°C
1 h	16°C
10 min	65°C

The reaction can be left at +4°C overnight.

d) Digestion of the ligation product with *Sal*I

Ligation product	20 µl
Buffer H	2.5 µl
*Sal*I	0.5 µl
H_2O	2.0 µl

- Incubate at 37°C for 15min
- Dilute the DNA 1/5 (i.e. 25 µl of ligation Mix + 100 µl of H_2O).

1.2 Amplification of the ligation product with the SALGD linker

	Initial conc.	1 Reaction	20 Reactions	Final conc.
H_2O	10 x	28.8 µl	576 µl	-
Taq buffer		5 µl	100 µl	1 x
DMSO		5 µl	100 µl	10 %
dNTP	10 mM (each)	1 µl	20 µl	200 µM
IS2	50 µM	1 µl	20 µl	1 µM
SALgd	50 µM	1 µl	20 µl	1 µM
$MgCl_2$	25mM	3 µl	60 µl	1.5 mM
Taq Cold	5 U/µl	0.2 µl	4 µl	1 U

Add 5 µl of DNA diluted 1 in 5 per reaction.

PCR Programme

Prog. 1	9 min – 94°C	
Prog. 2	30 sec – 94°C 30 sec – 55°C 3 min – 72°C	35 cycles
Prog. 3	10 min – 72°C	

Separate the products by electrophoresis for 1h ½ at 100V in 2.5 % agarose gel.
Load: 20 µl of the amplification product + 2 µl of loading buffer.
Load: 2 µl of 100 bp marker in the end lanes

1.3 Interpretation of the results

The number and size of fragments given by each sample is characteristic. If the results are ambiguous (different samples with very similar profiles), the technique should be repeated and/or another marker can be used.

1.4 Appendix ligation mediated PCR

a) Primers

Salgd TAG CTT ATT CCT CAA GGC ACG AGC
Salpt TCG AGC TCG TGC
IS 2 ACC CCA TCC TTT CCA AGA AC

b) Preparation of the linker

Reaction mix:

	Initial conc.	1 reaction	Final conc.
H_2O	-	148 µl	-
Taq buffer	10 x	20 µl	1 x
Salgd	500 µM	10 µl	25 µM
Salpt	500 µM	10 µl	25 µM
MgCl2	25 mM	12 µl	1.5 mM

Total volume of the MIX = 200 µl

Hybridisation:

Prog. 1 1 min at 80°C (denaturation)
Prog. 2 -1°C per min from 80 °C to 4°C

Divide the linker into 11 µl aliquots and store at -20°C.

1.5 Products ligation mediated PCR

Product name	Supplier	Reference
Agarose	Gibco BRL	15510027
Taq gold polymerase	Perkin-Elmer	M8080245
dNTP	Amersham	2703501
Buffer H	Roche diagnostic	1417991
Sal1	Gibco-BRL	15217-029
Neb 4 buffer	Biolabs	NEBUFFER 007-4
Ligase T4	Roche diagnostic	716359
Primers	Proligo	oligo@proligo.fr
TBE 10x	Sigma	T4415
Size marker	Invitrogen	156280.19

2. Spoligotyping

2.1 Sample preparation

- Place 150 µl of TE in 2 ml screw-cap tubes
- Using a disposable loop, collect a loop of culture medium containing sufficient colonies and suspend in the TE
- Incubate in the oven for 30 min at 100°C to extract the DNA and to kill the bacteria
- Store the DNA at -20°C after extraction.

Notes:
- The amplification step requires using very little DNA; it is therefore better to take fewer bacteria than to risk taking a large amount of medium.
- It is important that the bacteria are really heated to 100°C for at least 20 minutes to ensure that they have all been killed at this stage.

It is also possible to use DNA extracted by the phenol-chloroform method.

2.2 Preparation of the PCR mixture

The gene is first amplified with the Dra and DRb primers that anneal to sites within the DR and are directed outwards. DRs are direct repeat sequences of 36 bp
- DRa (GGTTTTGGGTCTGACGAC, 5' biotinylated)
- DRb (CCGAGAGGGGACGGAAAC).

The Dra primer is labelled with biotin. The biotin group binds to streptavidin, which is in turn coupled to peroxidase, thus making it possible to detect any amplification products.
Primer DRa is stored at 4°C and DRb is stored at -20°C.
In a sterile room, prepare the Mix for the numbers of reactions:

+ 1 TE control tube
+ 1 negative control tube

		For 1 reaction	For 10 Reactions	Final concentration
H_2O		14.9 µl	149 µl	
Tp chelating	5x	10.0 µl	100 µl	1 x
$MgCl_2$	50 mM	7.0 µl	70 µl	7 mM
Primer 1	5 µM	4.0 µl	40 µl	0.4 µM
Primer 2	5 µM	4.0 µl	40 µl	0.4 µM
dNTP	2.5 mM	8.0 µl	80 µl	0.4 µM
Tth polymerase	5U/ µl	0.1 µl	1 µl	0.5 U/µl

Place 48 µl of the mixture into each 0.5 µl sterile tube; in another room add 2 µl of DNA

PCR Programme:

Prog. 1	3 min – 96°C	
Prog. 2	1 min – 96°C 1 min – 55°C 30 sec – 72°C	30 cycles
Prog. 3	10 min – 72°C	

2.3 Hybridisation

The membranes must always be held by their edges.

1. Rinse the membrane twice in about 250 ml of 2xSSPE/0.1%SDS at 60°C for five minutes.
2. Place the membrane in the miniblotter that has previously been cleaned with soap and water and rinsed thoroughly, on a support cushion (that acts as a joint) such that the side with the oligunucleotides (spacer) faces the slots, ant so that the slots are perpendicular to the lines of oligonucleotides (spacers) on the membrane (use the date in the bottom tight corner as a marker).

Note: The membrane must be placed so that it is exactly aligned with the two ink lines in the upper and lower slots, so that the slots cross all the lines of oligonucelotide (spacers) fixed to the membrane.

3. Tightly shut the sic plastic screws to close the press
4. Dilute 20 µl of each of the PCR products (maximum of 43 / membrane) in 150 µl of 2 2xSSPE/0.1%SDS buffer. Include one negative control (TE alone) and two positive controls (DNAs from the strain H37Rv and from *M. bovis BCG*).
5. Heat each tube at 100°C for 10minutes, then immediately place on ice.
6. Use a vacuum pump to remove all the residual liquid from each to the slots of the miniblotter.
7. Fill the first slot of the miniblotter with 150 µl of buffer containing 2xSSPE/0.1%SDS.
8. Fill the remaining slots one by one (max 43) with 150 µl (approximately) of the diluted PCR products, kept at 4°C until loading. Note the loading order.
   Note: When filling the slots, take care not to introduce air bubbles (pipette slowly, in both directions, to avoid introducing bubbles). Each line must be completely filled, but care must be taken not to contaminate the slot underneath as this might lead to the contamination of neighbouring slots (use absorbent paper to eliminate excess).
9. Fill the last slot of the slot of the miniblotter with 150 µl of buffer containing 2xSSPE/0.1%SDS. The samples must always be surrounded by buffer to prevent leaking.
10. Incubate the membrane at 60 °C for one hour, keep the miniblotter horizontal throughout and do NOT shake to prevent overflow (and therefore contamination of one line by another).
11. Remove the samples from the slots by aspiration, in the same order as they were filled.
12. When all the slots are totally empty, dissemble the miniblotter and carefully remove the membrane.
13. Wash the membrane twice in250 ml of 2xSSPE/**0.5%**SDS at 60°Cfor 10minutesin a container with shaking.

(Possibly to interrupt the protocol

It is possible to interrupt the protocol at step 13. overnight for example. In this case, proceed to step 13'.

13'. Wash the membrane twice in 250 ml of 2xSSPE at room temperature for 10 minutes, in a container with shaking.
13''. Keep the membrane at 4°C until the following day, in a sealed plastic envelope or wrapped in Saran-wrap, to prevent it from drying out.

The following day, if interrupted at step 13:

13'''. Wash the membrane twice in 250 ml of 2xSSPE/**0.5%**SDS at 42°C for 10 minutes in a container with shaking.

Continue the protocol at step 14.)

14. Place the membrane in a hybridisation bottle, such that the side containing the oligonucleotides (spacers) is on the inside of the tube.
15. Check that the membrane is at a temperature below 42°C (so that the peroxidase is not inactivated).
16. Incubate the membrane in a solution of straptavidin-peroxidase conjugate diluted 1/4000 (3.5 µl of streptavidin-peroxidase conjugate in 14 ml of 2xSSPE/**0.5%**SDS), at 42°C, for 90 min.
17. Wash the membrane twice in 250 ml of 2xSSPE/**0.5%**SDS at 42°C fro 10 minutes, in a container with shaking.
18. Rinse the membrane twice in 250 ml of 2xSSPE fro at least 5 minutes at room temperature in a container with shaking.
19. Immediately before required, prepare 40 ml of E.C.L. detection liquid by mixing 20 ml of each of the solutions supplied in the kit.
20. Incubate the membrane in 40 ml of E.C.L. detection liquid for 2 minutes with gentle manual shaking so that the all of the membrane is in uniform contact with the liquid 8thee membranes repel water). Remove excess reagent with Whatman paper
21. Place the membrane in an autoradiography cassette inside a plastic sleeve. Remove any air bubbles, taking car not to create static electricity.
22. Expose an E.C.L film (Hyperfilm-ECL, Amersham) to the side of the membrane containing the oligonucleotides in a cassette for 1 minute
23. Develop the film
24. Expose another film for 5minutes and then another for 20minutes sot that even weak signals are all detected
25. Wash the membrane twice in 250 ml of 20 mM EDTA pH 8 for 15 minutes at room temperature in a container with shaking.
26. Until the dehybridisation step, store the membrane at 4°C in sealed plastic pouch or wrapped in Saran-wrap, to prevent it from drying.

2.4 Interpretation

The membrane is read by recording the presence or absence of signals at the sites of DNA/DNA hybridisations. Results can be interpreted using appropriate software.

2.5 Dehybridisation of the membranes

The membranes can be re-used if the oligonucleotides can be freed of the attached PCR products. As the oligonucleotides (spacers) are covalently fixed to the membranes, the removal method must be highly stringent.

1. Wash the membrane three times by incubating it in 250 ml of 1% SDS at 85°C for 30 minutes in a container with shaking
2. Wash the membrane twice in 250 ml of 20 mM EDTA pH 8 for 15 minutes at room temperature in a container with shaking.
3. Store the membrane at 4°C in a sealed plastic pouch or wrapped in Saran-wrap, to prevent it from drying out, until re-use.

2.6 Appendices to spoligotyping

a) Preparation of the buffers

All the buffers must be preheated to the desired temperatures just before use (42°C, 60°C or 80°C).

- 2xSSPE/0.1%SDS (60°C) 100 ml SSPE 20x + 5 ml –SDS 20% + H20 qsp 1 litre
- 2xSSPE/0.5%SDS (60°C) 100 ml SSPE 20 x + 25 ml SDS 20 % + H20 qsp 1 litre
Prepare 2 l for 2 membranes
- 2xSSPE/0.5%SDS (42°C) 100 ml SSPE 20 x + 25 ml SDS 20 % + H20 qsp 1 litre
Prepare 2 l for 2 membranes
- 2 SSPE (Room temperature) 200 ml SSPE 20 x + H20 qsp 2 litres
- EDTA 20 mM (Room temperature) 40ml EDTA 0.5 M + H20 qsp 1 litre

b) Materials

- membrane kit: positive and negative control, primer Dra and Drb and spoligo-membrane

Isogen Bioscience B.V. ; Industrieweg 68 ; Box 1179 ; BT Maarsen ; The Netherlands
- miniblotter MN45: Polylabo ref = 35209
- foam cushions: order number: PC200

Immunetics, 63 Rogers Street, Cambridge, Mass. 02139, USA
Sold by: Interchim s.a., BP 1140-03103 Montlucon, Cedex, France
-Tth DNA POLMERASE: (vials of 100, 250, 500 and 1000 units)
MgCl2- free 5x chelating amplification buffer is supplied with the enzyme.
Eurobio Ref 250U: 018191; Avenue de Sandinavie – 91953 Les Ulis Cedex B-France
- Streptavidin-POD conjugate, lyophilized, stabilized, 500U, Cat. N° 1089 153; Boehringer Manneim Biochemica
- ECL detection liquid; Cat N°RPN 2106 or 3000. Amersham International, Amersham France SA, Avenue des Tropiques, ZA Courtabeouf, Les Ulis. France.
- Hyperfilm ECL ; Cat. N° RPN 2103 Amersham International, Amersham France SA, Avenue des Tropiques, ZA Courtabeouf, Les Ulis. France.
- SSPE 20 x ; Gibco BRL Ref : 15595-035 (4l)
- SDS; Sigma Ref: L-4509

3. MIRU- and ETR-VNTR typing

Sample preparation and PCR amplification:
Each MIRU and ETR locus was amplified individually with primers specific for sequences flanking the MIRU and ETR units (Table 1). The reaction mixture for all loci contained a 1-µl DNA sample, 1x *Taq* PCR buffer, 0.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems), deoxynucleoside triphosphates (0.2 mM each; Amersham Pharmacia Biotech, Piscataway, N.J.), and a 0.5 µM concentration of the primer pair in a final volume of 20 µl. For the amplification a GeneAmp 9700PCR system (Perkin-Elmer Applied Biosystems) was used.

PCR Programme:

Prog. 1	1 min – 94°C	
Prog. 2	30 sec. – 94°C 30 sec. – 65°C 1 min. – 72°C	40 cycles
Prog. 3	10 min – 72°C	

Oligonucleotide	Sequence	Position[a]	Predicted size of amplicon containing 1 MIRU copy + size of additional copies (bp)
miru 2a	5'CATCGAATTGGACTTGCAGCAAT	153941	580 + 53
miru 2b	5'CGACGTCGTAGAGAGCATCGAAT	154521	
miru 4a	5'GTCAAACAGGTCACAACGAGAGGAA	580540	191 + 77
miru 4b	5'CCTCCACAATCAACACACTGGTCAT	580831	
miru 10a	5'ACCGTCTTATCGGACTGCACTATCAA	960130	273 + 53
miru 10b	5'CACCTTGGTGATCAGCTACCTCGAT	960508	
miru 16a	5'CGGGTCCAGTCCAAGTACCTCAAT	1644034	422 + 53
miru 16b	5'GATCCTCCTGATTGCCCTGACCTA	1644508	
miru 20a	5'GCCCTTCGAGTTAGTATCGTCGGTT	2059297	298 + 77
miru 20b	5'CAATCACCGTTACATCGACGTCATC	2059671	
miru 23a	5'CGAATTCTTCGGTGGTCTCGAGT	2531862	130 + 53
miru 23b	5'ACCGTCTGACTCATGGTGTCCAA	2532256	
miru 24a	5'CGACCAAGATGTGCAGGAATACAT	2686949	447 + 52
miru 24b	5'GGGCGAGTTGAGCTCACAGAA	2687395	
miru 26a	5'GCGGATAGGTCTACCGTCGAAATC	2995975	297 + 51
miru 26b	5'TCCGGGTCATACAGCATGATCA	2996373	
miru 27a	5'TCTGCGTGCCAGTAAGAGCCA	3006884	330 + 53
miru 27b	5'CTGATGGTGACTTCGGTGCCTT	3007319	
miru 31a	5'CGTCGAAGAGAGCCTCATCAATCAT	3192174	162 + 53
miru 31b	5'AACCTGCTGACCGATGGCAATATC	3192441	

miru 39a	5'CGGTCAAGTTCAGCACCTTCTACATC	4348555	712 + 53
miru 39b	5'CTCGGTGTTCCTTGAAGGTGGTTT	4349319	
miru 40a	5'GATTCCAACAAGACGCAGATCAAGA	802236	284 + 54
miru 40b	5'TCAGGTCTTTCTCTCACGCTCTCG	802519	

Locus name	Sequence of PCR primers (5'-3')	Location in H37Rv map (kb)	No. and size of repeat units in H37Rv	Size of PCR product in H37Rv
ETR-Aa	5'AAATCGGTCCCATCACCTTCTTAT	3820	(3x75)-23	420
ETR-Ab	5'CGAAGCCTGGGGTGCCCGCGATTT			
ETR-Ba	5'GCGAACACCAGGACAGCATCATG	4160	(3x57)-8	292
ETR-Bb	5'GGCATGCCGGTGATCGAGTGG			
ETR-Ca	5'GTGAGTCGCTGCAGAACCTGCAG	1480	(4x58)-21	276
ETR-Cb	5'GGCGTCTTGACCTCCACGAGTG			

Table 1: Primers of MIRUs (above) and ETRs (below) used for VNTR typing

[a]Refers to position in M. tuberculosis strain H37Rv unless otherwise indicated bay n alternative GenBank number

Electrophoresis:

The PCR products were analyzed on a 2.5% agarose (Gibco-BRL Products, Grand Island, N.Y.) gel in 1x Tris-borate-EDTA containing 1 µg of ethidium bromide/ml using the Sub-cell Model 192 apparatus (Bio-Rad, Hercules, Calif.), a 25- by 25-cm gel tray, and two rows of 51 wells (well width, 0.75 mm). 10 µl of a size marker was loaded in each side lane of the gel. For each amplification product, 5 µl of PCR product + 2 µl of loading buffer per well, such that samples form the same MIRU/ETR pair are side by side. Subject to electrophoresis for 5 hours at 120 V. After, the gel was observed under UV light and a photo taken. The number of MIRU repeats at each locus was determined by the size of the amplicon, using the convention described in Table 2.

Allele	**MI 2**	**MI 4**	**MI 4*a***	**MI 10**	**MI 16**	**MI 20**	**MI 23**	**MI 24**	**MI 26**	**MI 27**	**MI 31**	**Mi 39**	**Mi 40**	**ETR A**	**ETR B**	**ETR C**
0	527	114	61	220	369	221	77	395	246	277	109	659	230	195	121	44
1	580	191	138	273	422	298	130	447	297	330	162	712	284	270	178	102
2	633	268	215	326	475	375	183	499	348	383	215	765	338	345	235	160
3	686	345	292	379	528	452	236	551	399	436	268	818	392	420	292	218
4	739	422	369	432	581	529	289	603	450	489	321	871	446	495	349	276
5	792	499	446	485	634	606	342	655	501	542	374	924	500	570	406	334
6	845	576	523	538	687	683	395	707	552	595	427	977	554	645	463	392
7	898	653	600	591	740	760	448	759	603	648	480	1030	608	720	520	450
8	951	730	677	644	793	837	501	811	654	701	533	1083	662	795	577	508
9	1004	807	754	697	846	914	554	863	705	754	586	1136	716	870	634	566
10	1057	884	831	750	899	991	607	915	756	807	639	1189	770	945	691	624
11	1110	961	908	803	952	1068	660	967	807	860	692	1242	824	1020	748	682
12	1163	1038	985	856	1005	1145	713	1019	858	913	745	1295	878	1095	805	740
13	1216	1115	1062	909	1058	1222	766	1071	909	966	798	1348	932	1170	862	798
14	1269	1192	1139	962	1111	1299	819	1123	960	1019	851	1401	986	1245	919	856
15	1322	1269	1216	1015	1164	1376	872	1175	1011	1072	904	1454	1040	1320	976	914

Table 2: Number of MIRU and ETR-VNTR repetitions depending on the size of amplicons (bp);

a Number of MIRU-VNTR repetitions specific to locus 04 (53 bp unit missing).

4. IS6110-Restriction Fragment Length Polymorphism typing

4.1 Extraction of genomic DNA

Operations to be carried out in a Category 3 Laboratory

- Collect an adequate number of colonies on Lowenstein-Jensen medium and suspend them in 500 µl of TE (see appendix IS*6110*-RFLP)
- Place at 90°C for 30 min.

Operations NOT to be carried out in a Category 3 Laboratory

- Centrifuge the extracts
- Discard the supernatant and add 50 µl or 100 µl of 20 mg/ml lysozyme (see appendix IS*6110*-RFLP) to the pellet, according to the size of the pellet.
- Incubate for 1h at 37°C.
- Add 70 µl of 10% SDS + 5 µl of 10 mg/ml proteinase K (see appendix IS*6110*-RFLP), or twice as much if the pellet is large.
- Incubate for 1 h at 65 °C
- Add 100 µl of 5 M NaCl

Steps to be carried out under the Sorbonne flow hood

- Add 1 volume of aquaphenol containing 8-beta-hydroxyquinoline to the extract, mix thoroughly and then centrifuge for 15 min in a micorcentrifuge at 149000 rpm at 4°C.
- Transfer the aqueous phase into a clean Eppendorf tube.

Note: the aqueous phase is transparent and is generally the upper phase. The phenolic phase is yellow and is generally the lower phase. The phases may be inversed if the interface is very large. In this case, or if the aqueous phase is cloudy, take the aqueous phase and repeat the phenol extraction step.

- Add an equal volume of chloroform, mix and centrifuge for 1 min at 14900 rpm.
- Transfer the aqueous supernatant to a clean Eppendorf tube.
- divide the sample into two if its volume exceeds 600 µl.
- Add 1.5 volumes of absolute ethanol (-20°C) to precipitate the nucleic acids.
- Leave **overnight** at -20°C or for 30 min at -70°C

→ Possible to interrupt

- Centrifuge for 15 min at 14900rpm and 4°C
- Discard the supernatant by tipping the Eppendorf tube.
- Wash the pellet with 100 µl of 70% ethanol at -20°C.
- Centrifuge for 5 min at 14900 rpm and 4°C
- Discard the supernatant and dry the pellet in a speed-vac for 15 min or under the flow hook.
- Resuspend the DNA pellet in 20 µl of TE
- Incubate for 20 min in a 60°C water bath.
- Homogenise the sample with a pipette.
- To estimate the concentration of the DNA, run an aliquot in a 0.8% agarose electrophoresis gel (2 µl of extract + 2 µl of loading buffer per well)

4.2 Digestion of genomic DNA

- After estimating the concentration of the DNA, digest the equivalent of 2 µg of DNA with the restriction enzyme as follows (amount per reaction):

    - X µl of DNA
    - 3 µl of enzyme buffer
    - 2 µl of *Pvu*II
    - H_2O (ultrapure) to a final volume of 30 µl

- Leave the enzyme to act for at least 1 h at 37°C or **overnight** at 37°C.

→ Possible to interrupt

- Subject to electrophoresis in a 0.8% agarose gel in 1X TBE to check the digestion and to estimate the amount of DNA to load for the Southern blot.

Note: If the bands nearest the top of the gel (high molecular weights) are less intense than the bands corresponding to lower molecular weights, the digestion has been satisfactory.
(In the case of incomplete or no digestion:
- Dialyse the sample with a Millipore filter (0.05 µm pore size): place the entire sample on the filter, leave contact with the water for 30 min and collect the DNA.
- Repeat the digestion step with:
  - Dialysed DNA
  - 3 µl of enzyme buffer
  - 1 µl of *Pvu*II
- Leave to digest for at least 1 h at 37°C.
- Check the digestion on a 0.8 % agarose gel.)

4.3 Electrophoresis

- Load the equivalent of 2 µg of digested DNA + 2 µl of loading buffer, on a 1 % agarose gel in 1X TBE containing EtBr.
- Do not load samples in the first or last wells.
Load 10 µl of the lambda *Pst*I external marker (appendix IS*6110*-RFLP) in the first lane to monitor the migration.
- Every 5 wells, load the external molecular weight marker: DNA from *M. tuberculosis* strain MT 14323 digested with *Pvu*II
- Start the electrophoresis at 90V for 10 min (until the DNA has left the wells) and then continue **overnight** at 40V (i.e. 1-2 V/cm)
→ Possible to interrupt
- The following day, stop the electrophoresis when the highest λ molecular weight marker (i.e. the 11500-bp band) is 2 cm from the wells and take a photo.

4.4 Treatment of gels

a) Dupurination

- Wash the gel with a solution of 0.25 M HCl (appendix IS*6110*-RFLP) for 10 min, for depurination
- This treatment facilities the DNA transfer step by breaking up the large molecular weight molecules.
Warning: Prolonged treatment can lead to excessive hydrolysis of the DNA and generate DNA fragments that are too small to adhere to the membrane correctly.
- Rinse the gel with distilled water for 1-2 min.

b) Denaturation

-Cover the gel with the denaturing solution (appendix IS*6110*-RFLP) for 2 x 20 min periods with shaking.
- Rinse the gel with distilled water for 1-2 min.

c) Neutralisation

Cover the gel with the neutralising solution (appendix IS*6110*-RFLP) for two 20 min periods with shaking.

4.5 Transfer (Southern Blot) of nucleic acids onto membranes

a) Vacuum transfer

- Cut a piece of Hybond N+ membrane and a piece of Whatman paper to the size of the gel.
- Soak the Whatman with water and place it on the porous plate, then soak the Hybond N+ membrane with water and place on top, taking care not to form any air bubbles.
- Place the gel in the middle of this pocket.
- Adjust the clips on the apparatus
- Fill the gel wells with 1 % agarose, ensuring that no bubbles are formed
- Connect the vacuum pump. For the transfer of genomic DNA from an agarose gel, the recommended conditions are 45-60 mbar for 60-90min.
- Cover the gel with 20x SSC buffer.

b) After the transfer

- Before removing the transferred gel, use a pencil to mark the positions of the first and last wells.
- Remove the gel and collect the membrane
- Wrap the membrane in Saran Wrap.
- Fix the DNA to the membrane by exposing to UV light, either for 4 min at 365 nm or for 1 min at 254 nm (DNA side towards the UV light source)
- Check the transfer efficiency by examining the gel under UV light and ensuring that all traces of DNA have disappeared
- The fixed membrane can be stored at 4°C

4.6 Hybridisation and detection

a) Prehybrdisation

- Place the membrane in a glass hybridisation tube such that the DNA faces the inside of the tube.
- Prehybridise the membrane for 45 to 60 min at 42°C with rotation in hybridisation buffer (appendix IS*6110*-RFLP). Voulume of buffer required = 0.125 ml / cm^2 of membrane.

b) Labelling the probe

ECL Kit protocol:

- Dilute the probe to a concentration of 10 ng/µl with the water supplied in the ECL Kit.
- Heat to 100°C for 5 min, then leave at 0°C for 5 min
- Add an equal volume of labelling reagent
- Add the same volume of glutaraldehyde.
- Incubate for 10 min at 37°C.
- Keep the labelled probe on ice until use.

Note; The labelled probe can be stored in 50% glycerol at -20°C for 6 months.

c) Hybridisation

- Transfer the hybridisation buffer into a clean tube and add the labelled probe.
- Mix by rotating
- Place the buffer + probe in the hybridisation tube with the membrane
- Incubate overnight at 42°C with rotation

d) Washes

- The following day, discard the hybridisation buffer.
- Wash the membrane with buffer I (appendix IS*6110*-RFLP) at 55°C with shaking: 2 x 10 min
- Wash the membrane with buffer II (appendix IS*6110*-RFLP) at room temperature with shaking: 2 x 5 min

e) Detection

- With the ECL kit: mix 15 ml of detection reagent 1 with 15 ml of detection reagent 2 in a tube (in the dark).
- Dry the membrane on a sheet of Whatman paper
- Place the membrane in a container with the DNA side outwards
- cover the membrane with the detection mixture and swirl manually for 1 min
- Remove the membrane in an autoradiography film cassette and cover with a sheet of Saran Wrap
- Place a film (Hyperfilm) on the membrane, shut the cassette and leave for 1 min
- Remove the film and immediately replace with another.
- Develop the first film and estimate the exposure time necessary for the second

4.7 Appendix IS*6110*-RFLP

a) Preparation of the IS*6110* probe

The IS*6110* probe is generated from a PCR product.
An 868-bp fragment, corresponding to nucleotides 462 to 1330 of the IS*6110* sequence of a strain belonging to the M. tuberculosis complex, is amplified (MT 14323). The oligonucleotides used as primers as follows:

G1: 5' CTGACCGAGCTGGGTGTGCC
G2: 5' TCTGATCTGAGACCTCAGCC

Preparation of the MIX for 10 reactions

REAGENT	QUANTITIY	FINAL CONCENTRATION
H_2O	235 µl	
Taq Buffer 10 x	50 µl	1 x
DMSO	50 µl	10 %
$MgCl_2$ 25 mM	30 µl	1.5 mM
dNTP 10 mM	10 µl	200 µM (each)
G1 50µM	10 µl	1 µM
G2 50 µM	10 µl	1 µM
Taq 5U/µl	5 µl	2.5 U

- Place 40 µl of MIX into each PCR tube.
- Add 10 µl of MT 14323 DNA diluted 1/100 (extracted by the Phenol/Chloroform method)

PCR conditions:

Prog. 1	6 min – 94°C	
Prog. 2	1 min – 94°C 2 min – 55°C 2 min – 72°C	35 cycles
Prog. 3	10 min – 72°C	

- Subject the PCR product to electrophoresis on a 0.8 % agarose gel
- Electrophoresis conditions with a 50 ml miniprep: ½ h at 90 V.
- Load: 1 µl of DNA + 2 µl loading buffer
- Prepare a 1 % gel with LMP agarose for a Miniprep apparatus, i.e. with 50 ml of 1 x TBE
- Load 100 bp marker in the end lanes, and all of the amplified samples in the central lane, i.e. 500 µl of PCR product + 55 µl of loading buffer.
- Leave for 30 min to 1h30 at 100 V. (Monitor the migration of the 800-bp band)
- Identify the 800-bp band by use of the marker, cur the band out, taking care not to take too much agarose.
- Put the 800-bp fragment in a Petri dish.
- Weigh the agarose plug and place it in an eppendorf

With the QIAGEN extraction kit (Qiaquick Gel Extraction Kit protocol)

- Add 3 volumes of QG buffer to 1 volume of agarose (100 mg agarose = 100 µl)
- Incubate at 50°C fro 10 min (the gel must be completely dissolved), vortex gently every 3 min during the incubation period
- When the agarose is completely dissolved, the liquid becomes yellow
- Add 1 volume of isopropanol and mix
- Place 1Qiauick column on a 2 ml tube.
- Place the melted gel containing the DNA to be purified on the column and centrifuge for 1 min at 13000 rpm
- Discard the liquid in the 2 ml tube and place 0.5 ml of QG buffer on the column, centrifuge for 1 min at 13000 rpm
- Wash the column with 0.75 ml of PE buffer and centrifuge for 1 min at 13000 rpm
- Discard the liquid in the tube and centrifuge again.
- Place the column on a 1.5 ml eppendorf tube and elute the DNA with 50 µl of the H_2O provided in the ECL kit for labelling the probe (see below: labelling the probe)
- Subject to electrophoresis in a 0.8 % agarose gel in 1 x TBE for 30 min at 90 V
- Load:
    - lane n°1= 10 µl λ *Pst*I + 2 µl of loading buffer

- lane n°2= 1 µl IS6110 to be quantified + 10 µl H_2O + 2 µl of loading buffer
- lane n°3= 1 µl λ *Hin*dIII (10 ng) + 10 µl H_2O + 2 µl of loading buffer
- lane n°4= 5 µl λ *Hin*dIII (50 ng) + 10 µl H20 + 2 µl of loading buffer
- lane n°5= 10 µl λ *Hin*dIII (100 ng) + 10 µl H20 + 2 µl of loading buffer

Labelling the probe:

After calculating the concentration of the purified probe, calculate the amount of probe to be labelled.
Example: Amount of probe required for a 120 cm^2 membrane
Final concentration of the probe to be 1 ng/ cm^2
Thus 120 ng for a 120 cm^2 membrane
If for example, the concentration of the probe is 10 ng/µl, 12 µl of probe is required
For the labelling mix: 12 µl of the probe at a concentration of 10 ng/µl
+ 12 µl Labelling Reagent
+ 12 µl of Glutaraldehyde
Total= 36 µl of labelled probe.

b) Solutions and materials used

Product name	Supplier	Reference	Reference IP
Tris-Trizma base	SIGMA	T 1503	52150
Boric Acid	SIGMA	B-6768 (1 kg)	-
ETA	SIGMA	E-5134 (1 kg)	-
Lysozyme	SIGMA	L-6876 (5kg)	-
Proteinase K	ROCHE-Boehringer	1092766 (1 g)	90428
SDS	SIGMA	L-4509 (500 g)	
Sodium chloride (NaCl)	PROLABO	27 810 295 (500 g)	48700
Phenol	APPLIGENE	130181 (250 ml)	-
8-beta-hydroxquinoline	SIGMA	H6878	-
Chloroform	CARLO ERBA	438601	49520
Ethanol	MERCK	983	44656
*Pvu*II	GIBCO BRL	15 4120 18	95034
*Pst*I	GIBCO BRL	15 2150 23	90531
Hydrochloric acid (HCl)	PROLABO	20 252 290	44160
Tri-Sodium citrate	PROLABO	27 833 294	48740
Sodium hydroxide (NaOH)	PROLABO	28 245 298	48880
Filter 0.05 µM/25 mm	MILLIPORE	VMWP02500	-
Whatman paper	3 MM	3030 917	-
Hybond-N+ membrane	AMERSHAM	RPN 303 B	90803
Hyperfilm ECL	AMERSHAM	RPN 2103 K	96098
ECL Kit	AMERSHAM	RPN 3000	97059
Qiaquick Gel Extraction Kit	QIAGEN	28704	42792
Bromophenol Blue	SIGMA	B5525	-
Agarose	GIBCO BRL	15510027	52171 (500 g)
Ethidium bromide (EtBr)	EUROBIO	17589	99025
Taq	PERIKNELMER	M8080161	90085
dNTP	AMERSHAM	2703501	90005
TBE 10 x	SIGMA	T4415	52155 (4 l)
Lambda DNA size markers	PHARMACIA	27-4111-01(500 µg/ml)	-
100 bp size markers	GIBCO-BRL	156280.19	90637

- Lysozyme: 20 mg/ml stock solution, divided into aliquots and frozen
- Proteinase K: 10 mg/ml stock solution, divided into aliquots and frozen
- SDS 10 % in distilled H_2O
- NaCl 5m
- TE: 1 ml Tris 1 M (=> 89 mM), 0.2 ml EDTA 0.5 M (=>89mM), 100 ml H_2O qsp
- Phenol, saturated in water (pH 8) and coloured with 0.1 % 8-beta-hydroxyuinoline
- TBE 10 x pH 8: Tris 89 mM, Boric Acid 89 mM, EDTA 2 mM
- Bromophenol blue solution: 0.25 % bromophenol blue + 40 % sucrose
- Preparation of lambda-*Pst*I marker

Digest 100 µl of lambda DNA with *Pst*I in a final volume of 250 µl
Check the digestion by agarose gel electrophoresis
Add 75 µl of the bromophenol blue solution and 175 µl of TE buffer pH 8
Store the marker in 50 µl aliquots at + 4°C

- Depurination solution: HCl 0.25 M = 13 ml HCl + 500 ml of H_2O
- Denaturation solution: NaCl 1.5 M / NaOH 0.5 M
- Neutralising solution: NaCl 1.5 M / Tris 1 M pH 8
- SSC buffer 20 x: NaCl (=> 3 M) 175.3 g/l, Na-Citrate (=> 0.3 M) 88.2 g/l, H20 1 l qsp, Adjust the pH to 7 with 5M NaOH
- Hybridisation buffer (Amersham Kit)

Heat the hybridisation buffer supplied in the kit to 65°C.
Adjust the concentration of NaCl to 0.5 M
Add 5 % (w/v) blocking agent
Mix, taking care not to allow any lumps to form, using a magnetic stirrer for 1 h at 65°C
Store 30 ml aliquots of the buffer at – 20°C for up to 3 months
For 200 ml of ECL buffer: 5.84 g of NaCl + 10 g of blocking agent

- Washing buffer n°1 (SSC 0.5 x, SDS 0.4 %): 25 ml SSC 20 x, 20 ml SDS 20 %, 1 l H_2O qsp
- Washing buffer n°2 (SSC 2 x,): 100 ml SSC 20 x, 1 l H_2O qsp.

Curriculum vitae

Name/Prename: Hilty Markus
Date of birth: 30.01.1976
Nationality: Liechtenstein
Address: Josefstrasse 192, 8005 Zürich, Switzerland
Phone number: +41 1 2715439
E-Mail: Markus.Hilty@unibas.ch

Education

01/2003-03/2006 **Swiss Tropical Institute (STI) of Basel, Switzerland**

Ph.D., Microbiology, molecular-epidemiology

Thesis title: *Molecular epidemiology of mycobacteria: Development and refinement of innovative molecular typing tools to study mycobacterial infections.*

Supervisors: PD Dr. Jakob Zinsstag, Prof. Marcel Tanner

10/1996-05/2001 **Swiss Federal Institute of Technology (ETH) of Zurich, Switzerland**

Master degree of natural Science, Bio- and Organic chemistry

Master thesis title: *Benzophenones as photo activable surface markers for Haemagluthinin* (German).

Mark thesis: 5.5 (magna cum laude)

Supervisor: Prof. Josef Brunner

Post graduate research experience

01/2003-03/2006 **Swiss Tropical Institute (STI) of Basel, Switzerland**

Department of Public health and epidemiology

Supervisors: PD Dr. Jakob Zinsstag, Prof. Marcel Tanner

10/2001 **Swiss Federal Institute of Technology (ETH) of Zurich, Switzerland**

Institute of Biochemistry.
Post graduate research work title: Benzophenones as photo activable surface markers for Haemagluthinin

07/2001 - 09/2001 **University of Kiev, Ukraine**

Institute of Biochemistry.
Practical work title: Solubilisation of fibrin clots by specific enzymes

Fieldwork and -experience

01/2003-03/2006 **'Support en Santé Internationale (CSSI)' and 'Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha (LRVZ)' of N'Djaména, Chad**

Collection and processing of samples within the Ph.D. research project. Responsibility: Contribute to maintaining the mycobacterial unit of LRVZ.

03/2002 – 10/2002 **Total Community Mobilisation against HIV/AIDS of Francistown and Kasane, Botswana**

Employment within a non governmental organisation. Responsibilities: Presenting of courses about 'anti viral therapy', 'time management and teamwork' and 'health and hygiene' and supervision of the work of local collaborators.

Meetings and Seminars attended

6th International Meeting on Microbial Epidemiological Markers. Molecular epidemiology and transmission dynamics of human and bovine tuberculosis in Chad and Mauritania (Poster). Les Diablerets, Switzerland. August 27 - 30, 2003

Course and practical work: Molecular tools and epidemiology of tuberculosis. Paris, France. September 1-12, 2003

Swiss Meeting for Doctoral Students in Parasitology and Tropical Medicine. State of surveillance of tuberculosis (TB) transmission in Chad (oral presentation). Müncheswiler, Switzerland. October, 2003

63rd annual meeting of the Swiss Society for Microbiology. Molecular epidemiology and transmission dynamics of human and bovine tuberculosis in Chad and Mauritania (poster). Lugano, Switzerland. March 11-12, 2004

Annual Congress of the Swiss and the German Society of Tropical Medicine and Parasitology. Molecular epidemiology and transmission dynamics of human and bovine tuberculosis in Chad and Mauritania (poster). Würzburg, Germany. September 23-25, 2004

Mitgliederversammlung der Schweizerischen Vereinigung für Tierpathologie. Molecular epidemiology of *Mycobacterium bovis* in Chad: A concern for humans? (oral presentation). Bern, Switzerland. June 24, 2005

4th International Conference on *Mycobacterium bovis*. Evaluation of the discriminatory power of Variable Number Tandem Repeat typing of *Mycobacterium bovis* strains from Chad (poster). Dublin, Ireland. August 22-26, 2005

Annual Congress of the Swiss Society of Tropical Medicine and Parasitology.
- Evaluation of the discriminatory power of Variable Number Tandem Repeat typing of *Mycobacterium bovis* strains from Chad (poster).

- Epidemiology and economics of *Mycobacterium bovis* and its control (oral presentation). Ascona, Switzerland. November 2-3, 2005

International Mini-symposium on human and animal health. Molecular epidemiology of human and animal tuberculosis in the Sahel (oral presentation). Basel, Switzerland. December, 2005.

Publications

Hilty, M. +, D. Yeboah-Manu,+ D. Boakye, E. Mensah-Quainoo, S. Rondini, E. Schelling, D. Ofori-Adjei, F. Portaels, J. Zinsstag and G. Pluschke. Genetic diversity in *Mycobacterium ulcerans* isolates from Ghana revealed by a newly identified locus containing a variable number of tandem repeats. **Journal of Bacteriology** 2006 Feb;188(4):1462-5
+ contributed equally

Diguimbaye-Djaibé C.+, **Hilty M.+,** Ngandolo R, Mahamat HH., Pfyffer G. E., Baggi F., Hewinson G., Tanner M., Zinsstag J. and Schelling E. *Mycobacterium bovis* Isolates from Tuberculous Lesions in Chadian Zebu Carcasses. **Emerging Infectious Diseases** 2006;12(5):769-771
+ contributed equally

Hilty, M., C. Diguimbaye, E. Schelling, F. Baggi, M. Tanner, and J. Zinsstag. Evaluation of the discriminatory power of variable number tandem repeat (VNTR) typing of *Mycobacterium bovis* strains. **Veterinary Microbiology** 2005 Aug;109(3-4): 217-222.

Colette Diguimbaye, Markus Hilty, Richard Ngandolo, Hassane H. Mahamat, Gaby E. Pfyffer, Franca Baggi, Marcel Tanner, Esther Schelling, and Jakob Zinsstag. Molecular characterization and drug resistance testing of *Mycobacterium tuberculosis* isolates from Chad. **Journal of Clinical Microbiology** 2006 Apr;44(4):1575-7

Anthony Ablordey, **Markus Hilty,** Pieter Stragier, Jean Swings, and Françoise Portaels. Comparative nucleotide sequence analysis of polymorphic variable-number tandem-repeat Loci in *Mycobacterium ulcerans*. **Journal of Clinical Microbiology** 2005 Oct; 43(10):5281-4

C. Diguimbaye-Djaibé, V. Vincent, E. Schelling, **M. Hilty,** R. Ngandolo, HH. Mahamat, G. Pfyffer, F. Baggi, M. Tanner, and J. Zinsstag. Species identification of non-tuberculous mycobacteria from humans and cattle of Chad. **Schweizerisches Archiv für Tierheilkunde** 2006;148(5):251-6

Sebastien Gagneux, Kathryn DeRiemer, Tran Van, Midori Kato-Maeda, Bouke C. de Jong, Sujatha Narayanan, Mark Nicol, Stefan Niemann, Kristin Kremer, M. Cristina Gutierrez, **Markus Hilty**, Philip C. Hopewell and Peter M. Small. Variable host-pathogen compatibility in *Mycobacterium tuberculosis.* **Proceedings of the National Academy of Sciences of U S A** 2006 Feb 21;103(8):2869-73