Identification and genotyping of *Mycobacterium tuberculosis* complex infections at the human/domestic animals/wildlife interface in Nigeria and South Africa

By

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LIST OF ABBREVIATIONS

AE	Agarose electrophoresis
AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
BCG	Bacille Calmette Guerin
CE	Capillary electrophoresis
CERVA-CODA	Centre d'Etude et de Recherches Vétérinaires et Agrochimiques
CT	Cattle traders
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleotide triphosphate
DR	Direct repeat
EAI5	East Africa and India 5
EPTB	Extra pulmonary tuberculosis
ETRs	Exact tandem repeats
HiP	Hluhluwe iMfolozi Park
HIV	Human Immunodeficiency virus
IF	Infant feces
LAM10-CAM	Latin America 10-Cameroun
$MgCl_2$	Magnesium chloride
MIRUs	Mycobacterial Interspersed Repetitive Units
MLVA	Multiple Locus VNTR analysis
MOT	*Mycobacterium* Outside Tuberculosis Complex
MTBC	*Mycobacterium tuberculosis* Complex
MTUB	*Mycobacterium tuberculosis*
ND	Not determined

No Amp	No amplification
NTM	Non tuberculous mycobacteria
PTB	Pulmonary Tuberculosis
RD	Region of Difference
RNA	Ribonucleic acid
SB	Spoligopattern
SolDb4	International Spoligopattern database
SICTT	Single Intradermal Comparative Tuberculin Test
VNTR	Variable number of tandem repeats
ZN	Ziehl Neelsen

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SUMMARY

The relevance of the use of molecular tools in the global epidemiology of *Mycobacterium tuberculosis* complex (MTBC) cannot be undermined. Molecular epidemiological studies of the MTBC in Nigeria are not extensive, and to date, there has only been one detailed report. More strains are therefore needed to be genotyped in order to give a clear indication of disease transmission chains and to highlight routes of infection particularly with respect to zoonotic tuberculosis. This study therefore focuses on the identification and genotyping of MTBC isolates in south western Nigeria, with emphasis on interactions occurring at the human/livestock interface. The molecular epidemiology of *M. bovis* strains in Hluhluwe-iMfolozi Park in South Africa was also undertaken.

Prior to this study, a pilot study was initially done to establish techniques, using samples from Belgium. *Mycobacterium bovis* strains were first identified in Belgium using the Multiple locus [variable number of tandem repeats] (MLVA) technique and analysis was done using capillary electrophoresis. In this study, the Belgium isolates were repeated using MLVA and analysis by agaorse gel electrophoresis and the two analysis techniques compared.

Human isolates (136) and livestock isolates from cattle (50), pigs (12) and goats (5) isolated in Nigeria were also used and species identification of the members of the MTBC were done using the deletion analysis PCR technique amplifying RD1^{mic}, RD2^{seal}, RD4 andRD9 regions as well as spoligotyping. Seventy four positive MTBC strains (humans and livestock) were genotyped using 16 VNTR loci. The discriminatory ability of the 16 loci MLVA was compared with spoligotype data on 33 MTBC strains. *Mycobacterium bovis* isolates from buffalo in Hluhluwe-iMfolozi Park (HiP) South Africa, were also genotyped using the 16 loci MLVA and spoligotyping.

Results indicated that agarose based MLVA is as discriminatory as the capillary based MLVA. Furthermore, the relevance of molecular techniques in the rapid identification and genotyping of

members of the MTBC, especially in a tuberculosis endemic setting like Nigeria, is also highlighted. This was clearly seen in the identification of undescribed spoligopatterns of the LAM 10-CAM *M. tuberculosis* strains in humans as well as the identification of undescribed *M. bovis* spoligopatterns in livestock isolates. The prevalent *M. bovis* strain (SB0944) in Nigeria was also identified in a human isolate. Also, two classical *M. bovis* strains were identified in two human isolates obtained from cattle traders, thus suggesting the influence of close interaction between infected animals and man as a means of zoonotic tuberculosis transmission. *Mycobacterium tuberculosis* was also identified in three isolates, from cattle, pig and goat; with the goat isolate having a spoligopattern (EAI5) typical of strains indigenous to East Africa and India. This study demonstrated the prevalent strains of *M. bovis* and *M. tuberculosis* circulating in Nigeria with SB0944 the predominant *M. bovis* spoligotype and LAM10-CAM the predominant *M. tuberculosis* spoligotype. The MLVA results revealed the occurrence of interspecies transmission of mycobacterial species, which was seen as isolates from different animal species having identical VNTR profiles and thus belonging to the same genotype.

In the HiP, two strains of *M. bovis* were identified, a strain previously described in cattle and buffalo in other regions of South Africa and a new undescribed strain, thus giving an indication of the circulating strains in HiP and also suggesting possible sources of introduction of novel species in HiP.

The relevance of a detailed molecular epidemiological study was clearly demonstrated in both Nigeria and HiP. Strain relatedness and interactions occurring at human/livestock interface and domestic/wild life interface could also clearly be demonstrated.

CHAPTER ONE

Introduction and Literature review

➢ Identification, evolution and genotyping of *Mycobacterium tuberculosis* complex

➢ Epidemiology of *Mycobacterium tuberculosis* complex in Nigeria

1.1. Introduction

Tuberculosis (TB) remains the most prevalent infectious disease in the world with one-third of the humanity being infected with members of the *Mycobacterium tuberculosis* complex (MTBC) (Sola *et al.,* 2003). *Mycobacterium bovis* infections are of major importance in many developed and developing countries (Van Soolingen. 2001) and it was stated by Acha and Szyfres (1987) that the most common cause of human tuberculosis is *M tuberculosis* with an unknown proportion of cases due to *M. bovis. Mycobacterium bovis* has clearly been described as an organism that is pathogenic to most mammals; humans, livestock and wildlife in general hence its zoonotic relevance. There are also many susceptible animal species i.e. goats, rabbits, sheep, badgers and pigs which are spill over hosts of the infection and in which the infection is not confined (Allix *et al.,* 2006).

Bovine tuberculosis is a zoonosis and the transmission to humans constitutes an important public health problem (Thoen and Steele, 1995). The prevalence of human pulmonary tuberculosis in Nigeria is high because of the high level of poverty, the failure of the national tuberculosis control programme and the incidence of HIV/AIDS infections (Salami and Katibi, 2007) and possibly also, as a result of a lack of a bovine tuberculosis control policy and human to livestock interactions at various levels (Cadmus *et al.,* 2004). The true picture of the burden of tuberculosis in humans in Nigeria is not accurately presented despite the prevalent risk factors. This is because of the limitation of routinely used diagnostic methods i.e. detection of acid fast bacilli by Ziehl-Neelsen (ZN) staining and microscopy and less often mycobacterial culture. In research laboratories however, culture and biochemical tests are used to identify and differentiate members of the MTBC.

Recently, a more rapid and sensitive molecular technique which is based on the PCR amplification of the presence or absence of specific regions of the genome referred to as genomic regions of difference (RD) have been utilized in the identification of MTBC in clinical and field isolates (Parsons *et al.,* 2002; Warren *et al.,* 2006). This is however not currently being adopted for MTBC identification in Nigeria, but used routinely in many sophisticated laboratories.

The combination of conventional methods and molecular typing of isolates have yielded important insights into the epidemiology of tuberculosis in livestock (Supply *et al.,* 2000; Allix *et al.,* 2006), wildlife populations (Michel *et al.,* 2008) and in humans (Savine *et al.,* 2002; Cadmus *et al.,* 2006). These methods have permitted a more precise targeting and monitoring efficacy of conventional control measures. Newer molecular methods have been developed to differentiate strains of *M. bovis*. One of these methods include the polymerase chain reaction (PCR) based spoligotyping as described by Kamerbeek and others (1997). This has been widely used to genotype *M. bovis* isolates and it is highly reproducible, rapid and it produces reliable phylogenetic data (Monaghan *et al.,* 1994).

Analysis of multiple genomic regions that contain tandem repeats of different families of genetic elements (Frothingham and Meeker-O'Connell, 1998; Le Fleche *et al.,* 2002; Skuce *et al.,* 2002 and Smittipat, and Palittapongarnpim, 2000) has recently been proposed as an alternative tool for molecular epidemiological studies of *M. bovis*. Variable number tandem repeat (VNTR) markers were identified during the recently completed genome sequencing projects for members of the MTBC. They include exact tandem repeats (ETRs) (Frothingham and Meeker-O'Connell, 1998), mycobacterial interspersed repetitive units (MIRUs) as reported by Supply and others (Supply *et al.,* 2000) and VNTRs (Le Fleche *et al.,* 2002).

In the context of bovine tuberculosis (BTB), a reliable high resolution genotyping technique such as MLVA typing can be applied to the systematic study of epidemiological risk factors such as movement of cattle, spread within a herd, persistence and latency and in addition, the significance of the interactions between cattle, wildlife and humans can be investigated at a reproducibly high resolution. In this study the VNTR genotyping method will be utilized in the typing of *M. bovis* isolates from Nigeria thus providing insights into the molecular epidemiology of this disease in a country in which there are risk factors which can promote the spread of this neglected zoonosis. Two different approaches of genotyping based on VNTRs will be compared with each other to determine the reproducibility of the technique and the discriminatory ability on the same set of isolates. This study also highlights the relevance of deletion typing PCR

which is based on regions of difference namely RD1, RD4, RD9, RD 1^{mic} and RD2^{seal} in the identification of mycobacterial isolates obtained from cattle isolates and patients with diverse risk factors in Ibadan, southwestern Nigeria.

1.2. Literature review

1.2.1. Taxonomy

Mycobacterium is a gram-positive, acid fast, non-motile, non-spore forming slow growing organism which are known to be relatively resistant to chemical disinfectants. Attempts have been made to group *Mycobacterium* based on pigment production, growth rate, biochemistry, pathogenicity and currently genetic studies. However, with the exception of *M. leprae*, *Mycobacterium* species can be distinguished into two groups; *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium* outside tuberculosis complex (MOT) (Van Soolingen. 2001). The MTBC, comprising of *M. tuberculosis, M. bovis*, *M. africanum* and *M. bovis* subsp. *caprae* are closely related bacteria and are often difficult to distinguish on the basis of biochemical or growth characteristics. DNA fingerprinting has increasingly been applied to classify MTBC isolates into species and sub-species (van Embden *et al.,* 1993).

1.2.2. Diagnosis of bovine tuberculosis

There are quite a number of ways in which BTB in cattle can be diagnosed and several of these ways evolved as a result of lack of specificity and sensitivity of earlier forms of BTB detection. A presumptive diagnosis of TB in cattle and other susceptible species is often made on history and clinical findings (Acha and Szyfres, 1987), results of the tuberculin skin test (Monaghan *et al.,*1994) gamma interferon assay (Wood and Jones, 2001) and necropsy findings (Corner. 1994).

1.2.2.1. Antemortem diagnosis

1.2.2.1.1. Clinical signs

Due to a lack of specific clinical signs, the diagnosis of cattle based on clinical signs is usually difficult. The use of clinical signs as a presumptive diagnosis is limited due to low specificity

and sensitivity in the early stages of the infection (Acen. 1991). In advanced stages of TB however, the characteristics include, weakness, emaciation, anorexia, dyspnoea, enlargement of lymph node and coughing (Coetzer and Tustin, 2004).

1.2.2.1.2. *Single Intradermal Comparative Tuberculin Skin Test*

The Single Intradermal Comparative Tuberculin Test (SICTT) is also another commonly used diagnostic tool in BTB infections in cattle despite its limitations of low sensitivity and specificity in areas with low prevalence (Acen. 1991; Monaghan *et al.,* 1994). It is based on a delayed type of hypersensitivity reaction, which is maximal at approximately 72 hours after injection of tuberculin, a purified protein derivative (PPD) in the intradermal layer of the skin. Animals with prior exposure to the PPD respond by a measurable swelling at the injection site. The use of TST as a screening test for cattle in several countries where BTB control eradication programmes have been implemented has been documented by Cousins and Roberts (2001).

1.2.2.1.3. *Interferon Gamma test*

The gamma interferon (IFN-γ) assay is an *in-vitro* blood test that was developed in Australia in the late 1980's and it is used in combination with the SICTT. The IFN-γ assay measures interferon, a cytokine predominantly released by T-lymphocytes (de la Rua-Domenech. 2006) and is reported to detect a substantial proportion of infected cattle that escape detection by the tuberculin test (Wood and Jones, 2001). The most likely reason for this is that the IFN- γ test identifies infection at an earlier stage than the skin test (de la Rua-Domenech. 2006). The main limitation however is the lower specificity leading to important numbers of false positive reactions.

The skin test and the IFN- γ both share the disadvantage of having a low probability in detecting infected cattle in a state of depressed cell mediated immune response (anergy). The ability of natural killer cells to produce interferon in response to stimulation in the absence of any previous sensitization by *M. bovis, M. avium* subsp. *paratuberculosis* and *M. tuberculosis* also increase

the proportion of false positive test results. For these reasons Michel and co-workers (2006) developed a modified IFN- γ assay to overcome non-specific responses from non-tuberculous *Mycobacterium* in the environment.

1.2.2.2. Post mortem diagnosis- Isolation and bacteria culture

Isolation of agents by culturing can be considered as the gold standard of diagnosis as it is 100% specific. However, the sensitivity of bacteriological culture is low and isolation may be difficult in live animals due to difficulty in the collection of samples (Acha and Szyfres, 1987). *Mycobacterium* spp. may be isolated from tubercles at post-mortem. After sample collection, tissues are homogenized and then decontaminated and cultured in egg-based media such as Lowenstein-Jensen (with or without pyruvate) agar based 7H10 fluid medium or as 7H9 solid media (Acha and Szyfres, 1987; Ashford *et al.,* 2001). Within *M. bovis* species*,* the strains are even more uniform in their properties and cannot be distinguished from each other by traditional methods such as serotyping, phage typing and amino acid uptake (Collins *et al.,* 1994). Therefore the need exists for molecular tools to aid in the differentiation of species and strains.

1.2.3. Evolutionary scenario of the *Mycobacterium tuberculosis* complex

Following the whole genome sequence project, comparative genomics uncovered several variable genomic regions in the members of the *M. tuberculosis* complex. Differential hybridization arrays identified 14 regions of difference (RD1–14), ranging in size from 2 to 12.7 kb, that were absent from bacillus Calmette–Gue´rin Pasteur relative to *M. tuberculosis* H37Rv (Behr *et al.,* 1999).

The deletions (RD1, RD2, RD4, RD7, RD8, RD9, RD10, RD12, RD13, RD14, and TbD1) which flanking regions typically do not contain repetitive sequences are intact in strains of the *M.*

tuberculosis complex and have enabled the proposition of an evolutionary scenario for the members of this complex (Brosch *et al.,* 2002).

In the study by Brosch and co workers (2002), it was revealed that the majority of these polymorphisms did not occur independently in the different strains of the *M. tuberculosis* complex but, rather, occurred from ancient, irreversible genetic events in common progenitor strains. It was further observed in *M. canettii*, that all of the RD, RvD, and TbD1 regions except the prophages (phiRv1, phiRv2) were present and most *M. tuberculosis* strains are highly conserved with respect to RD1, RD2, RD4, RD7, RD8, RD9, RD10, RD12, RD13, and RD14. These RDs further represent regions that can differentiate *M. tuberculosis* strains independently of their geographical origin and their typing characteristics from certain other members of the *M. tuberculosis* complex. Thus, identifying *M. tuberculosis* and/or *M. canettii* as most closely related to the common ancestor of the tubercle bacilli whilst *M. canettii* remains a fascinating tubercle bacillus, whose detailed genomic analysis may reveal further insights into the evolution of the *M. tuberculosis* complex.

Successive loss of DNA as presented in Figure 1.1, reflected by loss of RDs and other subsequent deletions was identified for an evolutionary lineage represented by *M. africanum*, *M. microti*, and *M. bovis* that diverged from the progenitor of the present *M. tuberculosis* strains before TbD1 occurred. Based on either the presence or absence of an *M. tuberculosis* specific deletion (TbD1), *M. tuberculosi*s strains can be divided into ancestral and 'modern' strains. In the same regard, *M. africanum* was divided into East African and West African *M. africanum*.

Further loss of genes identified 5 groups of *M. bovis* with the *M. bovis* BCG having the most deletions.
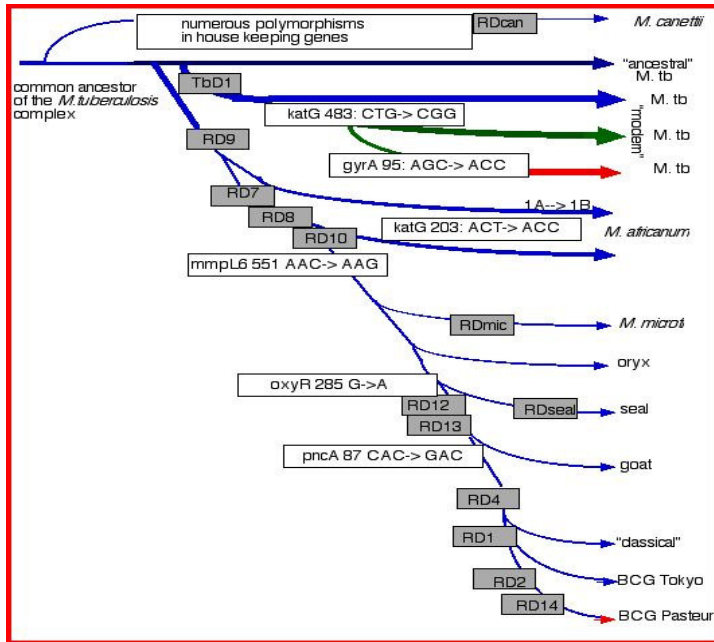
Figure 1.1: A proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (grey boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes (Source: Brosch *et al.,* 2002).

1.2.4. Variable minisatellite-like regions in the Mycobacterial genome

1.2.4.1. *Exact tandem repeats*

Exact tandem repeats were the first established VNTR set used for typing strains of the MTBC and were identified by searching existing literature as well as early versions of the *M. tuberculosis* genome sequence data (Frothingham and Meeker-O'Connell, 1998). According to a previous comparative study, the resolution provided by this first set of five loci was found to be lower than both IS*6110* RFLP typing and spoligotyping (Kremer *et al.,* 1999). It was observed when typing *M. bovis* isolates from Ireland that most of the discriminatory power of these loci resided in ETR-A and ETR-B (Roring *et al.,* 2004), whilst these two loci ranked third and fifth in their discriminatory power respectively in a comparative study by Skuce and others (2005).

1.2.4.2. Mycobacterial Interspersed Repititive Units

Mycobacterial interspersed repititive units are minisatellites because of their size of 40-100 bp and sequence repeats, are homologous DNA sequences found as tandem repeats and dispersed in intergenic regions on the MTBC and *M. leprae* genomes (Savine *et al.,* 2002). There are three major types (types I, II and III) of these MIRUs and according to Supply and others (2000) they are dispersed within 41 loci throughout the *M. tuberculosis* H37Rv genome, and subsequent investigations revealed that 12 of these MIRUs are polymorphic.

Most MIRU-VNTRS have been discovered to be relatively stable and to evolve slowly in mycobacterial populations. This was concluded when the MIRU loci of three different genealogically distant BCG strains (Pasteur, Glaxo and Japan) were investigated and the presence of only one polymorphic locus could be showed (locus 4). Others were monomorphic i.e. showing no variability amongst the BCG strains. It then was discovered that the copy numbers of the repeats and number of alleles within the MIRU loci are lower when compared with those found within the minisatellites of samples of human origin, which have repeats of up to 100 copies or more, probably due to a higher functional constraint resulting from the higher gene density and the smaller size of mycobacterial chromosomes compared to eukaryotic chromosomes (Supply *et al.,* 2000).

The currently available collection of polymorphic tandem repeats for the typing of *M. tuberculosis* comprises 27 loci (Le Fleche *et al.,* 2002). This MIRU method relies on PCR amplification of multiple loci using primers specific for the flanking regions of each repeat locus and subsequently on the determination of the sizes of the amplicons, which reflect the numbers of the targeted MIRU-VNTR copies (Supply *et al.,* 2000). The results of Savine and others (2002) and of Skuce and others (2005), suggest that VNTR-type loci are sufficiently stable and robust and allow transmission chains to be traced effectively.

1.2.5. Molecular identification and genotyping of MTBC isolates

The application of molecular techniques together with traditional epidemiological approaches has provided insights into the source of infection and the identification of risk factors which enhance the spread of tuberculosis. All molecular genotyping techniques capitalize on the mutations which occur over time within specified regions of the bacterial genome, these mutations are responsible for the differences within strains. The discriminatory power of any molecular epidemiology technique therefore depends on the ability to detect the frequency at which the targeted gene is present in the bacterial genome.

1.2.5.1. *Identification of MTBC isolates by molecular analysis of genomic regions of difference (RD)*

The differentiation of the members of the MTBC by traditional methods such as culture, smear microscopy and biochemical tests are labor intensive, low in sensitivity, time consuming and not practical for surveillance purposes (Warren *et al.,* 2006). The outcome of the comparative genomic analyses of *M. tuberculosis* and *M. bovis*, amongst other things, identified certain genes in the *M. tuberculosis* genome that were absent in the genome of *M. bovis*, despite the 99.95 % homogeneity of these genomes. These regions were dubbed as regions of difference (RD) and 16 regions, RD1-RD16, have been identified (Parsons *et al.,* 2002).

The presence or absence of these genes was further exploited as a species differentiating tool by adopting a PCR based technique. Parsons and co-workers (2002) recommended the PCR amplification of 6 regions of difference; RD1, RD3, RD5, RD9, RD10 and RD11 for the identification of MTBC isolates. However, in a more recent study, a two step multiplex PCR approach was suggested in which two primer sets were utilized. The first primer set targeting the RD1, RD4, RD9 and RD12 was found to sufficiently discriminate between *M. tuberculosis, M. bovis, M. canetti, M. caprae* and *M. bovis* BCG whilst the second primer set, targeting the RD 1$^{\text{mic}}$ and RD2 $^{\text{seal}}$ differentiates between *M. africanum, M. microti* and *M. pinnipedii.* The vaccine strain (*M. bovis* BCG) differs from the pathogenic *M. bovis* strain by the presence of RD 1 in pathogenic *M. bovis* strains (Warren *et al.,* 2006).

1.2.5.2. *Restriction Fragment Length Polymorphism (RFLP) with hybridization (IS6110 RFLP)*

DNA fingerprinting using IS*6110* as a probe is a standardized and widely applied molecular typing method for the comparison of *M. tuberculosis* isolates (Hernandez *et al.,* 1999, Warren *et al.,* 2000). This transposable sequence belongs to the IS*3* family which is found in virtually all members of the MTBC and is apparently restricted to this group of organisms. This technique is based on the differences in copy numbers (ranging from about zero to 25) and its variability of the IS*6110* sequence in the chromosomal positions on the genome (Van Soolingen. 2001).

Ten to 12 IS*6110* copies are present in various strains of *M. tuberculosis* while some strains of *M. tuberculosis* and *M. bovis* contain none or single to a few copies. The limited discriminatory ability of the IS*6110* RFLP technique for *M. bovis* strains is due to low copy numbers of IS*6110* found in most *M. bovis* strains which makes it a good technique for most *M. tuberculosis* as they have several *IS*6110 copies (Allix *et al.,* 2006). Although they are discriminatory for some *M. bovis* isolates RFLP is difficult to reproduce consistently, making it difficult to compare strains either in long-term studies or between laboratories (Kremer *et al.,* 1999). Another limitation of the IS*6110*RFLP is the requirement for relatively large amounts of high quality DNA which is difficult to obtain for slow growing *Mycobacterium* (Le Fleche *et al.,* 2002).

1.2.5.3. *Spoligotyping*

This typing method is based on DNA polymorphism present at one particular chromosomal locus, the Direct Repeat (DR) region, which is present in members of the MTBC (Kamerbeek *et al.,* 1997). Spoligotyping 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 MTBC strains. Spoligopatterns are obtained by PCR amplification, of the DR region followed by the hybridization 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 (Kamerbeek *et al.,* 1997).
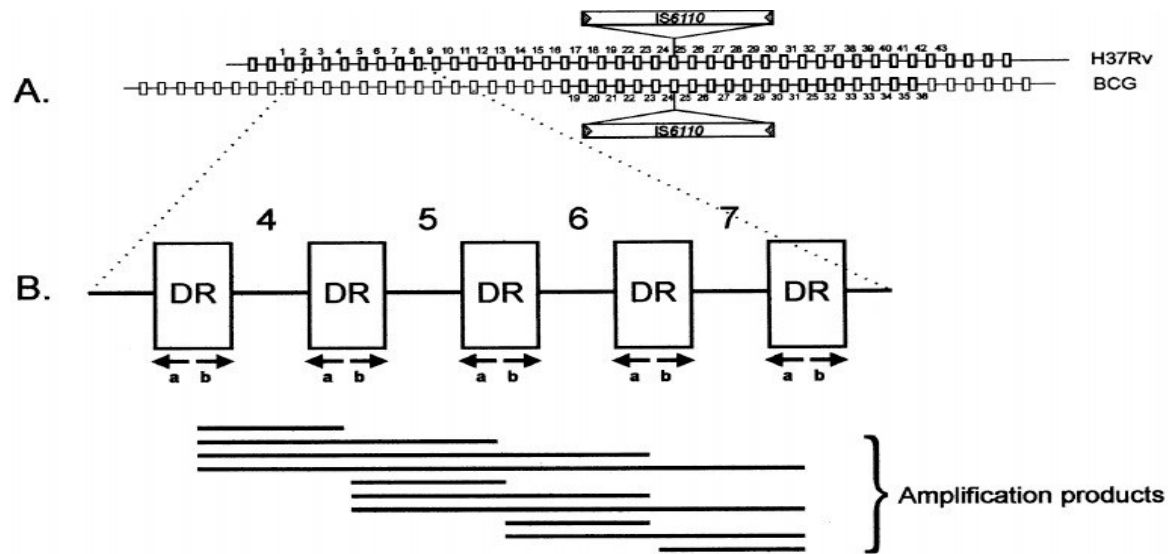
Figure 1.2.: Structure of the DR locus in the mycobacterial genome. *M. tuberculosis* H37Rv and *M. bovis* BCG contain 48 and 41 DRs, respectively (depicted as rectangles), which are interspersed with unique spacers varying in length from 35 to 41 bp. The (numbered) spacers used correspond to 37 spacers 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. Here a combination of fragments is shown that would be produced by *in vitro* amplification of a DR target containing only five contiguous DRs. (Source: Kamerbeek *et al.,* 1997).

Spoligotyping is the first technique of choice for *M. bovis* typing, not only because it is simple and rapid, but also because a free online database (www.mbovis.org) is available, which ensures easy exchange of information all around the world (Brudey *et al.*, 2006). It is simple and robust and highly reproducible generating results which can be read as a digital code even in a word processor. This method can be used to classify MTBC in taxons or sub-species (Haddad *et al.*, 2001) and to simultaneously detect and type MTBC bacteria in one assay (Kamerbeek *et al.*, 1997). Although it is powerful with many advantages, spoligotyping suffers from a lack of resolution compared to the gold standard used for the identification of *M. tuberculosis* namely, IS*6110* typing (Kremer *et al.*, 1999) and the VNTR genotyping technique (Allix *et al.*, 2006; Kremer *et al.*, 1999; Le Fleche *et al.*, 2002; Skuce *et al.*, 2002).

1.2.5.4. 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. The VNTR typing of isolates of the MTBC has been described by Kremer and others (1999). The development of polymorphic markers is now further facilitated by the availability of whole genome sequences for bacterial genomes which show that members of the MTBC randomly contain 41 MIRUs (Supply *et al.*, 2000).

The typing of tandem repeats is relatively straight forward and the resulting data can easily be coded and exchanged between laboratories independently of the technology used to measure PCR fragment sizes (Le Fleche *et al.*, 2002). Furthermore, the resolution of tandem repeats typing is cumulative i.e. the inclusion of more markers in the typing assay can when necessary, increase the identification resolution (Le Fleche *et al.*, 2002). However, the density of tandem repeats in bacterial genomes varies from species to species and not all tandem repeats are polymorphic (Le Fleche *et al.*, 2001). In a sample of 47 isolates from Northern Ireland, these markers were shown to be more discriminatory than spoligotyping (Roring *et al.*, 2002). The results obtained by Savine and others (2002) and of Skuce and others, (2005), suggested that

VNTR-type loci are sufficiently stable and robust and allow transmission chains to be traced effectively. Variable number of tandem repeats analysis are high-throughput PCR based techniques, which work well from boiled cell lysates without the need for sub-culturing, unlike RFLP based typing which require several manipulations and analysis (Le Fleche *et al.,* 2002). Furthermore, VNTR typing has the potential for further automation of PCR product analysis and allele calling (Supply *et al.,* 2000; Lindstedt. 2005).

There are two methods which are currently adopted for VNTR typing i.e. the multiplex PCR approach with three or more primer pairs and subsequent separation by capillary electrophoresis (Allix *et al.,* 2006) and another approach in which one set of primer pair amplifies a locus and separation is done by agarose gel electrophoresis (Le Fleche *et al.,* 2002). The latter is thought to be less expensive and as discriminatory as the former but there is no sufficient data to support its discriminatory ability relative to the former.

1.2.6. Epidemiology of tuberculosis infection in Nigeria

1.2.6.1. *Burden of <u>M. bovis</u> infections in humans in Nigeria*
Nigeria is recorded to have the fourth highest burden of human tuberculosis (TB) in the world, with an incidence of 304 cases per 100,000 in 2002 and a mortality rate of 89 in every 100,000 cases (Cadmus *et al.,* 2006). This status has been attained by the co-infection of tuberculosis patients with the human immunodeficiency virus (Salami and Katibi, 2006).

The burden of tuberculosis caused by members of the MTBC in humans in Nigeria is largely unknown or under diagnosed due to the lack of adequate laboratory equipment but its presence has been proven and infections in humans due to *M. bovis* have been described in various African countries (Cosivi *et al.,* 1998;, Kazwala *et al.,* 2001; Cadmus *et al.,* 2006). Alhaji and Schnurrenberger in 1976 documented a 10% incidence rate of *M. bovis* in human pulmonary tuberculosis in four of the then northern states of the country (Ayanwale. 1984) and Idigbe and

co-workers documented a 3.9% incidence rate in Lagos (Idigbe *et al.,* 1986). Reports have also been documented of the incidence of BTB infections in the eastern part of the country (Itah and Udofia, 2005). In a more recent study in Jos, a North-central part of the country, a 15.4% prevalence rate of *M. bovis* in human pulmonary infections was observed (Mawak *et al.,* 2006). This higher prevalence in the North of Nigeria as compared to the South can be explained by the communal lifestyle of the Fulani nomads because of their close proximity with their livestock as well as certain cultural practices.

1.2.6.2. *Risk factors associated with zoonotic tuberculosis in Nigeria*

The prevalence of bovine tuberculosis is yet to be fully elucidated as there is currently no national control strategy in place to control the disease. According to a preliminary molecular study using PCR based VNTR analysis and spoligotyping on isolates from humans and animals undertaken in Ibadan, South-west Nigeria, it was discovered that approximately 13% of tuberculosis in humans was caused by *M. africanum* and *M. bovis* (Cadmus *et al.,* 2006). This percentage can be directly linked to the prevailing risk factors and the persistence of cultural practices which have the potential to facilitate transmission between cattle and humans. There have been several workers' reports on bovine tuberculosis in Nigerian abattoirs (Ayanwale. 1984; Cadmus *et al.,* 1999). Abattoir workers often do not wear protective gear and hence they are a high risk catergory with respect to BTB infections.

With the increase in HIV/AIDS infection in the country a 5% increase in the prevalence rate is also suggested to be attributed to human to human transmission of zoonotic bovine tuberculosis (Nigerian Federal Ministry of Health. 2004). One epidemiological report has estimated that one third of the sub-saharan Africans are carrying the latent *M. tuberculosis* infection which can be activated by HIV infection and thereby increasing the pool of HIV pulmonary TB co-infection in the region (Saunder. 2001).

Another possible factor is the consumption of a local delicacy in northern Nigeria; *"fura de nunu"* which is made from unpasteurised milk as well as drinking of unpasteurised milk by the nomadic herdsmen who also live in close contact with their herd. There is also the lack of bovine tuberculosis control programmes and failure of implementation of proper meat inspection procedures at almost all the abattoirs in the region (Cadmus *et al.,* 1999). Socio-economic factors such as poverty and poor health care systems are also contributing to the transmission of tuberculosis infections as revealed by studies in Ibadan, South-west Nigeria, between 1966 to 1995 (Nwachokor and Thomas, 2000). Since the potential sources for transmission of infection remain uncontrolled, the economic and public health importance of zoonotic bovine tuberculosis infections will remain relevant.

1.2.6.3. *Molecular epidemiology of zoonotic tuberculosis in Nigeria*

Molecular epidemiology is a powerful approach to monitoring infectious diseases and it is based on the notion that patients infected with strains which are similar are linked epidemiologically (Savine *et al.,* 2002). Studies on the prevalence of tuberculosis in Nigeria have relied thus far on the basic diagnostic techniques such as culture of the organism, ZN staining of the acid fast mycobacteria and phenotypic tests to categorize them.

The first and only molecular report of MTBC infections in Nigeria was by Cadmus *et al.,* (2006). This study was based on the combination of 6 VNTR loci as reported by Frothingham and Meeker-O'Connell (1998) and spoligotyping on *M. bovis and M. tuberculosis* isolates from the same geographical location. The results of the study showed that 18 different spoligopatterns were identified among the 51 *M. tuberculosis* with one pattern, characteristic of the Cameroon family of *M. tuberculosis* strains, being the most dominant in 69% of the isolates tested. Furthermore, all the isolates of this unique group except four isolates had an identical VNTR pattern. From the isolates obtained from cattle, a "modern" *M. tuberculosis* strain was identified with a spoligopattern identical to *M. tuberculosis* and with a deletion of the TbD1 locus but the presence of the RD9, RD10, RD4 and RD7 suggesting a human to cattle transmission of *M. tuberculosis* (Cadmus *et al.,* 2006).

From the literature it is clear that the use of molecular techniques is essential in the characterization and genotyping of MTBC species in Nigeria. This study will highlight the use of molecular tools in rapid species identification in humans as it is not currently adopted in Nigeria. Furthermore, the report on molecular genotyping of MTBC in Nigeria (Cadmus *et al.,* 2006) gives an indication of the level of differentiation that can be achieved with 6 VNTR loci, however, with the inclusion of more markers, a higher definition can be achieved and the circulating strains can be properly classified. This will ultimately serve as background information on the prevalent strains as well as giving an indication of strain variation and genetic diversity which have occurred within particular strain types. This study used MTBC strains isolated from humans and livestock within the same geographical location, South-west Nigeria with a view to studying disease transmission dynamics between different animal species as well as utilizing molecular genotyping as an epidemiological tool.

1.3. Aims and objectives of the study

The objectives of this study were:

• To compare the resolution between two methods of electrophoresis for MLVA genotyping.

• To determine a suitable MLVA panel for use in the molecular analysis of MTBC isolates from the study sites.

• To determine the array of different species of the members of the MTBC in humans and livestock as well as molecular genotyping of MTBC species in Ibadan, South-west Nigeria by the use of rapid, reproducible and sensitive molecular techniques.

CHAPTER 2

Comparing the resolution of the capillary and agarose electrophoresis based Multiple locus [variable number of tandem repeats] analysis (MLVA) on *Mycobacterium bovis* isolates

2.1. Introduction

The molecular genotyping of *Mycobacterium* isolates has received a lot of attention in the last decade. The need has arisen as a result of a reported increase of the infection in certain countries. Hence, several genotyping techniques have been developed which have added valuable information to the epidemiology of *M. bovis*. The global approach to VNTR genotyping of MTBC is yet to be standardized, i.e. there is no selected panel of loci for VNTR genotyping. This is clearly seen in reports by different workers where different combinations of loci and different typing techniques are used for their genotyping work (Allix *et al.,* 2006; Le Fleche *et al.,* 2002; Skuce *et al.,* 2002; Roring *et al.,* 2004; Supply *et al.,* 2006; Yokoyama *et al.,* 2007).

The MLVA method is currently a key method in MTBC genotyping and there are two methods of electrophoresis currently employed in MLVA; the agarose based gel electrophoresis (AE) and the capillary based electrophoresis (CE) (Section 1.2.5.5.). The former is less expensive and able to sufficiently discriminate *Mycobacterium* spp. (Frothingham and Meeker-O'Connell, 1998; Le Fleche *et al.,* 2002). The latter on the other hand has a high throughput and requires expensive instrumentation which requires PCR products to be electrophoresed via capillaries and fluorescent labelled primers (Lindstedt. 2005; Allix *et al.,* 2006; Yokoyama *et al.,* 2006). A previous study by Yokoyama and co-workers suggested that the CE VNTR analysis provides better resolution and is more epidemiologically useful when compared to the AE VNTR analysis (Yokoyama *et al.,* 2006), thus suggesting that MLVA cannot be used in laboratories lacking this equipment.

The AE VNTR analysis has been reported to be limited when compared to the CE VNTR due to its inability to cater for certain ambiguities occurring at specific loci in terms of copy numbers (Le Fleche *et al.,* 2002; Yokoyama *et al.,* 2006). Suggestions have been raised regarding the standardization of the AE VNTR technique that copy numbers should be rounded up from decimal fractions to integers (Le Fleche *et al.,* 2002). This however has been reported to lead to incorrect cluster assignments (Yokoyama *et al.,* 2006). Errors may also occur by visual assignment of band sizes as it is with the AE VNTR due to distortions which may occur during

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electrophoresis. Likewise, with the capillary based CE VNTR analysis errors may also occur due to internal size markers which are used in capillary electrophoresis influencing data obtained from peaks during electrophoresis leading to wrong copy number assignments (Lindstedt. 2005).

This experiment was carried out to serve as a pilot study in getting familiar with MLVA genotyping of mycobacterial isolates and also to confirm the findings of the study by Yokoyama *et al.,* (2006). The obtained data was compared against another MLVA technique, which in this instance serves as a reference. This study therefore focused on the resolution and discriminatory ability of these two methods of electrophoresis of MLVA on 19 *M. bovis* isolates obtained from Belgium. The comparison was done using 14 VNTR loci which are common to both studies. The aim was to highlight the relevance of the AE VNTR technique as a sufficiently discriminatory method of MLVA genotyping *M. bovis* isolates and to justify its capability in providing epidemiologically relevant information.

2.2. Materials and Methods

2.2.1. History of strains

Isolates from 19 cases of bovine tuberculosis diagnosed in Belgium during the period 1995 to 2003 were used in this study. The isolates had previously been genotyped using spoligotyping, IS*6110* RFLP and CE VNTR genotyping. The collection was maintained at the Belgian National Reference Laboratory (CERVA-CODA, Brussels, Belgium). DNA was prepared as described by Allix *et al.,* (2006) and was obtained for use in a pellet form. PCR ready DNA was then diluted in 100 µℓ of TE buffer, and then stored at -20 °C until used for PCR.

2.2.2. Tandem repeats PCR amplification

The isolates were amplified using primers targeting 20 loci. Minisatellite PCR amplification was performed as previously described by Le Fleche *et al.,* (2002). PCRs were performed in 25 µℓ containing 2 µℓ of the DNA, 12.5 µℓ of 2 X Fermentas PCR Mastermix, 9.5 µℓ of RNA free

water and 0.5 µl of each flanking 20 pM primer. The PCR cycles were 94 °C for 5 minutes, 62 °C for 1 minute and 72 °C for 1.5 minutes (40 cycles). A final elongation step of 72 °C for 10 minutes and then hold at 4 °C till samples were electrophoresed on an agarose gel.

The PCR products were loaded in the standard MLVA format on 2% agarose gels for most loci and 3% agarose gels for MTUB02 and MTUB12. Fifty wells were cast on a 20 cm agarose gel and PCR products were loaded in a systematic manner in which the reference strain, which was added as a control for size assignments, was loaded next to the molecular marker after which 6 isolates were subsequently loaded. The gel was allowed to run at 200V-250V for 2 hours. This was to ensure that the bands were properly separated for easy base pair size assignments and to minimize errors, which might arise when converting band sizes to copy numbers. Allele sizes were estimated using a 100 bp plus ladder (Fermentas) as a size marker. The gel images obtained were stored as a TIFF file and copy numbers were visually analysed by importing the TIFF image into Microsoft excel.

2.2.3.   Conversion of band sizes to copy numbers and genetic relationship analysis

To convert to copy numbers an Allele Naming Table was used (Table 1 APPENDIX). The table was made available courtesy of Gilles Vergnaud and co-workers of the Institut de Génétique et Microbiologie, Université Paris-Sud Orsay. This was done by visual estimation of the band sizes which were then compared with band size estimates of the Allele Naming Table. The data was stored in an Excel file and then exported into the Bionumerics software for data analysis, genetic relationship analysis between the isolates and construction of a phylogenetic tree.

The data, which was exported into the Bionumerics software, was converted to categorical character and dendograms were plotted using unweighted pair group method with arithmetic averages (UPGMA) to align branch tips for better visualization.

2.2.4. Allelic and genotypic diversity

The allelic and genotypic diversity were calculated using the following equation: $h = 1 - \sum x_i^2$ [(n/n-1)], where n is the number of tested isolates and x_i is the frequency of the ith allele for allelic diversity and for genotypic diversity, n is the total number of genotypes obtained and x_i is the frequency of the ith genotype.

2.3. RESULTS

2.3.1. Comparison between CE and AE VNTR genotyping

A panel of 14 loci was used to analyze 19 *M. bovis* strains which were genotyped using the AE VNTR and compared with the CE VNTR technique. The CE VNTR technique had been done previously in Belgium and the results were compared with those of the AE VNTR technique.

Figure 2.1 shows a typical gel image obtained after electrophoresis using agarose based electrophoresis. The genotypic diversity index of the two techniques was h^d=0.83 for AE VNTR and CE VNTR respectively. There were no observable copy number differences at all loci tested when both techniques were used (Table 2.1) except strain 96427 which presented with a copy number of 10 when AE VNTR analysis was done, but from the data set obtained from CE VNTR analysis, a copy number of 9 and 10 was presented at QUB11A.

ETRA-2165_75bp_397bp_3U
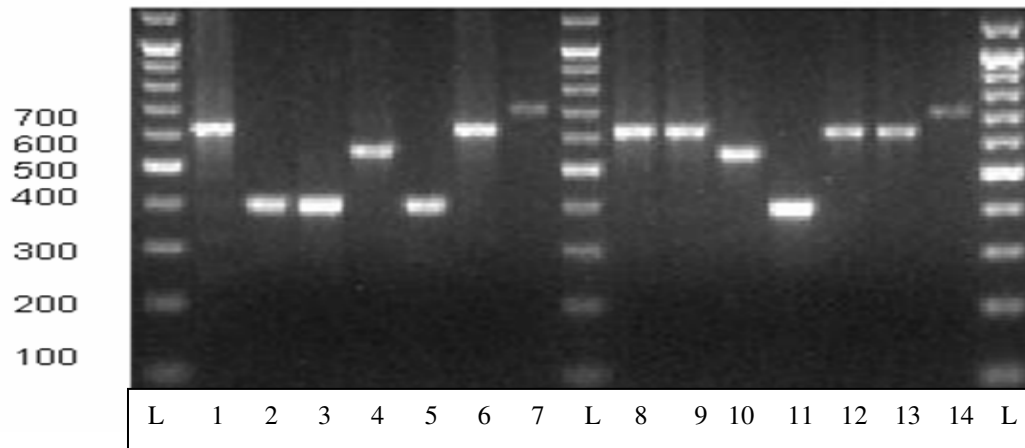
Figure 2.1: Gel image showing 12 *M. bovis* samples lanes 2-7 and lane 9-14 and the *M. bovis* reference strain in lanes 1 and 8. The ETR A locus is according the *M. tuberculosis H37Rv* reference system (2165_75bp_397bp) which defines 3 repeat units at this locus when a 397 bp band is obtained.

Table 2.1: Copy numbers obtained at 14 VNTR loci from 19 *M. bovis* strains. Copy numbers were identical in both techiques

Strain ID	MIRU 02	MIRU 40	MIRU 10	MTUB 21	QUB 11a	MTUB 30	MIRU 23	MIRU 27	MTUB 39	MIRU 39	ETR-A	ETR B	ETR C	ETR-D	ETR E
01076T1	2	2	2	3	10	4	4	3	2	2	3	3	5	3	3
01158	2	2	2	3	10	4	4	3	2	2	3	3	5	3	3
03017T1	2	2	2	3	10	4	4	3	2	2	3	3	5	3	3
03017T2	2	2	2	3	10	4	4	3	2	2	3	3	5	3	3
03002	2	2	2	3	10	4	4	3	2	2	4	3	5	3	3
00106T	2	2	2	1	10	4	4	2	2	2	5	5	5	3	3
01042T1	2	2	2	3	6	4	5	3	2	2	1	2	5	2	3
96411	2	2	2	3	6	4	5	3	2	2	3	2	5	3	3
03050T2	2	2	2	3	9	4	4	5	2	2	6	4	5	3	3
99074	2	2	2	3	10	4	4	3	2	2	6	4	5	3	3
97128	2	2	2	3	7	4	4	3	1	2	5	4	3	3	3
96453	2	2	2	3	11	4	4	3	1	2	7	4	3	3	3
00206T4	2	2	2	1	10	4	4	2	2	2	5	5	5	3	3
03062T9	2	2	2	1	11	4	4	2	2	2	5	5	2	3	3
03044	2	2	2	1	10	4	4	2	2	2	5	5	2	3	3
03081	2	2	2	1	11	4	4	2	2	2	5	5	2	3	3
96169	2	2	2	1	10	4	4	2	2	2	5	5	5	4	3
96427*	2	2	2	3	**9 and 10**	4	4	3	2	2	7	5	5	3	2
00012	2	2	2	3	10	4	4	3	2	2	5	4	5	3	3

* Strain 96427 presented with a copy number of 10 when AE VNTR analysis was done, but from the data set obtained from CE VNTR analysis, copy number of 9 and 10 was presented at QUB11A.

2.4. Discussion

Separation of VNTR amplification products is done either by agarose or capillary electrophoresis. A previous report however stated that the latter which is a higher throughput method is capable of providing better resolution than the agarose gel electrophoresis method (Yokoyama *et al.,* 2006). Nineteen *M. bovis* strains from Belgium strains were genotyped using the AE VNTR technique and the copy numbers obtained were compared to those obtained in a previous experiment in which the CE VNTR technique was used. A comparison of both methods done in this study indicated that the use of CE VNTR technique does not improve the sensitivity of the assay when compared to the AE VNTR technique. This was confirmed by the identical genotypic diversity index that was obtained for both techniques. Also, based on the identical copy numbers obtained from both techniques (Table 2.1), no relevant inferences could be made; hence there was no need to generate a dendogram as a means of comparison. Strain 96427 presented with copy numbers of 9 and 10 at QUB11A with CE VNTR analysis and a copy number of 10 when AE VNTR analysis was done, this may be due to artifactual peaks which was consequently labeled as occurs using CE VNTR analysis (Supply. 2005). In such instances, the use of AE VNTR analysis is hereby recommended as it will clarify the precise copy number that should be allocated.

It is believed that the sensitivity of CE VNTR analysis is due to its ability to express tandem repeats copy numbers as decimal fractions, which increases the level of discrimination between two strains, leading to precise clustering (Yokoyama *et al.,* 2006). This is opposed to AE VNTR analysis where it is conventionally acceptable to round off to whole integers which may result in incorrect cluster assignments (Le Fleche *et al.,* 2002). The use of a uniform reference system in sizing copy numbers often prevents incorrect copy number assignments, especially when these two techniques are compared. Hence, the quality of the DNA fragment resolution and the use of a control for possible migration smiling effects are critical for sizing accuracy (Supply. 2005).

The sensitivity of CE VNTR over AE VNTR can however be apparent only when certain loci with small differences between repeats are treated like markers with larger base pair differences

between repeats. Examples of such loci are MTUB02 which has a 9 bp difference between alleles and MTUB12 which has a 15 bp difference (Le Fleche *et al.,* 2002). Such markers are best separated on 3% agarose gels or on Nu-Sieve agarose gels which offer high resolution for small DNA fragments together with the use of molecular markers which can capture these small base pair differences between repeats (Supply. 2005). Furthermore PCR amplicons must be allowed to properly separate on long gels and sufficient electrophoresis time should be ensured for precise band size estimates to be obtained (Supply. 2005). The length of the gel and duration of electrophoresis is therefore crucial in determining the actual band sizes especially when loci with small base pair differences between alleles are used as this will ultimately ensure that the polymorphic ability of such loci can be captured, hence the documentation of accurate data.

The methodology of the CE VNTR, lends itself to a higher throughput of samples, is more amenable to advances in technology i.e. suitable for complete automation and capable of multiplexing (Lindstedt. 2005), but it is however not practical in laboratories with limited instrumentation. However, the possibility exists, that the use of multiplex primers, targeting different loci, may affect the deterimination of accurate amplification peaks by the internal size markers used as well as optimization problems associated with proper primer set combinations (Lindstedt *et al.,* 2004). This is capable of resulting in allocation of incorrect copy numbers. Likewise, the possibility of accurate band size determination and subsequently proper copy number assignment can also occur due to distortions that happen during agarose gel electrophoresis (Lindstedt *et al.,* 2004), especially when one relies on visual estimations. Nevertheless, the use of different software e.g. Bionumerics, special transilluminators which can analyse gel images and can help with distorted gel images, can ensure that actual band sizes are obtained.

The resolution obtained by AE VNTR analysis has been proven in this study to provide exactly the same resolution as the CE VNTR technique and this resolution is higher than existing techniques such as IS*6110* RFLP and spoligotyping, provided a sufficiently discriminatory panel is used (Le Fleche *et al.,* 2002; Roring *et al.,* 2002; Roring *et al.,* 2004; Skuce *et al.,* 2005). The level of discrimination of AE VNTR combined with spoligotyping and or IS*6110* RFLP can provide sufficiently informative epidemiological data in bovine tuberculosis endemic countries,

whilst still relatively inexpensive when compared to studies carried out by CE VNTR analysis, especially in developing countries.

This study suggests that the discriminatory ability of the CE VNTR technique is not higher than the AE VNTR technique and that AE VNTR is a technique which can be adopted in laboratories that cannot afford the expensive instrumentation needed for the CE VNTR analysis (Supply. 2005). These results also suggest that AE VNTR analysis is a reliable technique capable of providing sufficient epidemiological data. Furthermore, the training of personnel and maintenance of equipment are some of the many problems limited funded laboratories may face with CE VNTR analysis. Thus, the AE VNTR technique is hereby recommended as the reference technique for *M. bovis* genotyping in laboratories which run a few samples, as it is an epidemiologically reliable and sensitive technique. Furthermore, the VNTR profile generated can be compared with those obtained in laboratories which utilize the CE VNTR technique for MLVA analysis, provided they are based on the same reference system.

CHAPTER THREE

Molecular identification of *Mycobacterium tuberculosis* complex isolates in Nigeria

3.1. Introduction

Nigeria with its population of 140 million has an incidence of approximately 450,000 cases of all forms of tuberculosis in humans (WHO report 2008). This is further compounded by the high incidence of HIV/AIDS infection, poverty and a myriad of factors including the lack of specificity of routinely employed diagnostic tools used in clinical settings as well as in research (Salami and Katibi, 2007).

Tuberculosis in humans is caused by *Mycobacterium tuberculosis* as well as other members of the MTBC. These members are highly homogenous species having a 99.95% nucleotide sequence homology which makes sufficient species differentiation of the MTBC by sequence analysis difficult. The use of molecular techniques as a diagnostic tool in the routine identification of human tuberculosis in clinical settings in Nigeria does however not occur. Sputum and cervical aspirates samples are currently used in the routine detection of human tuberculosis in Nigeria (Kehinde *et al.,* 2005; Cadmus *et al.,* 2005). Smear microscopy as a diagnostic tool for MTBC diagnosis in humans is however not a sensitive tool, as acid fast bacilli negative sputum samples have yielded positive growths on Lowenstein-Jensen culture medium. This has resulted in the misdiagnosis of smear negative tuberculosis patients (Kehinde *et al.,* 2005). A previous study indicated that smear negative tuberculosis patients are responsible for 17% of TB transmissions (Behr *et al.,* 1999).

National reports submitted to the OIE and WHO by Nigeria do not mention the importance of *Mycobacterium bovis* in human TB cases, mainly because of the current limitations of techniques employed in routine identification of MTBC. Hence there is a need to provide information on the true burden of zoonotic tuberculosis due to *M. bovis* infection in Nigeria. This study utilizes molecular tools such as deletion analysis and spoligotyping in identifying members of the MTBC responsible for tuberculosis in humans of different ages, exposed to different risk factors as well as livestock in the same geographical location in Ibadan, South- west Nigeria.

3.2. Materials and Methods

3.2.1. Origin and history of Nigerian Isolates

This study location, Ibadan, the capital of Oyo State, is the third largest city in Nigeria by population (after Lagos and Kano), and has the largest geographical area. It is located in South-western Nigeria, 78 miles inland from Lagos and is a prominent transit point between the coastal region and the areas to the North of the country where cattle is reared. Its population is 2,550,593 (National Bureau of Statistics, 2008) according to the 2006 census results, including 11 local government areas. The population of central Ibadan, including five local government areas (LGAs), is 1,338,659 according to census results for 2006, covering an area of 128 km².

One hundred and thirty five samples (135) were analysed and they were obtained from sputum from humans of different ages. These samples were obtained from patients with pulmonary tuberculosis (PTB), extra-pulmonary tuberculosis (EPTB), HIV patients, cattle traders and infants' faeces. Also from the same geographical location, samples were obtained from cattle at abattoirs (50), pigs (12) and goats (5). The samples (tissues and sputum) were processed according to the Becton Dickinson digestion procedure and then cultured on Lowenstein-Jensen media for 8-12 weeks at the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Oyo State, Nigeria. The positive samples on culture were further subjected to smear microscopy using ZN stain. No further differentiation was done on the mycobacterial isolates. Thermolysates were prepared by heating pure colonies at 80 °C for 30 minutes and then centrifuged at 12,000 rpm to remove the cellular debris. Ethanol precipitation was performed on the supernatant and the resultant pellet was then dissolved in TE buffer and stored at -80 °C for future use.

3.2.2. Identification of MTBC isolates by deletion analysis of the regions of difference

The two sets of primers used were obtained from previously published data (Warren *et al.,* 2006). Each primer set consists of two flanking primers and an internal primer. The first set of primers was the RD4 and RD9 primers and the second set were the RD 1^{mic} and RD 2^{seal} primers.

The PCR mix contained 12.5 µℓ of the Hotstart Taq multiplex master mix (Qiagen), 5 µℓ of the Q-solution (Qiagen), 3.5 µℓ of the RNAse free water and 0.5 µℓ of each 50 pM primer. The reaction was run at a denaturation temperature of 95 °C for 15 minutes, and 40 cycles of 94°C for 1 minute, 62 °C for 1 minute and 72 °C for 1 minute, a final elongation step at 72 °C for 10 mins and a holding step at 4 °C until used. The PCR products were then separated by electrophoresis using a 3% agarose gel at 10V/cm for 2 hours.

The RD9 deletion analysis was done to discriminate *M. tuberculosis* from other MTBC. Those with a deletion at this region were then further investigated with primers targeted at RD4. This then discriminated between *M. bovis, M. caprae* and the other MTBC. The other unidentified MTBCs were then further differentiated by using primers targeting the RD 1[mic] and RD 2[seal] regions. These primers were designed to differentiate *M. africanum* from *M. pinnipedii* and *M. microti*. The isolates without amplification or with abnormal amplification were then regarded as non tuberculous mycobacteria (NTM) (Warren *et al.,* 2006). The positive controls included a known *M. bovis* isolate (AN5) and a known *M. tuberculosis* isolate provided by the Medical Research Council, Center for Molecular and Cellular Biology, University of Stellenbosch, whilst the negative control was water. The resulting gel images were analyzed on the basis of their alignment on the gel i.e. same band size with either of the controls.

3.2.3. Spoligotyping analysis

Spoligotyping was done according to a standardized international method described by Kamerbeek and co-workers, using a commercially available kit (Isogen Biosciences BV). It was adopted in this study as a confirmatory method of identification for representative isolates after deletion analysis typing.

3.2.4. Sequencing analysis of the RD2 $^{\text{seal}}$ band

After RD1$^{\text{mic}}$ and RD2$^{\text{seal}}$ deletion analysis on human and cattle MTBC isolates an unexpected band was obtained from a number of isolates. Direct sequencing was then done to investigate the presence of the particular band (293 bp) which according to Warren and co workers (2006) confirms the presence of the RD2$^{\text{seal}}$ region.

The PCR products were purified with a commercial available PCR purification kit (Qiagen, Hilden, Germany). Sequencing reactions were performed using the BigDye Cycle sequencing kit V3.1 (Applied Biosystems) and samples were run on an Applied Biosystems 3130XL sequencer. Nucleotide sequences of the RD2$^{\text{seal}}$ were assembled and edited using gap4 of the Staden software suite (Staden. 1996). Multiple sequence alignments were performed using the Clustal X sotware. The alignments were adjusted manually using the Bioedit (version 7.0.5.2) program (Hall. 1999). Searches of databases for homologous sequences were performed using BLASTN (Altschul *et al.,* 1990).

3.3. Results

3.3.1. Deletion analysis

For species identification of *Mycobacterium,* deletion analysis targeting the RD1$^{\text{mic}}$, RD2$^{\text{seal}}$, RD4 and RD9 was carried out on 135 samples from humans, 50 from cattle, 12 from pigs and 5 from goats. Of the 135 human strains 44 strains presented with multiple banding patterns, hence were classified as MOT according to Warren *et al.* (2006). Table 3.1 shows the frequency of the species of MTBC which were isolated from the different categories of patients and species of animals after the species identification.

Deletion analysis amplifying the RD9 region was initially done, 64 of the MTBC isolates showed a band size of 235 bp indicating the presence of the RD9 region typical of *M.*

tuberculosis. These were mainly isolates of human origin (61) with three isolates of one from cattle, pig and goat respectively. Further analysis with primers targeting the RD4 region was done on the isolates in which RD9 region was deleted, the absence of RD4 region as indicated by a 268 bp band size was observed in 2 human and 6 cattle MTBC isolates. These isolates were classified as 'classical' *M. bovis* (Brosch *et al.,* 2002).

The presence of the RD4 region indicated by a 172 bp band was observed in 30 human isolates, 43 cattle isolates, 11 pig isolates and 4 goat isolates. To further differentiate this group in which RD9 was absent and RD4 region was present, the RD1^{mic} and RD2^{seal} regions were amplified using a multiplex PCR approach. It was discovered that 18 of the 30 human isolates, 3 cattle isolates as well as 1 pig isolate were *M. africanum* species i.e. presence of a 195 bp band size indicating the presence of the RD1^{mic} region. The other isolates in this category had an extra band of 293 bp, hence could not be further identified using the deletion analysis protocol as described by Warren *et al.* (2006).

The isolates in the group that presented with the additional band size (293 bp) which could not be further identified were subjected to tests by other molecular techniques to confirm their status. Spoligotyping analysis and sequencing analysis were performed on representative isolates as explained in Sections 3.2.3 and 3.2.4. Reference strains (*M. bovis* from Belgium, *M. bovis* AN5, *M. bovis* ATCC19210 alongside three of the test isolates, one "classical" *M. bovis* and one query group from cattle and humans) were tested with primers amplifying RD1^{mic} and RD2^{seal}. The resultant gel image after electrophoresis was similar to the query group that possessed the extra 293 bp band (Figure 3.1). The complete system that was used to classify the members of MTBC identified in this study is summarized in Table 3.2.
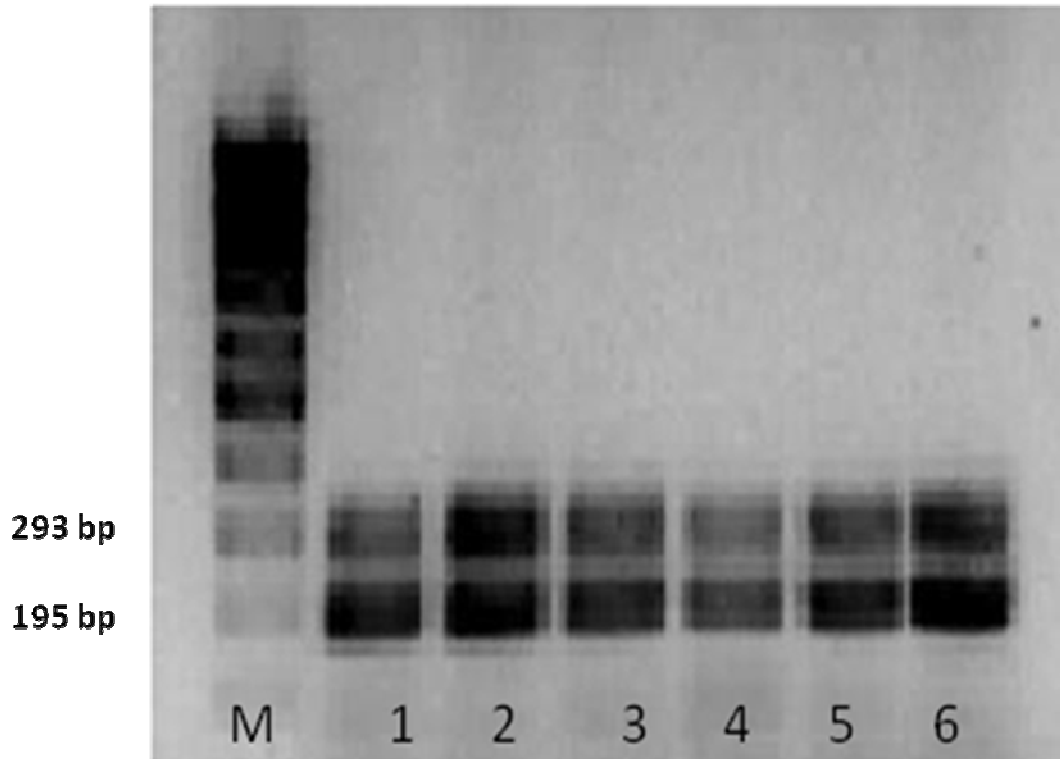
Figure 3.1: Gel image showing the two band sizes 195 bp and 293 bp which indicates the presence of RD1[mic] and RD2 [seal] regions respectively. Reference strains in lane 1-AN5, lane 2-Belgian *M. bovis* and lane 3-A44 *M. bovis* isolated from Buffalo in South Africa. Lanes 4 – 6 represent representative strains in which the RD4 region was present; 2 cattle isolates, c34 and c35 (lane 4 & 5) and 1 human isolate h2 (lane 6) were used. Lane M is the 100 bp molecular marker used from Fermentas.

Table 3.1: Species classification of MTBC and their frequency in different animal species and in humans

Origin of isolate	M. tuberculosis	M. bovis	"Classical" M. bovis	M. africanum	MOT**	Total
HIV	7	1	-	3	29	40
IF	9	-	-	-	-	9
CT	9	-	2	8	4	22
PTB	29	4	-	4	11	48
EPTB	-	-	-	1	-	1
Human*	7	6	-	2	-	15
Cattle	1	40	6	3	-	50
Goat	1	4	-	-	-	5
Pig	1	10	-	1	-	12
Total	64	65	8	21	44	202

* Isolates of human origin without patient history

** Isolates classified as MOT in this study on the basis of deletion analysis PCR, further analsysis with 16S DNA

will need to be done to confirm these isolates as MOT

Table 3.2: Classification of the members of the MTBC identified in this study

RD4 +172bp - 268bp	RD9 +235bp -108bp	RD1^{mic} +195bp*	RD2^{seal} +293bp*	Spoligopattern Spacers 39-43	Species
+	+	ND	ND	Present	*M. tuberculosis*
-	-	+	+	Absent	*"Classical" M. bovis*
+	-	+	+	Absent	*M. bovis*
+	-	+	No amp	ND	*M. africanum*

ND - no analysis was carried out

No amp - no amplification was obtained at that region of difference

- sign before the band sizes in bp means RD region absent whilst a + sign indicates the presence of RD region

3.3.3. Sequencing analysis

Sequence analysis of the RD2^{seal} band revealed nucleotide sequences homologous to a number of *M. bovis* and *M. tuberculosis* sequences after a BLASTN nucleotide search was done (Figure 3.2). No nucleotide variation was observed when 250 bp of 5 sequences obtained from Genebank were compared with isolates H2 and 27. As earlier mentioned H2 is a human isolate whilst 27 is from cattle origin.

```
                     10        20        30        40        50        60        70        80        90        100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

M_bovis                      GGGAAGAGCTGCCATGGGTGAGGCGAACATCCGCGAGCAGGCGATCGCCACGATGCCACGGGGTGGCCCCGACGCGTCTTGGCTGGATCGTCGATTCCAG
M_tuberculosis_              GGGAAGAGCTGCCATGGGTGAGGCGAACATCCGCGAGCAGGCGATCGCCACGATGCCACGGGGTGGCCCCGACGCGTCTTGGCTGGATCGTCGATTCCAG
M_bovis_del2_mpt64           GGGAAGAGCTGCCATGGGTGAGGCGAACATCCGCGAGCAGGCGATCGCCACGATGCCACGGGGTGGCCCCGACGCGTCTTGGCTGGATCGTCGATTCCAG
H2_edit                      GGGAAGAGCTGCCATGGGTGAGGCGAACATCCGCGAGCAGGCGATCGCCACGATGCCACGGGGTGGCCCCGACGCGTCTTGGCTGGATCGTCGATTCCAG
c27_1                        GGGAAGAGCTGCCATGGGTGAGGCGAACATCCGCGAGCAGGCGATCGCCACGATGCCACGGGGTGGCCCCGACGCGTCTTGGCTGGATCGTCGATTCCAG
M_tuberculosis_F11_          GGGAAGAGCTGCCATGGGTGAGGCGAACATCCGCGAGCAGGCGATCGCCACGATGCCACGGGGTGGCCCCGACGCGTCTTGGCTGGATCGTCGATTCCAG
gi|148503909_2230795-2231047 GGGAAGAGCTGCCATGGGTGAGGCGAACATCCGCGAGCAGGCGATCGCCACGATGCCACGGGGTGGCCCCGACGCGTCTTGGCTGGATCGTCGATTCCAG


                     110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

M_bovis_                     ACCGACGCACTGGAGTACCTCGACCGCGACGATGTGCCCGATGAGGTCAAACAGAAGATCATCGGGGTGCTCGACCGGGTGGGCACCCTGACCAACCTGC
M_tuberculosis_              ACCGACGCACTGGAGTACCTCGACCGCGACGATGTGCCCGATGAGGTCAAACAGAAGATCATCGGGGTGCTCGACCGGGTGGGCACCCTGACCAACCTGC
M_bovis_del2_mpt64           ACCGACGCACTGGAGTACCTCGACCGCGACGATGTGCCCGATGAGGTCAAACAGAAGATCATCGGGGTGCTCGACCGGGTGGGCACCCTGACCAACCTGC
H2_edit                      ACCGACGCACTGGAGTACCTCGACCGCGACGATGTGCCCGATGAGGTCAAACAGAAGATCATCGGGGTGCTCGACCGGGTGGGCACCCTGACCAACCTGC
27_1                         ACCGACGCACTGGAGTACCTCGACCGCGACGATGTGCCCGATGAGGTCAAACAGAAGATCATCGGGGTGCTCGACCGGGTGGGCACCCTGACCAACCTGC
M_tuberculosis_F11_          ACCGACGCACTGGAGTACCTCGACCGCGACGATGTGCCCGATGAGGTCAAACAGAAGATCATCGGGGTGCTCGACCGGGTGGGCACCCTGACCAACCTGC
gi|148503909_2230795-2231047 ACCGACGCACTGGAGTACCTCGACCGCGACGATGTGCCCGATGAGGTCAAACAGAAGATCATCGGGGTGCTCGACCGGGTGGGCACCCTGACCAACCTGC


                     210       220       230       240       250
                ....|....|....|....|....|....|....|....|....|....|...

M_bovis_                     ACGAGAAGTACGCCCGGATAGCCCTGAAACTTGTTTCTGACATTCCCAACCCG
M_tuberculosis_              ACGAGAAGTACGCCCGGATAGCCCTGAAACTTGTTTCTGACATTCCCAACCCG
M_bovis_del2_mpt64           ACGAGAAGTACGCCCGGATAGCCCTGAAACTTGTTTCTGACATTCCCAACCCG
H2_edit                      ACGAGAAGTACGCCCGGATAGCCCTGAAACTTGTTTCTGACATTCCCAACCCG
27_1                         ACGAGAAGTACGCCCGGATAGCCCTGAAACTTGTTTCTGACATTCCCAACCCG
M_tuberculosis_F11_          ACGAGAAGTACGCCCGGATAGCCCTGAAACTTGTTTCTGACATTCCCAACCCG
gi|148503909_2230795-2231047 ACGAGAAGTACGCCCGGATAGCCCTGAAACTTGTTTCTGACATTCCCAACCCG
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Figure 3.2: Nucleotide sequences of RD2^{seal} region of 27 and H2 and identical sequences obtained from the NCBI database

3.3.4. Spoligotyping analysis

To confirm the MTBC status of human and livestock isolates in which RD9 was absent as well as in which RD4 and RD1^{mic} were present and RD2^{seal} absent (293 bp), spoligotyping was done on 57 strains as described by Kamerbeek *et al.*, (1997). Of the representative isolates tested, only three human isolates were *M. bovis* (h2, hb74 and hb83) whilst the other human strains were *M. tuberculosis*. The spoligopattern of h2 presented with deletions at spacers 4-12, 18, 33-36 which did not correspond to any spoligopattern in the www.mbovis.org database. It has since been submitted and designated as SB1475. Three other strains which had spoligopatterns similar to the patterns observed in the LAM10-CAM family were identified in the human isolates. Two human isolates (h114 and h116) had an identical pattern as indicated by the absence of spacers whilst the third isolate (h110) differed. The spoligopattern, common to strains (h114 and h116), lacked spacers 10 and 14 as well as spacers 23-25 and 33-36 which is absent in most LAM10-CAM strains, whilst h110 lacked only spacer 10 and typical LAM10-CAM deleted spacers (Table 3.3).

All the cattle, goat and pig isolates were confirmed as *M. bovis* by spoligotyping except c15, g49 and p44 which were *M. tuberculosis*. When the *M. bovis* spoligopatterns were compared with the existing www.mbovis.org database, the following patterns were identified; SB1027, SB0944, SB1105, SB1025 and SB0951. The strains with these spoligopatterns are however yet to be reported in the SpolDB4 database. The strain with the spoligopattern of SB1105 was isolated from white Fulani cattle and was first reported in a dromedary in Chad. Furthermore, SB0944 appears to be the dominant spoligopattern and it was found in 38.5% of the *M. bovis* strains that were spoligotyped. It was also identified from other non bovine isolates i.e. pig and a human isolate (hb74). Spoligotyping data of two cattle isolates, c4 and 20 also revealed two new spoligopatterns common to *M. bovis* strains which are yet to be available in the international database www.mbovis.org and SpolDb4 database. These two novel spoligopatterns have been submitted to www.mbovis.org and designated SB1472 and SB1473 (Table 3.2). The *M. bovis* spoligopattern obtained from c4 presented with an unique spoligopattern which showed deletions at spacers 3, 6, 7, 9, 14, 16, 29-34, 36, 37 and 39-43 whilst that of c20 presented with deletions at spacers 3, 9, 16, 21, 30 and 39-43 (Table 3.3).

Table 3.3: Spoligopatterns of representative strains from human and livestock isolates

Host	Spoligotype	SpolDB4 type	SpolDB4 Family	Strain ID
PTB		Nil	New spoligotype	h2
PTB		119	X1	h7
PTB, HIV, IF, cattle		53	T1	h5, h10, h25, h26 h33, h40 h103, c15
PTB, CT		54	MANU 2	h11, h84
PTB		50	H3	h15 , hb75, hb77
PTB		1498	U	h17, h4
PTB		83	T1	h22
HIV			T family	h41
HIV		1166	T1	h42
HIV		838	LAM10_CAM	h48, h49,
PTB, CT		403	LAM10_CAM	h77, h79 ,h128
IF		52	T2	h102
IF		774	T1	h106
IF		New spoligotype	LAM10_CAM	h110
IF		358	T1	h111
PTB, HIV, IF, pig		61	LAM10_CAM	h112, h113, h122, h123, h126, h19, h67 h105, h107,p44
PTB		New spoligotype	LAM10_CAM	h114, h116
human		37	T3	hb76, hb82
human		SB1432*	Nil	hb83
WF		SB1027	Nil	c2
WF, pig human		SB0944	BOVIS1	c3 , c24, c34 p36, hb74
RB			New spoligotype	c4
WF		SB1105	Nil	c6
WF		SB1025	Nil	c17
WF, pig		SB0951	Nil	c18, p35
WF		New SB	New spoligotype	c20
goat		342	EAI5	g49

WF-White fulani; RB-Red Bororo, CT- Cattle traders, PTB-Pulmonary TB; IF- Infant feces; HIV-Human Immunodeficiency syndrome

3.4. Discussion

Despite bacterial culture being regarded as the gold standard for the identification of the members of the MTBC, it is often incapable of differentiating between members of this complex. The deletion analysis technique has been described as a rapid method of identification of *Mycobacterium* isolates (Parsons *et al.,* 2002; Warren *et al.,* 2006), whilst spoligotyping is a well established molecular genotyping and identification tool (Brudey *et al.,* 2006).

Nigeria is a country whose reports to international health authorities on the detection and prevalence of MTBC are based on data obtained from smear microscopy, a technique which is not sensitive enough for species identification and discrimination of the members of this complex (Kehinde *et al.,* 2005). The results in this study indicated the presence of an array of *M. tuberculosis* as well as *M. bovis* strains, responsible for human TB. This was illustrated by the spoligopatterns obtained from the different strains of *M. tuberculosis* species.

About 65 isolates from humans and livestock in this study could not be identified as described by Warren *et al.* (2006). After deletion analysis these isolates presented with a deletion at the RD9 region, presence of RD4 and RD1^{mic} regions and an extra 293 bp band indicating the presence of the RD2^{seal} genomic region of difference. If the latter was absent these group of isolates would have been defined as *M. africanum,* which according to Warren *et al.,* (2006) do not present with a band at the RD2^{seal} region. To further confirm the identity of these isolates, four *M. bovis* reference strains (*M. bovis* ATCC19210, *M. bovis* AN5, and one *M. bovis* strain isolated from cattle and buffalo in Belgium and South Africa, respectively) were subjected to deletion analysis multiplex PCR, using primers targeting the RD1^{mic} and RD2^{seal} regions. The reference strains had the same banding pattern as the query group as they presented with the additional 293 bp band (RD2^{seal} region present) thus confirming the identity of this group of isolates as *M. bovis*. Although sequence analysis of the RD2^{seal} region of a human and a cattle isolate resulted in the aligning with several *M. bovis* and *M. tuberculosis* strain sequences from Genbank (Figure 3.2), these group of isolates could not have been *M. tuberculosis* because they earlier presented a deletion at the RD9 region, which is present in all *M. tuberculosis* strains (Brosch *et al.,* 2000).

Sequence analysis also suggests that these strains could not have been *M. africanum* either as nucleotide sequences corresponding to the RD2^{seal} region are deleted in *M. africanum.*

Previous studies indicated that *M. tuberculosis* isolates have most of the regions of difference present (Brosch *et al.,* 2002). In this case, isolates tested, had the RD4 and RD9 regions and were classified as *M. tuberculosis*, thus, suggesting that the query group, in which the RD9 region was deleted, could not be *M. tuberculosis*. There are however, strong indications that the query group can be categorized as *M. bovis*. A report by Brosch *et al.,* (2002) stated that a group of *M. bovis* isolates bear identical deletions with the "classical" *M. bovis* isolates at all regions except at the RD4. These strains also exhibited an adenosine at position 285 of the *oxyR* pseudogene, which is also found in "classical" *M. bovis* (Aranaz *et al.,* 1999). The application of deletion analysis in routine identification of MTBC should hence be structured in a manner which accommodates identification of certain strains of *M. bovis* species which present with deletions not described by the protocol initially adopted in this study. This will ensure the stringency of this technique in species differentiation of the members of this complex, especially in tuberculosis endemic regions. Furthermore, based on results obtained in this study, a table (Table 3.2) summarising markers which can be used in the rapid identification of some of the members of the MTBC was generated.

The relevance of the future use of molecular techniques in the identification of *Mycobacterium* in Nigeria cannot be stressed enough. In this study different species of *Mycobacterium* were isolated from humans and of particular reference is "classical" *M. bovis* which was observed from isolates obtained from 2 cattle traders. The cattle trader in Nigeria often lives in close proximity with his herd whilst migrating from the North of the country to the South for trade. Furthermore, 9.6% of all the human MTBC isolates in this study were characterized as *M. bovis*. This forms 8.3% of the total isolates obtained from PTB patients. These values are consistent with previously published data in Nigeria where *Mycobacterium* culture was mainly used. Idigbe *et al.,* (1986) found 3.9% of isolates and Mawak *et al.,* (2006) found 15.38% of isolates to be *M. bovis.* Cadmus *et al.,* (2006) also found in the first molecular survey 5% of human isolates to be

M. bovis. Furthermore, a significantly large number of MOT was identified in this study as described by the adopted protocol (Warren *et al.,* 2006) where the presence of inappropriate bands suggests the presence of non-MTBC DNA. This was observed to be the highest amongst isolates obtained from HIV-positive or AIDS patients (Table 3.1), and it may be directly linked to immunosuppression due to the virus.

Spoligotyping is a more discriminatory technique when compared with deletion analysis as it is capable of both species identification and strain differentiation (Brudey *et al.,* 2006). The use of spoligotyping on representative strains further validated the deletion analysis technique as it helped in confirming the query group as *M. bovis*. It also provided epidemiologically useful information in this study as seen in the strain with the spoligotype SB1105, which had been previously identified in a dromedary in Chad and in a cattle isolate in this study. Cattle and camels are often brought into Nigeria for sale via border countries north of Nigeria; the country of isolation suggests the origin of the strain and the movement of animals explains its mode of transmission. Three new *M. bovis* spoligotypes were identified and have since been submitted to the international database www.mbovis.org. Two of the 3 *M. bovis* strains were isolated from cattle (SB1472 and SB1473) whilst the third one (SB1475) was a human isolate (h2) from a PTB patient. The distribution of the loss of spacers indicates peculiar mutational events suggesting that these strains evolved differently. Subsequent epidemiological studies will therefore be useful in validating the transmission of these strains particularly strains of human origin with respect to its zoonotic potentials.

Previous molecular reports indicated that the most prevalent strains of *M. tuberculosis* found in West Africa are the LAM 10, H and T strains (Brudey *et al.,* 2006; Cadmus *et al.,* 2006; Njanpop-Lafourcade *et al.,* 2001). Our spoligotyping results also confirmed this finding as 42.5% of the *M. tuberculosis* isolates which were spoligotyped were of the LAM10-CAM family. Furthermore, 2 new spoligopatterns were identified from three isolates i.e. one pattern from isolates h114 and h116 and the other pattern from isolate h110 (Table 3.2). Their spoligopatterns have spacer deletions typical of the 61 LAM10-CAM spoligotype but each

spoligopattern has one peculiar spacer deletion. It is therefore possible to assume, based on the distribution of the spacer deletion that these new spoligopatterns may have evolved from 61 LAM10-CAM (Table 3.2) which is the predominant spoligotype amongst the LAM10-CAM family.

Another interesting finding is the identification of one *M. tuberculosis* strain from goat with spoligopattern EAI5, which is typical of strains isolated in East Africa and India (Brudey *et al.,* 2006). The isolation and identification of a strain with the spoligotype EAI5 in Nigeria remains an interesting finding as it is a strain commonly found in East Africa and India (Brudey *et al.,* 2006). The identification of foreign *M. tuberculosis* strains and new subtypes of *M. tuberculosis* and *M. bovis* in humans suggests high diversity amongst the circulating MTBC strains in Nigeria. This also justifies the need for a thorough molecular epidemiological survey of MTBC with a view of identifying the prevalent strains and detecting newer ones.

Ibadan, the study site is a highly populated South-western city of Nigeria as well as a confluence point between northern Nigeria and South-west Nigeria, particularly for cattle trade. Since the currently adopted method of MTBC identification in most parts of Nigeria i.e. smear microscopy is not sensitive enough in detecting members of this complex, especially in the era of the increasing incidence rate of HIV/AIDS and TB, a more rapid and sensitive method of detection is hereby recommended. Improper identification of the members of this complex together with the resultant improper drug administration can promote increased drug susceptibility and a high genetic diversity amongst the circulating strains.

This study therefore highlights the relevance of using molecular techniques in the identification of MTBC in high density settings where there is a high risk of human TB as well as a huge potential for zoonotic TB. Routine species identification using deletion analysis as a first test is hereby recommended because it is a rapid and simple method of species differentiation when compared to traditional methods of species identification. The latter requires several weeks of

bacterial growth and biochemical tests which are not as sensitive as PCR based techniques. However, in the instance of a more thorough screening process, spoligotyping should be applied as it is capable of both species and strain differentiation and would aid in epidemiological studies as well as exchange of information between laboratories.

CHAPTER 4

Multiple locus [variable number of tandem repeats] analysis of *M. bovis* and *M. tuberculosis* in Nigeria using a 16 VNTR loci

4.1. Introduction

Molecular epidemiology of bovine tuberculosis is very important in the light of this disease as a re-emerging zoonosis in developed countries and in developing counties like Nigeria which has an increasing incidence of HIV/AIDS infection and other factors which promote the transmission of tuberculosis (Salami and Katibi, 2006; Salami and Katibi, 2007).

The incidence of zoonotic TB is of great concern as its degree of transmission in Nigeria is unknown despite reports of BTB in human tuberculosis infections (Cadmus *et al.,* 2006). Cattle to cattle transmission of BTB on the other hand can be assumed to be the reservoir portal of the introduction of novel strains into Nigeria as suggested in an earlier report in which cattle migration from neighboring countries was implicated (Cadmus *et al.,* 2006).

As discussed in the previous Chapter, previous studies on BTB in Nigeria were based on mycobacterial culture (Idigbe *et al.,* 1986; Mawak *et al.,* 2006) while the first molecular report involving the application of spoligotyping and 6 loci VNTR analysis (VNTR A-F) on BTB was done by Cadmus and co-workers (2006). This study revealed that spoligotype profiles of certain strains isolated in Nigeria have been previously reported by earlier studies in Cameroon (Njanpop-Lafourcade *et al.,* 2001). Also, in a recent study in Mali, a strain with a similar spoligopattern to SB0944 which has been reported previously in Nigeria, Chad and Cameroon was reported in 13 of the 20 strains studied (Muller *et al.,* 2008). This gave strong indications regarding clonal distribution of *M. bovis* strains in West Africa (Cadmus *et al.,* 2006).

It is therefore imperative that an epidemiological study be carried out using a panel of loci which is able to provide sufficient discrimination and subsequently more reliable information on the circulating strains. This can also provide an insight into the transmission chains responsible for the spread of BTB as well as its zoonotic potential. In this study, a 16 VNTR MLVA assay was done on 74 MTBC isolates obtained from livestock tissues from the abattoir and human samples with relevant epidemiological information with a view of possibly detecting zoonotic TB

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amongst the human isolates included. The resolution of the 16 VNTR loci was further determined on some of the strains which had previously been spoligotyped.

4.2. Materials and Methods

4.2.1. Samples

Seventy three strains of human and animal origin (24 human isolates and 49 isolates of animal origin) were subjected to the 16 loci MLVA analysis. These samples had been previously identified using deletion analysis (Chapter 3) as described by Warren *et al.*, (2006) and 33 of these samples were spoligotyped as described by Kamerbeek *et al.*, (1997).

4.2.2. Multiple locus variable number of tandem repeats assay

As described in Chapter 2.

4.3. Results

4.3.1. Allelic diversity and MLVA-16

Allelic diversity was calculated using the Simpsons index option in the Bionumerics software. For the *M. bovis* strains, 9 loci were regarded as highly discriminatory in this assay i.e. $h^a \geq 0.30$, whilst 3 loci (ETRD, MTUB21 and MTUB30) were classified as moderately discriminatory loci where $0.3 \geq h^a \geq 0.20$, whilst one locus (MTUB38) provided very little polymorphism and had the value $h^a = 0.041$. Two loci were clearly non discriminatory $h^a = 0$ (Table 4.1). The genotypic diversity of the 16 loci on these set of samples was 0.969. The allelic diversity index values for the *M. tuberculosis* on the other hand revealed 10 highly polymorphic loci when $h^a \geq 0.30$ whilst 2 loci, ETRD and MTUB38 were moderately polymorphic $0.3 \geq h^a \geq 0.20$. Only one locus (MTUB12) was monomorphic with these strains.

In the combined dendogram i.e. *M. tuberculosis* and *M. bovis* strains, 5 VNTR loci provided 100% typability, however, MIRU10 failed to amplify with 14 isolates, hence only 81% typability and subsequently no copy number was assigned to these isolates at this locus. Ten of these isolates were *M. tuberculosis* and were isolated from PTB patients.

4.3.2. Cluster analysis

The MLVA technique was carried out by amplifying 16 tandem repeat loci using 73 MTBC isolates (*M. bovis* and *M. tuberculosis*) obtained from livestock and humans. The obtained copy numbers were analyzed using Bionumerics software. Because of the absence of copy numbers at certain loci, characters that could not be assigned copy numbers due to lack of PCR amplification at certain loci, presumably for technical reasons rather than the unlikely absence of the target locus were not treated as "0" character states. Hence at such loci, for instance, Bionumerics assumed that data there could have been a copy number present. A dendogram was drawn by UPGMA using the copy numbers as categorical characters which resulted in three major cluster groups; A, B and C (Figure 4.1).

Group A was further divided into two subgroups; Ai and Aii and 17 different VNTR profiles. All the strains in group A consists of only *M. tuberculosis* strains with Ai being mainly strains of the T, H, LAM10-CAM and U spoligopatterns, whilst Aii were predominantly the LAM10-CAM family. Group Ai consists of 8 *M. tuberculosis* strains which clustered together. Isolates were from 6 human samples; cattle isolate (c15) and as well as one pig isolate (p44). All the isolates in this group presented with different VNTR profiles. Group Aii isolates on the other hand were divided into two sub groups, group Aiia consisting of 10 *M. tuberculosis* isolates which were isolated from patients diagnosed with pulmonary tuberculosis (PTB) whilst Aiib consisting of 6 *M. tuberculosis* isolates obtained from infant feces and PTB patients (Figure 4.1).

All the isolates in group B which were confirmed as *M. bovis* by the deletion analysis PCR are predominantly of cattle origin. There were however 4 goat, 8 pig isolates and 2 isolates of human origin; hb74 and hb83 clustering in different subgroups of group B. The spoligotype of the human strain hb74 is SB0944 and it has been previously described in Nigeria, Cameroon, Chad and Mali whilst strain hb83 with a spoligotype identical to SB1432 was also been previously described in cattle in Nigeria (Cadmus *et al.*, 2006). Group B was resolved into 30 VNTR profiles consisting of only *M. bovis* strains and further divided into 8 subgroups, consisting of 49 isolates.

A particular genotype was found in 6 strains which were of cattle origin. One of the 6 cattle strains possessed a SB0944 spoligopattern which is fairly ubiquitous in West Africa. Identical VNTR profiles were found in strains isolated from different species of animals i.e. g47, c59, c60 and c63 all had a similar profile, hence the same genotype; and these were isolated from cattle and a goat. Four other strains (b3, p41, p43 and p45) also shared the same VNTR profile and were isolated from pigs and cattle (Figure 1). One cattle isolate (c6) however presented with a spoligotype of SB1105 which had been previously isolated from the tissues of a dromedary in Chad (www.mbovis.org).

Group C consists of a single *M. tuberculosis* strain obtained from a goat. It possessed a unique VNTR profile hence it clustered alone. Spoligodata confirmed it as having the pattern EAI5 (Figure 4.1).

4.3.3. Comparison of different 16 VNTR loci and ETR A-E loci panels

The resolution of 2 loci combinations consisting of ETRs A, B, C, D, E and the 16 loci combination were compared. The genotypic index of the different loci combinations were determined for the 49 *M. bovis* and 23 *M. tuberculosis* isolates (Table 4.1). The 16 VNTR loci combination had the highest genotypic diversity whilst the ETR A-E loci combination had the lowest genotypic diversity for the *M. tuberculosis* and *M. bovis* isolates (Table 4.1). Fourteen *M.*

tuberculosis isolates all had an identical ETR A-E profile, *42433*; hence belonging to the same genotype according to ETR A-E loci panel (Figure 4.2), whilst the 16 VNTR loci panel resulted in 9 different VNTR profiles. The fourteen strains were of the LAM10-CAM spoligopattern with 13 having the 61 Shared International type (SIT) number and h128 having the 403 SIT.

4.3.4. Resolution of spoligopatterns by the 16 loci VNTR analysis

The MLVA profiles of 33 MTBC strains from humans and animals which had been subjected to spoligotyping were compared (Figure 4.3). Nineteen of these were *M. tuberculosis* whilst 14 were *M. bovis*. The dendogram suggests the MLVA-16 has the ability to split up strains of identical spoligopatterns. Five *M. bovis* strains (c24, p36, c34, hB74, c3) with the same spoligopattern (SB0944) were further differentiated by the MLVA 16 analysis. There were however two strains, c3 and c4, with an identical MLVA profile and different spoligopatterns. The spoligopattern of c4 possessed spacer deletions at exactly the same regions as c3 (SB0944) with additional loss of spacers 5, 6, 14, 2, 31-34, 36 and 37. This loss of spacers may have occurred as a result of a spoligotyping error. The *M. tuberculosis* on the other hand was mainly of the LAM10-CAM family. Three *M. tuberculosis* strains all isolated from PTB patients had identical spoligopatterns and MLVA profiles. Strains h128 (403LAM10-CAM) and h112 (61LAM10-CAM) were assigned to the same genotype despite their differences in spoligotypes. In strain h128 however, there were 3 loci to which copy numbers could not be assigned, hence there is a chance that this strain may have different MLVA profiles (Figure 4.1). On the other hand, hb76 and hb82 which had the same spoligotype had different VNTR profiles differences at 2 loci, MTUB02 and QUB11A.

Table 4.1: Allelic and genotypic diversity of 16 VNTR loci in Nigerian *M. bovis* and *M. tuberculosis* strains

	M. bovis n=49		*M. tuberculosis* n=23	
locus	allele number	allelic diversity	allele number	allelic diversity
ETRA-2165	4	0.29	5	0.603
ETRB-2461	4	0.628	4	0.297
ETRC-0577	5	0.39	4	0.497
ETRD-0580	3	0.192	3	0.157
ETRE-3192	4	0.581	3	0.41
MIRU10-0959	1	0	4	0.47
MIRU27-3006	3	0.429	3	0.46
MIRU40-0802	4	0.442	5	0.723
Mtub21-1955	2	0.153	2	0.08
Mtub30-2401	3	0.224	3	0.477
Mtub39-3690	3	0.081	7	0.823
Mtub02-0079	2	0.474	6	0.62
Mtub12-1121	1	0	1	0
Mtub29-2347	4	0.412	3	0.62
Mtub38-3663	2	0.041	2	0.153
Qub11a-2163	8	0.456	7	0.72
	total genotype number	Genotypic index	total genotype number	Genotypic index
MLVA 16	30	0.969	18	0.97
ETR A-E	17	0.35	8	

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ETRA-2165	ETRB-2461	ETRC-0577	ETRD-0580	ETRE-3192	MIRU10-0959	MIRU27-3006	MIRU40-0802	Mtub21-1955	Mtub30-2401	Mtub39-3690	Mtub02-2079	Mtub12-1121	Mtub29-2347	Mtub38-3863	Qub11a-2163	Strain ID	Spoligotype	Host
3	2	3	3	4	3	3	4	3	4	4	9	4	3	3	6	hB76	37T3	humans
3	2	3	3	4	3		4	3	4	4	8	4	3	3	7	hB82	37T3	humans
3	2	3	3	4		3	1	3	2	3	8	4	4	3	3	p44	61 Cameroon	pig
3	2	3	3	2		3	2	3	4	4	9	4	4		2	hB77	50H3	humans
3	2	3	3	3	5	3	2	3		4	7	4	4			N4	U	humans
3	2	3	3	2	5	3	1	3	4	4	10	4	2	2	11	c15	53T1	cattle
3	2	3	3		5	3	1	3		4	7	4	2			NB75	50H3	humans
3	2	2	3			3				6		4				N111	358T1	humans
4	2	4	3	3	3		3	3		6	4	4				N8	61 Cameroon	humans
4	2	4	3	3	3	3	3			6	4	4				N115	Cameroon	humans
4	2	4	3	3	3		3	3	11	6	4	5				N48	838 cameroon	humans
4	2	4	3	3	3	3	3	4	11	7	4	3	3		6	hB81		humans
4	2	4	3	3	3	3	3	2	6	5	4	3	3		6	h107	61 Cameroon	humans
4	2	4	3	3	3		3	2	6	5	4	3	3			h108		humans
	2	4	3	3	3	3		3	2	5		4	3			h109		humans
4	2	4	3	3	3	3		3	2	6	5	4	3	3	7	h110	Cameroon	humans
4	2	4	3	3			3	2	6	9	4	4	3			h122	61 Cameroon	humans
4	2	4	3	3	3		3	2	6	9	4	4	3		6	h123	61 Cameroon	humans
4	2	4	3	3	3	3		3	2	6	9	4	4	3	6	h126	61 Cameroon	humans
4	2	4	3	3	3	1		3	2	5	9	4	4	3	6	h112	61 Cameroon	humans
4	2	4	3	3	3			3	2		9	4	4			h128	403 Cameroon	humans
3	2	4	3	3			3		2		9	4	4	3	6	h116	Cameroon	humans
4	1	4	3	3	3	3		3	2	11	9	4	4	3		h49	838 Cameroon	humans
4	2	4	3	3	3	1		3	2	5	9	4	2	3	6	h113	61 Cameroon	humans
5	4	5	4	3	2	3	1	3	4	3	10	4	3	3	9	p36		pig
5	4	5	4	3	2	3	1	3		3	10	4	3	3	9	p37		pig
5	4	5	4	3	2	3	1	3		3	10	4	3	2	9	p38		pig
5	4	5	4	3	2	3	2	3	4		10	4	3	3	8	c20		cattle
5	5	5	4	3	2	3	2	3	4	3	10	4	3	3	9	c8		cattle
5	5	5	4	3	2	3	2	3	4	3	9	4	3	3	9	c19		cattle
5	5	5	4		2	3		2	3	3	10	4	3	3	9	c24		cattle
5	5	5	4		2		2	3		3	10	4		3	9	c27		cattle
5		5	4		2		2	3		3	10	4	3	3	9	c30		cattle
5		5	4		2		2	3		3	10	4	3	3	9	c31		cattle
5	5	5	4	4	2		2	3	4	3	10	4		3	9	c22		
5	5	5	4	3	2	3	2	3		3	8	4	3			NB83	SB1432	humans
5	5	5	4	3	2	2	1	3	4	3	10	4	3	3	9	c33		cattle
5	5	5	4	3	2	2	1	3		3	10	4	3	3	9	c34		cattle
5	5	5	4	3	2	3	2	3	4	3	9	4	3	3	9	c25		cattle
5	5	5	4	3	2	3	2	3	4	3	9	4	3	3	9	g48		goat
5	5	5	4	3	2	3	3	3	4	3	9	4	3	3	9	p39		pig
5		5	3	3	2	3		3	4		9	4	3	3	9	g51		goat
5	4	5	4	3	2	3	2	3	4	3	9	4	3	3	9	g47		goat
5	4	5	4	3	2	3	2	3	4	3	9	4	3	3	9	c59		cattle
5	4	5	4	3	2	3	2	3	4	3	9	4	3	3	9	c60		cattle
5	4	5	4	3	2	3	2	3	4	3	9	4	3	3	9	c63		cattle
5	4	5	4	3	2	1	3	4		3	9	4	3	3	9	p35		pig
5	4	5	4	2	3	2	3	4		3	9	4	3	3	9	b3		cattle
5	4	5	4	2		3		4	3	9	4	3	3	9		p41		pig
5	4	5	4	4	2		3	4	3	9		3	3	9		p43		pig
5	4	5	4	2		3	2	3	4	3	9	4	3	3	9	p45		pig
5	4	6	4	3	2	3	2	3	4	3	9	4	3	3	9	b1		cattle
5	4	5	4	3	2	3	2	3	4	3	9	4	3	3	9	p40		pig
5	4	5	4	3	2	3	2	3		4	9	4	2	3		hB74	1037BOVIS1	humans
5	5	5	4	2	2	3	3	3	4	3	9	4	1	3	9	c3		cattle
5	5	5	4	2	2	3	3	3	4	3	9	4		3	9	c4		cattle
4	5	5	4	2	2		3	3	4	3	9	4		3	9	c6		cattle
5	5	5	4	2	2		3	3	4	3	10	4	3	3	9	c32		cattle
4	5	5	4	2		3	2	3	4	3	9	4	3	3	6	b7		cattle
4	5	5	4	3	2	3	2	3	4	3	9	4	3	3	6	c2		cattle
5	5	5	4	3	2	3	2	3	4	3	9	4	3	3	6	b5		cattle
3	5	5	4	4	2	3	2	3	4	3	9	4	3			b9		cattle
4	5	5	4	3	2	3	2	2	3	4	9	4	3	3	9	c12		cattle
5	5	5	4	3	2	3	2	3	4	3	9	4	3	3	12	b6		cattle
5	5	4	4	3	2	3	2	3	4	3	9	4	3	2	3	b10		cattle
6	4	7	4	4	2		3	2	3	4		9				b8		cattle
5	5	3	5	3	2	3	2	3	4	3	9	4	3	3	11	c9		cattle
5	5	3	5	3	2	3	2	3	4	3	9	4	3	3	11	g50		goat
5	5	3	5	3	2	3	2	3	4	3	9	4	3	3	10	c57		cattle
4	3	4	4	3	2	2	2	2	4	3	10	4	3	3	9	c16		cattle
4	3	4	4	3	2	2	2	2	4	3	10	4	3	3	9	c17		cattle
5	3	4	4		2	2		2	3	3	10	4	3	4	9	c18		cattle
5		5	5		2		2	2		3	10	4		3	9	c26		cattle
8	5	4	6	2	5	3	3	6	1	9	9	4	3	3	12	g49	342EAI5	goat

Ai

A

Aii

B

C
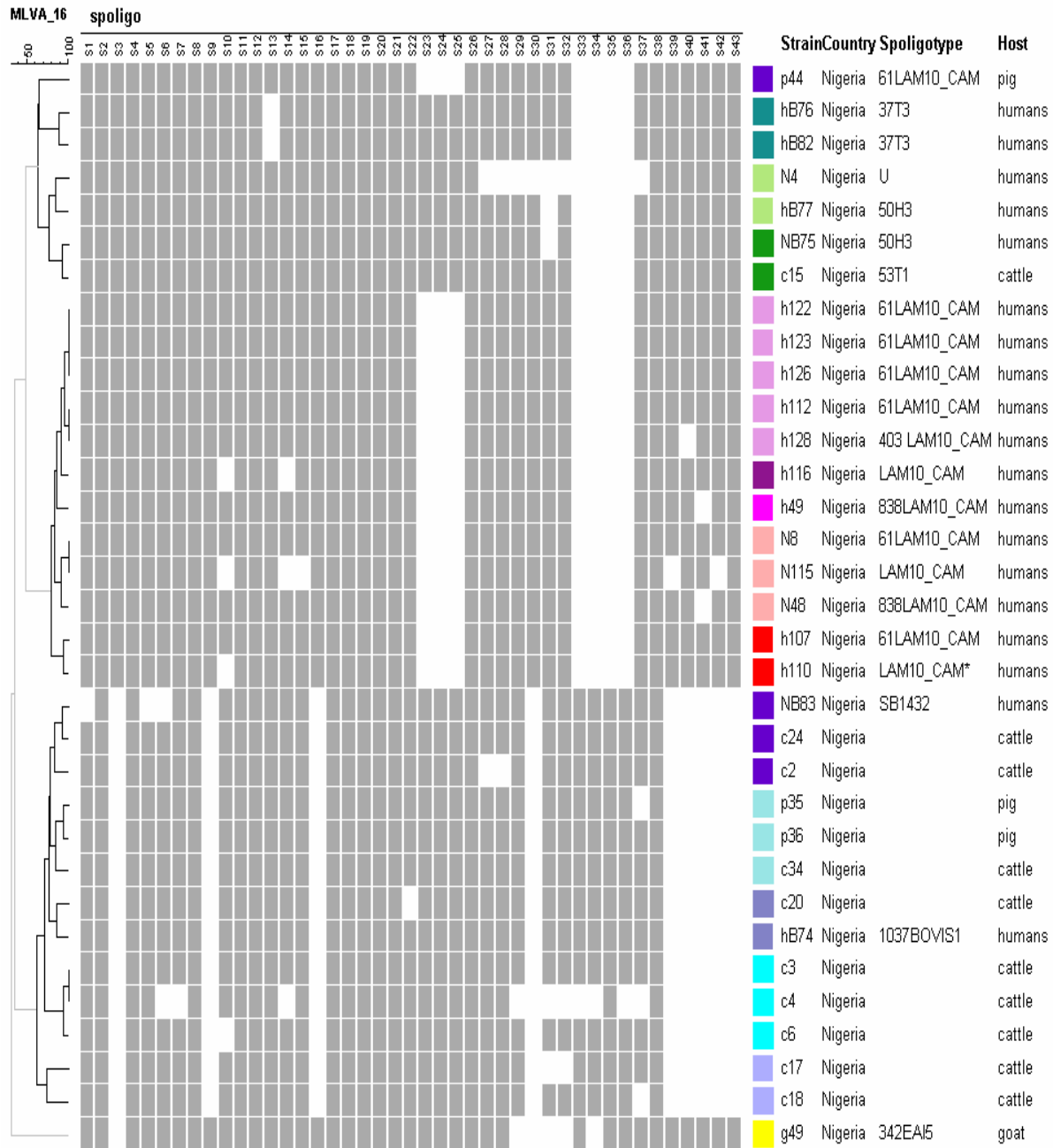
M. tuberculosis

M. bovis

M. tuberculosis (EAI 5)

Figure 4.2: Dendogram showing the resolution ability of MLVA 16 compared with Spoligotyping on 33 *M. bovis* and *M. tuberculosis* strains obtained from humans and animals

4.4. Discussion

The use of multiple locus variable number of tandem repeats assay (MLVA) as a genotyping and epidemiological tool for *M. bovis* studies has been well documented (Cadmus *et al.,* 2006; Digumbiaye *et al.,* 2006, Muller *et al.,* 2008, Roring *et al.,* 2002; Roring *et al.,* 2004). In this study 16 VNTR loci were genotyped in 73 MTBC isolates and 13 VNTR loci were regarded as highly polymorphic, $h^a \geq 0.44$, two had a low polymorphism index $h^a \geq 0.11$ whilst one was monomorphic. The 16 loci VNTR profiles identified 49 *M. bovis* isolates from the 73 strains typed and these were resolved into 30 different VNTR profiles with a genotypic diversity of 0.97 whilst the 24 *M. tuberculosis* strains identified were resolved into 18 different VNTR profiles.

The previous molecular study in Nigeria was done using the VNTR A-F loci. However, the utilization of ETR A-F loci has previously been reported to be less discriminatory than spoligotyping (Roring *et al.,* 2002). This was also apparent in this study where 14 *M. tuberculosis* stains which were of the LAM-10 family could not be further differentiated by the ETR A-E loci panel. Furthermore, 2 of the 13 strains were of different subtypes of the LAM-10 family, yet they all had the same ETR A-E profile; *42433*. However, the discrimination provided by the 16 VNTR loci set on these 14 LAM10-CAM *M. tuberculosis* strains resulted in all 14 strains having different VNTR profiles. Comparing the ETR A-E and the 16 loci VNTR panel on the *M. bovis* strains also resulted in the splitting of 2 genotypes, comprising of 10 and 11 strains, respectively into genotypes with a smaller number of strains and several unique VNTR profiles (Figures 4.1 and 4.2).

Human to animal transmission of *M. tuberculosis* has previously been reported to occur in Nigeria (Cadmus *et al.,* 2006). A similar observation has also been made in this study in which *M. tuberculosis* was isolated from cattle (c15) and pig (p44) samples. Spoligopattern results confirm these isolates, c15 and p44, as *M. tuberculosis* of the T and LAM 10-CAM family, respectively and in a dendogram obtained from the MLVA analysis they clustered with other members of the *M. tuberculosis* strains. This is the second time such an observation is reported in isolates obtained from Nigeria, strongly supporting the possibility of human to livestock

transmission of *M. tuberculosis* (Cadmus *et al.,* 2006). It was also observed in a previous study by Cadmus and co workers (2006) that 69% of the *M. tuberculosis* isolates from their study were found to be of the LAM 10-CAM family. Strains with the LAM 10-CAM spoligopattern originate from Latin America and had been previously described in Cameroon (Njanpop-Lafourcade *et al.,* 2001). These strains have however been isolated from Niger, Ivory Coast as well as in several parts of Europe particularly France (Haddad *et al.,* 2001) and they appear to be the dominant strain circulating in Nigeria.

Furthermore, another *M. tuberculosis* strain with spoligopattern EAI5, typical of strains commonly found in East Africa and India (Brudey *et al.,* 2006) was isolated from a goat (g49) in this study. Cluster analysis proved unequivocally that this is a foreign strain in Nigeria as it formed an out group in the tree generated by UPGMA using the Bionumerics software. In a further analysis, when this strain was compared with other strains from Africa, it grouped closely with other strains with the EAI spoligopattern isolated from Sudan and Djibouti (personal communication Institute de Genetiqué et Microbiologie, Université Paris-Sud Orsay). This is an interesting finding which strongly suggests a transmission from countries in the East African block, probably via humans or through transportation of animals to West Africa.

The primary reason for screening human samples in this study was to identify zoonotic tuberculosis due to *M. bovis*. Zoonotic tuberculosis in Nigeria had been established previously by Cadmus and co workers (2006) using molecular techniques. The relevance of molecular techniques in elucidating zoonotic tuberculosis is again emphasized in this study as it reports 2 *M. bovis* strains isolated from humans. These *M. bovis* strains (hb74 and hb83) had clearly distinct spoligopatterns and VNTR profiles, suggesting that they originated from different sources. The strain hb83, with a spoligopattern similar to SB1432 in the www.mbovis.org database is yet to be reported in the SpolDb4 database whilst hb74 has a spoligopattern of SB0944 which has been previously reported in cattle in Nigeria (Cadmus *et al.,* 2006), Chad, Cameroon (Njanpop-Lafourcade *et al.,* 2001) and Mali (Muller *et al.,* 2008). The spoligopattern SB0944 is quite ubiquitous as it had been isolated previously in Cameroon (Njanpop-Lafourcade

et al., 2001), France (Haddad *et al.,* 2001), and USA (Driscoll *et al.,* 1999) as well as in cattle in Nigeria (Cadmus *et al.,* 2006). This strain diversity of *M. bovis* induced tuberculosis in humans as highlighted by the two different *M. bovis* strains causing infection in two different patients further justifies the need for the adoption of molecular techniques in routine diagnosis of MTBC in humans in Nigeria.

A high allelic diversity was obtained amongst the *M. bovis* strains from the 13 of the 16 used loci. This is suggestive of the level of genetic variability amongst the circulating *M. bovis* strains in Nigeria. The 3 loci which were not polymorphic in the *M. bovis* strains were MIRU10, MTUB12 and MTUB38. Incidentally, in previous studies on *M. bovis* strains isolated in Northern Ireland (Roring *et al.,* 2004) and Chad (Hilty *et al.,* 2005) MIRU10 had been described as monomorphic. On the other hand, it was illustrated to be highly polymorphic in *M. tuberculosis* in this study as well as in other *M. tuberculosis* strains isolated from South Africa, France and the United States (Hilty *et al.,* 2005). In this study, it is strongly suggested that this locus is not a discriminatory marker and hence should not be included in the diagnosis of *M. bovis* but may be used in epidemiological studies in which a record of polymorphism at this locus will indicate a significant mutational event in the strain.

This high genetic diversity amongst the *M. bovis* strains obtained from livestock in Nigeria is illustrated in this study in which 30 different VNTR profiles from 49 *M. bovis* isolates have a genotypic diversity index of 0.969. Four different *M. bovis* genotypes were observed in different animal species. This was identified when 5 isolates obtained from cattle, pig and one goat isolate (c59, c60, c63, g47 and p35) had the same VNTR profile (Figure 4.1.). Two other isolates (c25, g48) again isolated from cattle and goat also presented with the same VNTR profile (Figure 4.1.). One cattle isolate (b3) also had an identical VNTR profile with 3 pig isolates (p41, p43 and p45) whilst the final group (b1 and p40) also had an identical VNTR profile. Another strain with a spoligopattern of SB1105, which was previously isolated in the tissues of a dromedary in Chad (Diguimbaye-Djaibe *et al.,* 2006), was also identified from a tissue sample of a female

White Fulani breed of cattle (c6) in Nigeria. This may have occurred as a result of migration of cattle and camels from Chad to cattle markets in the northern states of Nigeria.

The presence of similar strains in different host species is quite an intriguing scenario as it highlights the relevance of the interaction of different susceptible host species and the circulation of *M. bovis* strains among them. The interaction of different animal species in the same environment as occurs in the extensive system of rearing can thus be regarded as a factor which promotes this interspecies transmission of *M. bovis* strains.

The results presented in this study are indicative of the resolution provided by using a larger panel of markers when compared with a smaller loci set. The use of the ETR loci alone in epidemiological studies of MTBC is not capable of providing sufficiently reliable information when compared with spoligotyping (Roring *et al.,* 2002). An MLVA analysis with a larger panel of loci has however been found to provide more reliable epidemiological information (Roring *et al.,* 2002). It was also observed in this study that improper clustering of members of the MTBC occurs when the ETR A-E loci alone are used, especially with respect to strain differentiation. This was observed when strains with different spoligopatterns have the same ETR A-E profile, hence they cluster together when dendograms are constructed based on these 5 VNTR loci. Hence, in the absence of standardized molecular techniques like spoligotyping, clusters generated by using the ETR A-E loci panel cannot be relied upon in providing information regarding epidemiologically related or unrelated strains, it can however be used to differentiate between *M. bovis* and *M. tuberculosis* as observed in this study.

Possible reasons for the high genetic diversity in Nigeria can hence be adduced to a lack of BTB control policies together with livestock migration from neighboring countries (Cadmus *et al.,* 2006). Further epidemiological studies are hence recommended in Nigeria as it will shed more light on the transmission dynamics of BTB infections. This study therefore suggests the epidemiological relevance of the use of a larger VNTR loci combination on *M. bovis* isolates

from Nigeria, where there is currently no nationwide molecular epidemiological survey in place. Furthermore, a careful selection of markers is capable of explaining in a more reliable way, the epidemiological relatedness of the strains. This was illustrated when groups of strains, which would have otherwise been regarded as having a common VNTR profile or genotype, were properly differentiated.

CHAPTER 5

Molecular analysis of *M. bovis* isolated from Buffalo in Hluhluwe-iMfolozi Park, South Africa

5.1. Introduction

Mycobacterium bovis is particularly noted for its diverse host tropism which includes humans, livestock and several wild animal species. The first report of bovine tuberculosis in cattle in South Africa was made by Hutcheon in 1880 (Hutcheon. 1880), whilst bovine tuberculosis was first diagnosed in buffalo (*Syncerus caffer*) in Hluhluwe-iMfolozi Park (HiP) in 1986 (Jolles. 2004). Hluhluwe-iMfolozi Park is situated in the Kwazulu-Natal Province of South Africa and its 100,000 ha area is almost entirely surrounded by communal farmland and livestock population. It is the third largest game reserve in South Africa and has a buffalo population of approximately 3000 heads with subsequent reports of spillover of BTB to other species documented (Michel *et al.,* 2006).

The molecular characterization of *M. bovis* is essential in understanding disease transmission between species and the spatial distribution of this infection in wildlife populations. Furthermore, it can assist in establishing an epidemiological link of *M. bovis* isolated from buffalo and cattle kept on adjacent farms close to the south of the Kruger National Park (Michel. 2002). The previous molecular characterization of *M. bovis* isolated from wildlife at the KNP was based on restriction length polymorphism (RFLP) (Michel *et al.,* 2006)**.** Earlier reports indicated that two epidemiologically unrelated strains are in circulation in HiP whilst the strains in the KNP and HiP are epidemiologically unrelated (Michel *et al.,* 2006).

On the basis of the above information, the aim of this study was to investigate the genetic nature of the circulating strains of *M. bovis* in HiP buffalo by using the regions of difference (RD) deletion analysis PCR was used for the identification of the isolates and the MLVA and spoligotyping techniques for genotyping.

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5.2. Materials and Methods

5.2.1. Sample collection

Buffaloes of the HiP are routinely submitted to the tuberculin skin test (TST) by the South-African National Park authorities. Animals are captured in a kraal, anesthetized and inoculated with tuberculin. The buffaloes are kept enclosed for 3 days to allow the reading of the TST 72 later. Animals testing positive are culled as a control measure. Bronchial lymph node tissues from 12 positive buffaloes were collected and stored in formalin before mycobacterial culture.

5.2.2. Bacteriology

The tissue samples were processed at the Tuberculosis Laboratory of the Bacteriology Section, Onderstepoort Veterinary Institute by first decontaminating with 2% HCl and 4% NaOH and then homogenized. The homogenate was later neutralized with an equal volume of distilled water. The resultant solution was then inoculated onto Lowenstein-Jensen media with pyruvate and glycerol and incubated at 37 °C. Nucleic acid for PCR amplification was obtained by boiling pure culture colonies at 80 °C for 30 minutes, centrifugation at 8,000 rpm for 3 minutes and harvesting of the supernatant.

5.2.3. Deletion analysis

As described in Chapter 3

5.2.4. Tandem repeats PCR amplification

As described in Chapter 2.

5.2.5. Spoligotyping

As described in Chapter 3

5.3. Results

5.3.1. *Mycobacterium bovis* isolation

The 12 bronchial lymph node tissues were cultured and after 10 weeks of incubation, only 4 were positive for bacterial growth. ZN staining of culture smears confirmed the presence of tubercule bacilli. Deletion analysis using the multiplex PCR targeting the RD4 and RD9 further confirmed the isolates as *M. bovis*, (presence of two specific bands of 268 bp and 108 bp as shown in Figure 5.1).

5.3.2. Multiple locus VNTR analysis

To determine the level of differentiation between the four isolates, tandem repeats analysis was performed on the isolates. By comparing the copy numbers of the MLVA profile of each isolate, two distinct profiles were observed. Three of the 4 isolates have an identical MLVA profile at all 16 loci tested while the fourth isolate had a different copy number at 7 of the 16 loci tested (Table 5.1)**.** The loci that provided this discrimination were ETR-A, ETR-B, ETR-C, ETR-E, QUB11A, MTUB12 and MTUB21 (Le Fleche *et al.,* 2002).

5.3.3. Spoligotyping

The results obtained by spoligotyping (Table 5.2) revealed two different spoligopatterns. The 3 isolates that had the same MLVA pattern had the same spoligopattern whereas that of the 4th isolate was different. The spoligopattern of the latter, according to the international spoligotype database nomenclature, belongs to the family 481 BOVIS1 as delineated by the absence of the spacers 39-43, which is typical of *M. bovis* and the absence of spacers 3, 9, 16 and 21. This pattern is similar to *M. bovis* BCG except that spacer 21 is present in *M. bovis* BCG. The

spoligopattern of the three other isolates is marked by the absence of spacers 3, 6, 8-12, 16, 22 and 23, as well as spacers 39-43, which is typical of *M. bovis* isolates. This spoligopattern was compared with existing spoligopatterns on the database and it appears closest to the BOVIS2 family but not specific.

Table 5.1: MLVA profile of 4 *M. bovis* isolates collected from buffaloes at HiP, at 16 VNTR loci

	VNTR loci tested															
	MTUB 38	MIRU 27	MTUB 39	MIRU 23	MTUB 21	MTUB 12	MTUB 2	MTUB 29	QUB 11A	ETR A	ETR B	ETR C	ETR D	ETR E	MIRU 10	MTUB 30
Isolates																
1	3	3	3	4	3	3	8	1	13	4	5	5	3.5	3	2	4
2	3	3	3	4	3	3	8	1	13	4	5	5	3.5	3	2	4
3	3	3	3	4	3	3	8	1	13	4	5	5	3.5	3	2	4
4	3	3	3	4	2	5	8	1	8	6	4	3	3.5	4	2	4

The MTUB loci were previously described by Le fleche *et al.,* (2002), MIRU loci described by Supply *et al.,* (2000), the ETR loci described by Frothingham and Meeker O' Connel (1998) and QUB11A by Skuce *et al.,* (2002). Alternative names for the individual locus according to their position on the *M. tuberculosis H37Rv* genome was described by Le Fleche *et al.*, (2002).

Table 5.2: Spoligotyping patterns of the *M. bovis* isolates, with two reference strains

Isolate	Spoligopattern	SpolDB4	Family
1			BOVIS2_HiP
2			BOVIS1
3			BOVIS2_HiP
4		481	BOVIS2_HiP
BOVIS2		1856	BOVIS2(ref.)
BCG		482	BOVIS1_BCG
H37Rv		451	*H37Rv*

Spoligopatterns of the 4 *M. bovis* isolates and 2 reference strains; *M. bovis* BCG and *M. tuberculosis H37Rv* showing deletion of spacers 39-43 which is typical of *M. bovis* strains relative to *M. tuberculosis* and 1856 BOVIS2, a member of the BOVIS2 which had been described previously. The SpolDB4 is the international spoligotyping database described by Brudey *et al.,* (2006) with numbers indicating strain differentiation amongst the family to which they belong i.e. BCG is 482 and isolate 4 is 481 of the BOVIS1 family. Isolates 1, 2, 3 according to SpolDB4 database belong to BOVIS2 as marked by the distribution of their spacers.
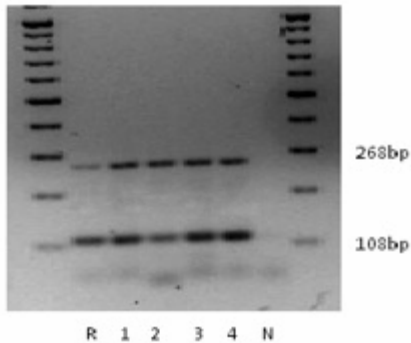
Figure 5.1: Confirmation of *M. bovis* status of Hluhluwe *M. bovis* isolates 1, 2, 3, 4 by deletion analysis (multiplex PCR) as indicated by two band sizes (108bp and 268bp) corresponding to deletions at the RD9 and RD4 regions.

5.4. Discussion

All mycobacteria isolated from the buffaloes in this study were confirmed to belong to the *M. bovis* species. Molecular tools have been utilized to study the transmission dynamics of mycobacterial infections and spoligotyping has been reported to provide sufficient discrimination between *M. bovis* strains. Even more discriminatory is the use of VNTR analysis with an appropriate panel of loci (Le Fleche *et al.,* 2002).

This study, despite the relatively small sample size, presents data which suggest that at least 2 *M. bovis* strains not decribed earlier are currently circulating among buffaloes in HiP. It is important to note that spoligotype SB0121 was previously identified in cattle in South Africa as well as in buffaloes in KNP, but not in HiP. Our observation therefore suggests that, spoligotype SB0121 has entered HiP, originating from communal cattle around the park or other unknown sources, possibly wildlife including buffaloes from other locations. It is also possible that, at one point in time, strains present in buffaloes sampled in this studied area (western part of HiP) may not have been present in buffaloes sampled in the previously studied areas (eastern part of HiP), if there

were multiple points of entry of different strains. Indeed, earlier behavioral studies indicated that HiP buffaloes have relatively stable and small home ranges (compared to KNP) due to the abundant food and water availability in HiP, even in winter (Dora. 2004). It was also shown that there was very little if any mixing of breeding herds with adult bulls implicated as the main source of infection between herds. This finding did explain why there could be dramatic differences in BTB prevalence in herds in close proximity with even overlapping home ranges (Dora. 2004).

The spoligotype patterns we obtained indicated that the strains isolated in 2007 have different origins and clearly did not evolve from each other. The new strain, however, seemed to have evolved from the BOVIS 2 family because all strains from this family have deletions at the spacers 3, 6, 8-12 and 16 in the direct repeat region. In addition to the typical BOVIS 2 spacer deletion, spacers 22 and 23 were lost in this strain; this pattern is suggestive of its origin. On the basis of its unique pattern and origin, this strain was given the nomenclature BOVIS2_HiP and subsequently included on the SpolDB4 database as well as on the international *M. bovis* database (www.mbovis.org) where it is designated as SB1474 (Table 5.2). Spoligopatterns of strains isolated before 2000 in HiP (spoligotype SB 0130) and strains isolated in 2007 in HiP (spoligopatterns SB0121 and SB1474) show clear differences suggesting that they have different origins and did not evolve from each other.

The MLVA suggests that an identical strain was the cause of infection in 3 buffaloes, whereas an unrelated strain infected the 4th one (Table 5.1). Furthermore, MLVA analysis based on ETR A-E (Frothingham and Meeker-O'Connell, 1998) highlighted variations at 4 of the 5 ETR-loci, as well as at 2 other loci (QUB 11A and MTUB 12) which have been previously reported to be discriminatory (Roring *et al.,* 2004) (Table 5.1). These loci sufficiently discriminated the 2 strains from the 16 loci panel. However, in a recent molecular study on *M. bovis* from cattle in 6 provinces in South Africa, the ETR A-F loci previously described by Frothingham and Meeker O'Connel (1998) when compared to other tools such as *IS6110* RFLP and spoligotyping was reported to be less discriminatory (Michel *et al.,* 2008).

The present study utilized 16 tandem repeat loci, which presented with an identical VNTR profile in 3 of the 4 isolates; the possibility of loci polymorphism and increased strain variation at certain loci could not be established because of the sample size, thus indicating that the novel strain is the cause of the infection in the 3 buffaloes. When comparing ETR A-E patterns, strains isolated in HiP buffaloes in 2007 do not match patterns from strains isolated in cattle in 1993, 1994 and 1999 by Michel and co-workers. These strains should be further tested by a MLVA genotyping assay including more loci in order to increase the resolution of the assay (Le Fleche *et al.,* 2002) and to assess the relatedness of these strains. Given that no VNTR patterns are yet available for SB0121 strains isolated in buffaloes in KNP, we cannot rule out that strains isolated in buffaloes from HiP and KNP may be related.

Our results have been generated from only 4 isolates and show one pattern not described before in HiP and one pattern not described before in international databanks. This suggests that new strains were introduced tin HiP, via multiple ports of entry. These strains may originate from livestock around the Park or other unknown sources, possibly wildlife including buffaloes from other locations. Given the behavior of buffaloes in HiP, our report suggests that a more extensive survey is needed within and around the HiP borders, as well as in the wildlife reservoir to characterize the different strains of *M. bovis* and identify sources of infection. Our study emphasizes on the need to include molecular tools in the study of *M. bovis* epidemiology in South Africa.

CHAPTER 6

GENERAL CONCLUSIONS

RECOMMENDATIONS

6.1. General discussion and recommendations

This study focuses on the identification of MTBC and genotyping of *M. bovis* and *M. tuberculosis*. Rapid molecular identification using deletion analysis technique and spoligotyping were adopted for species identification and genotyping using 16 loci MLVA was carried out on MTBC isolates from cattle in Belgium, humans and livestock in Nigeria and buffaloes from South Africa. This concluding chapter attempts to highlight the most important findings of the preceding chapters as well as suggesting opportunities for future research.

6.1.2. Rapid identification of members of the MTBC

Molecular tools aid in the rapid identification of MTBC species and should thus be routinely adopted in clinical settings in Nigeria. About 9.6% (13) of all human isolates were *M. bovis*, and 15.4% (2 of 13) of all the *M. bovis* isolates identified in the human isolates were from cattle traders. These isolates obtained from cattle traders were the only "classical" *M. bovis* species i.e. RD4 region deleted, which were obtained from human samples. Besides theses "classical" *M. bovis* strains, number of *M. bovis* strains which did not show the RD4 deletion were isolated from human, cattle, pig and goat. Other species of MTBC which were also identified from the human isolates were *M. africanum* (13.2%), M. *tuberculosis* (45%) and MOT (32.3%). This array of species responsible for human tuberculosis is of great importance especially in drug administration, in which case proper drugs are administered, ensuring efficient therapy and thus preventing the evolution of drug resistant species.

Spoligotyping is an effective tool in the identification of species of MTBC as well as in strain differentiation, it helps in identifying strains which are novel to a particular area. This was illustrated by the identification of a *M. tuberculosis* strain with a spoligopattern EAI5 typical of East African countries in Nigeria where the dominant strain is LAM10-CAM. Even amongst the LAM-CAM, spoligotyping enabled us to identify the most prevalent subtype which is the 61LAM10-CAM. Two novel subtypes of the 61LAM10-CAM were also identified from PTB patients and infant feces, suggesting evolution of new strains from the dominant strain in circulation. Spoligotyping is however less discriminatory than MLVA analysis, hence for an active and economical epidemiological surveillance, a simplified MLVA assay is hereby

recommended for adoption by the Ministry of Health for routine genotyping and identification of MTBC.

The use of molecular tools particularly the MLVA, is hence recommended in hospitals located in regions where tuberculosis is endemic and where there is a high risk of human to livestock interaction. This will help to quickly identify zoonotic tuberculosis and in the instance where molecular genotyping is done, disease monitoring can be effected. In the same regard, the application of molecular tools in rapid detection of MTBC and/or MOT in HIV endemic areas should be implemented. This will give an indication regarding the particular species which is most likely to be responsible for lesions and clinical symptoms in humans as well as perhaps suggesting (or excluding) the implication of zoonotic tuberculosis in HIV patients.

6.1.3. Molecular genotyping of human, livestock and wildlife MTBC

6.1.3.1. Comparison between AE VNTR and CE VNTR analysis

No difference in copy numbers was observed by AE VNTR analysis and CE VNTR analysis, except at QUB 11A where one strain was assigned two copy numbers with the CE VNTR analysis. This can be attributed to artifactual peaks which are often picked up by the CE VNTR analysis. Markers with small differences in repeats sizes however need to be run on 3% agarose gels and with lower molecular weigh ladders which will ensure that the slight differences in band sizes can be captured. Alternatively, certain transilluminators i.e. Biorad which can measure precise band sizes can be used as this will ultimately ensure accuracy of copy number assignments at such loci. Thus, as earlier mentioned, AE VNTR analysis is a reliable method of MLVA genotyping and is the preferred method in underfunded laboratories wich cannot afford the expensive instrumentation needed for CE VNTR analysis.

6.1.3.2. Performances of the AE VNTR

The use of a 16 VNTR loci in this study suggested a high genetic diversity among *M. bovis* and *M. tuberculosis* strains. A particular VNTR, MIRU10 was found to be monomorphic on all *M.bovis* strains in this study (Nigeria, Belgian and South African). This suggests that this locus is not a discriminatory marker for *M. bovis*. The stability of this locus should be further investigated as the occurrence of polymorphism may be indication of minor evolutionary or

mutational changes occurring within a strain type. Strains which would have otherwise been assumed to be of the same genotype if the ETR A-E loci had been used were discriminated further by the 16 VNTR genotyping.

Interspecies transmission of *M. bovis* was also observed in this study in which strains with identical genotypes were observed in isolates obtained from cattle, pig and goat. Zoonotic tuberculosis was also established in this study in which one strain with spoligopattern SB0944 was isolated from one human isolate and livestock in this study. The human strain clustered with other strains isolated from livestock. Furthermore, the identification of *M. tuberculosis* from different species of animals i.e. goat, pig and cattle was an interesting feature in this study, the goat *M. tuberculosis* isolate with spoligotype EAI5, however proved to be a foreign *M. tuberculosis* strain and hence formed an outgroup. The results obtained from the genotyping of South African *M. bovis* isolates on the other hand also highlighted a novel *M. bovis* strain and a previous strain which had been earlier documented in cattle from communal farms around the HiP.

These results strongly emphasize the need for a continuous application of molecular tools to aid in epidemiological surveys in humans, livestock and wildlife as the interspecies interaction and disease transmission dynamics can be properly explained.

6.2. Future research

6.2.1. MLVA and SNPs
The activity of SNPs in certain stable VNTR loci such as ETRD have been studied and discovered to be discriminatory markers for species identification of the members of the MTBC. The monomorphism of MIRU10 in *M. bovis* strains is an indication of its stability. It will be worthwhile to study the SNPs activity in the MIRU10 gene sequences of several *M. bovis* strains, as this might prove to be a useful marker and serve as a one step method in *M. bovis* strain differentiation.

6.2.2. Molecular epidemiology

The interactions between humans, livestock and wildlife are represented in this study with these interactions occurring at different levels. These results have hence provided a background which gives insight into future studies. It will be interesting to study the factors responsible for zoonotic transfer of mycobacterial agents in different settings in Nigeria by the use of molecular techniques such as Spoligotyping and MLVA. This will aid in providing a significant amount of information on the strains which are circulating between animal species as well as strains which are shared between humans and animals. Furthermore, a simplified MLVA assay comprising of a subset of (about 8-10) loci from the panel of 16 which was used in this study is proposed for a large scale epidemiological surveillance in Nigeria.

Unpasteurized milk when consumed can pose a serious risk to humans especially when it contains the tubercule bacilli. It will be interesting to attempt to isolate and characterize mycobacterial pathogens in unpasturised goat and cow milk, from "fura de nunu", a local delicacy made from raw milk and also from sputum samples of Fulani herdsmen or those who consume fura de nunu. The aim will be to establish the zoonotic impact of consumption of unpasteurised milk and to further highlight the circulating strains transferred between animals and humans and vice versa.

Species interaction and disease transmission sould be the focus in HiP. Molecular genotyping of mycobacterial isolates obtained from different species of animals which co-exist together in HiP must be done. This should be aimed at identifying the source animal species which ensures inter-species transmission of *M. bovis* strains.

APPENDIX: Table 1: Allele Naming Table for converting band sizes into repeat units. Shaded boxes represent the repeat unit of the reference strain H37 Rv

ETR	N° oligo										
ETRA-2165_75bp_397bp_3U	1824 / 1825	(1) 247	(2) 322	**(3) 397**	(4) 472	(5) 547	(6) 622	(7) 697	(8) 772	(9) 847	
ETRB-2461_57bp_292bp_3U	1850 / 1851	(1) 178	(2) 235	**(3) 292**	(4) 349	(5) 406	(6) 463	(9) 634			
ETRC-0577_58bp_346bp_4U	1772 / 1773	(2) 230	(3) 288	**(4) 346**	(5) 404	(6) 462	(7) 520	(10) 684			
ETRD-0580_77bp_330bp_3U	1828 / 1829	(1) 176	(2) 253	**(3) 330**	**(3,5) 353**	(4) 407	(5) 484	(6) 561	(7) 638	(8) 715	
ETRE-3192_53bp_651bp_3U	1844 / 1845	(1) 545	(2) 598	**(3) 651**	(4) 704	(5) 757	(6) 810	(7) 863			
MIRU											
MIRU02-0154_53bp_508bp_2U	1826 / 1827	(1) 455	**(2) 508**	(3) 561	(4) 614						
MIRU10-0959_53bp_643bp_3U	1830 / 1831	(1) 535	(2) 590	**(3) 643**	(4) 696	(5) 749	(6) 802	(7) 855	(8) 908	(10) 1013	
MIRU16-1644_53bp_671bp_2U	1832 / 1833	(1) 618	**(2) 671**	(3) 724	(4) 777	(5) 829	(6) 882	(11) 1147			
MIRU20-2050_77bp_591bp_2U	1834 / 1835	(1) 514	**(2) 591**	(3) 668							
MIRU23-2531_53bp_873bp_6U	1836 / 1837	(1) 607	(2) 661	(3) 714	(4) 767	(5) 820	**(6) 873**	(7) 926	(8) 979		
MIRU26-2996_51bp_613bp_3U	1840 / 1841	(1) 511	(2)562	**(3) 613**	(4) 664	(5) 715	(6) 766	(7) 817	(8) 868	(9) 919	
MIRU27-3006_53bp_657bp_3U	1842 / 1843	(1) 551	(2) 604	**(3) 657**	(4) 709						
MIRU39-4348_53bp_646bp_2U	1846 / 1847	(1) 593	**(2) 646**	(3) 699	(4) 752	(5) 805					
MIRU40-0802_54bp_407bp_1U	1848 / 1849	**(1) 407**	(2) 461	(3) 515	(4) 569	(5) 623	(6) 677	(7) 731	(8) 785	(9) 839	
Mtub											
Mtub01-0024_18bp_328bp_10U	1762 / 1763	(7) 274	(8) 292	(9) 310	**(10) 328**						
Mtub02-0079_9bp_230bp_6U	1764 / 1765	(3) 203	(4) 212	(5) 221	**(6) 230**	(7) 239	(8) 248	(9) 257	(10) 266	(11) 275	
Mtub02-0079_B_9bp_300bp_28U	1764 / 2838	(6) 282	(8) 300	(9) 309							
Mtub12-1121_15bp_215bp_4U	1790 / 1791	(3) 200	**(4) 215**	(5) 230	(6) 245						
Mtub21-1955_57bp_206bp_2U	1810 / 1811	(1) 149	**(2) 206**	(3) 263	(4) 320	(5) 377	(6) 434	(7) 491	(8) 548	(9) 605	(10) 662
Mtub29-2347_57bp_350bp_4U	1854 / 1855	(1) 179	(2) 236	(3) 293	**(4) 350**	(5) 407					
Mtub30-2401_58bp_319bp_2U	1856 / 1857	(1) 261	**(2) 319**	(3) 377	(4) 435	(5) 493					
Mtub38_37bp_310bp_3U	2041 / 2042	(1) 236	(2) 273	(3) 310	**(4,5) 373**	(5,5) 400					
Mtub39-3690_58bp_515bp_6U	2043 / 2044	(1) 225	(2) 283	(3) 341	(4) 399	(5) 457	**(6) 515**	(7) 573	(8) 631	(9) 689	(10) 747
Mtub49-4156_59bp_223bp_3U	2051 / 2052	(1) 105	(2) 164	**(3) 223**	(4) 282	(5) 341	(6) 400				
Qub											
Qub11a-2163_69bp_305bp_3U	2059 / 2060	**(3) 305**	(4) 374	(5) 443	(6) 512	(7) 581	(8) 650	(9) 719	(10) 788	(11) 857	(12) 926
		(14) 1064	(24) 1754	(25) 1822	(26) 1892	(27) 1961	(28) 2030				

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PRESENTATIONS CONNECTED WITH THIS DISSERTATION

A.O. Jenkins, L. Streicher, S.I.B. Cadmus, E.H. Venter, J. Godfroid. Molecular identification of *Mycobacterium tuberculosis* complex in livestock and humans in Nigeria (Oral presentation Faculty day, Faculty of Veterinary Science, University of Pretoria, September 2008)

A.O. Jenkins, L. Streicher, T. Marcotty, D. Cooper, E.H Venter, J. Godfroid. Molecular analysis of *Mycobacterium bovis* isolated from African buffaloes (*Syncerus caffer*) in the Hluhluwe-iMfolozi Park in Kwazulu Natal, South Africa (Award winning poster presentation, Faculty day 2008, Faculty of Veterinary Science, University of Pretoria, September 4, 2008)