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Mapping of IS6110 insertion sites in *Mycobacterium bovis* isolates in relation to adaptation from the animal to human host

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Abstract

The physiological role and impact of IS6110 insertions on the biology of *Mycobacterium tuberculosis* complex is not well understood. Insertion of IS6110 in coding regions can cause loss of gene activity, while homologous recombination between two copies of IS6110 can result in the deletion of genes or in rearrangement of genomic regions involved. In addition to these genomic changes, IS6110 can also activate flanking genes through acting as a mobile promoter.

In order to determine the possible role of IS6110 transposition in the adaptation to humans, we selected *Mycobacterium bovis* isolates from endogenous reactivation cases in elderly people in The Netherlands. The human isolates contained higher number of IS6110 copies in comparison to the bovine *M. bovis* strains. These additional integration sites of IS6110 were sequenced and analyzed. From 12 of such IS6110 insertion sites, 6 loci were located in the intergenic regions, and 6 other occurred within coding regions. IS6110 was inserted in a position where it might serve as a promoter in two cases. We conclude that IS6110 transpositions in *M. bovis* may be a driving force in the adaptation from the animal to the human host.

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Keywords: *Mycobacterium bovis*; Transposition; IS6110

1. Introduction

In general, the genome of *Mycobacterium tuberculosis* complex bacteria is highly conserved (Fleisch-

mann et al., 2002), however, insertion sequence IS6110 is associated with a high level of DNA polymorphism (Brosch et al., 1999). Therefore, IS6110 RFLP typing has gained recognition as a molecular epidemiological tool to examine transmission of tuberculosis (van Embden et al., 1993).

Insertion of IS6110 in coding regions of the genome of *M. tuberculosis* complex bacteria can result

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in the loss of genes or of gene activity (Brosch et al., 1999). In addition to these genomic changes, IS6110 can also activate flanking genes through acting as a mobile promoter, similar to the described for several insertion elements in other bacteria (Mahillon and Chandler, 1998).

It is clear that some of the IS6110 transpositions have influence on the biology of the bacterium. Increased expression of the two-component system PhoP/R implicated in mycobacterial virulence (Perez et al., 2001) was found in a multi-drug resistant (MDR) *Mycobacterium bovis* strain causing the largest outbreak of MDR tuberculosis described in Europe (Soto et al., 2004). So far, it remains unclear whether transposition of IS6110 is a result of a stochastic process or whether it might be a part of evolutionary adaptation driven by a selective force.

It is therefore conceivable that the persistence and spread of the IS6110 element in the genome of *M. tuberculosis* complex bacteria may also be related to the evolutionary adaptation of the bacteria to the intracellular stress during the dormant state, or the reactivation process thereafter. The fact that low-intensity bands in IS6110 RFLP patterns, caused by transposition of IS6110 in a sub-population of a *M. tuberculosis* isolate, is correlated with higher patients age (de Boer et al., 1999) supports this hypothesis.

During the application (from 1993 to date) of routine IS6110 RFLP typing in The Netherlands it was noticed that *M. bovis* isolates from older Dutch patients, obviously suffering from endogenous reactivations from remote infections, had more copies of IS6110 present than in *M. bovis* usually isolated from cows in the Netherlands. Latter strains almost invariably contain one copy of IS6110 at a fixed position as in BCG (van Soolingen et al., 1993), while *M. bovis* isolates from older Dutch patients contained up to 5 copies of the insertion sequence. This observation suggests that transposition of IS6110 in the genome of *M. bovis* may be associated with the adaptation of these bacteria to other host.

In this study we determine the IS6110 insertional sites in relation to the possible association of IS6110 transposition with the adaptation of *M. bovis* to humans. Five *M. bovis* isolates from older Dutch patients, harbouring three to five copies of IS6110 were selected to compare with two *M. bovis* strains isolated from cows and containing a single copy of IS6110.

2. Materials and methods

2.1. Bacterial strains

Seven *M. bovis* isolates were analyzed; five were isolated from older Dutch patients and two from cows in The Netherlands (Table 1). All seven strains shared nearly identical polymorphic GC-rich sequence (PGRS) profiles (van Soolingen et al., 1993). The five clinical isolates had different IS6110 RFLP pattern, containing three to five copies of IS6110, while the two *M. bovis* isolates from cows had one copy of the insertion sequence. For comparison, *M. bovis* BCG Pasteur strain was also used.

2.2. Isolation of genomic DNA

Genomic DNA was isolated according to a previously described method (van Soolingen et al., 1994). Mycobacterial strains were grown in Middelbrook 7H9 broth supplemented with albumin–dextrose–catalase (Difco Laboratories, Detroit, MI) and 0.05% Tween 80. All strains were grown at 37 °C.

2.3. IS6110- and PGRS RFLP typing

2.3.1. Briefly

The seven *M. bovis* selected for this study were typed by standard RFLP typing (van Embden et al., 1993). Extracted DNA was digested by PvuII (Boehringer-Mannheim), separated in 0.8% agarose gels in Tris-borate-EDTA buffer, and vacuum blotted onto nylon membranes. Blotted DNA was hybridized with a 0.8 kb fragment corresponding to the PCR amplified fragment between the PvuII restriction site and the 3' end of IS6110. The probe was labeled and RFLP patterns were visualized by use of an ECL direct

Table 1
M. bovis strains used in the study

Bacterial strain	Species	Origin
NL1	<i>M. bovis</i>	Cow
NL2	<i>M. bovis</i>	Cow
NL3	<i>M. bovis</i>	Human
NL5	<i>M. bovis</i>	Human
NL6	<i>M. bovis</i>	Human
NL7	<i>M. bovis</i>	Human
NL8	<i>M. bovis</i>	Human

nucleic acid labeling and detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England).

The PGRS typing was performed according to earlier description (Kremer et al., 1999).

2.3.2. Spoligotyping

The oligonucleotides Dra (5' biotinylated) and Drb, were used as primers for PCR amplification (Kamerbeek et al., 1997). Approximately 10 ng of purified mycobacterial DNA was used as the target. The DNA was added in 50 µl of a reaction mixture containing *Taq* buffer, 200 mM (each) deoxynucleoside triphosphate, 20 pmol (each) of primers Dra and Drb, and 0.5 U of *Taq* polymerase (PerkinElmer). Thermal cycling was performed. Twenty microliters of the reaction mixture was hybridized to 43 different spacer oligonucleotides, which were covalently linked to a filter (Kamerbeek et al., 1997).

2.4. LMPCR

Ligation-mediated PCR was used as described by Prod'hom et al. (1998) to amplify one or both ends of each copy of *IS6110* and its flanking sequence. Briefly, genomic DNA was digested with *SalI* (Boehringer-Mannheim) and ligated to a linker containing a *SalI* restriction site. The resulting template was thereafter digested by *SalI*. PCR was performed using ISA1 and ISA3 (Table 2), specific primers for *IS6110* and directed outwards from this element (Mendiola et al., 1992), and the linker primer Salgd. The template was initially denatured by incubation at 95 °C for 9 min and amplified by 35 cycles of PCR (95 °C for 30 s, 70 °C, and 72 °C for 90 s) followed by a final extension at 72 °C for 10 min in a Gene Amp PCR System 9700 thermocycler (PE Applied Biosystems, Foster City, CA). Amplified products were separated by standard horizontal gel electrophoresis in a 1.5% agarose gel in

Table 2
Oligonucleotides used in the study and their sequences

Oligonucleotide	Sequence	Reference
Salgd	TAGCTTATTCCTCAAGGCACGAGC	Prod'hom et al. (1998)
ISA1	CCTGACATGACCCCATCCTTTCC	Mendiola et al. (1992)
ISA3	GAGGCTGCCTACTACGCTCAACG	Mendiola et al. (1992)
ISA4	CGGTTGATGTGGTCGTAGTAGG	This work
1765c	CGGTTCCAACCTTGAGCGCGGTC	This work
1765-ISA1	GTTGGTGCATCATTCCGACC	This work
PPE28-ISA1	GGACAATGGCCGAGATGTC	This work
1800-PPE28	CACCATCGAATACGACGGC	This work
1883-ISA1	ACGATAATGCCAATTGTTCCACCG	This work
1883-ISA3	GTTGCCAATCGGGTTCTCGCC	This work
PPE34-ISA1	GTTGCCGAAGCCGGATGC	This work
34-ISA3	ACTGAGCGTCCAAGTGAATGG	This work
1947-1948c	GTGCTGGAGCAAGACCTCGC	This work
1947-ISA3	ACTGCGGATATGGAAGCTGGG	This work
2016	GTATGTTTTTCAGAGCCTTATCCG	This work
2016-ISA1	CCGATGCTCCAGAGTCTTCACC	This work
PE22-ISA1	ACTGGCCAGAGCGTTCCATCC	This work
2107	GCAGCTGCGACACTGGAGTCC	This work
2407-ISA1	ATCCATCGCATTGAGCAGGC	This work
2407	AGCTATGACCAACCAGCCC	This work
DR-ISA1	GGGACGGAAACCTTGAATAACG	This work
DR-ISA3	CCTGTATTTTCGCTGGTTTCCGTC	This work
3126-ISA1	GGTCGATGAGCTGAGCGC	This work
3126-ISA3	CCAACGCGGCCTCAAACG	This work
3189-ISA1	GCCGCGATCTTGCTCTTTATCC	This work
3189	GACGAAACAGATCTTCTGTCCG	This work
rmlB2-ISA1	AACACATGCAGCCACCATGAGAG	This work
rmlB2-ISA3	CTGCGCGGCGTTGAGGTG	This work

Tris-borate-EDTA buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and stained with ethidium bromide. PCR products were purified using GFX PCR DNA gel band purification kit (Amersham Pharmacia Biotech).

2.5. Sequencing

The amplified products were sequenced by using the fluorescence-labeled dideoxy nucleotide technology on an Applied Biosystems Instruments automatic sequencer (model ABI 3700). The primers used for sequencing the LMPCR products were Salgd, ISA1 and ISA3 (Table 2).

Each PCR product was sequenced and analyzed for homology with the TubercuList (<http://genolist.pasteur.fr/TubercuList>) and BoviList (<http://genolist.pasteur.fr/BoviList>) database Blast analysis.

2.6. PCR test to identify insertion sites

Flanking primers (Table 2) were generated from sequence data of LMPCR products. When a match was found for a flanking region in the data bases,

additional primers were designed to verify the site of insertion on the right and the left sides. Each of these primers was paired with the outbound IS6110 specific primers ISA1 or ISA3.

The temperature profile for PCR was an initial denaturation at 94 °C for 2 min followed by 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, for 25 cycles, followed by 72 °C for 5 min.

Most PCR products were in the range from 300 to 600 bp included approximately 70–100 bp of IS6110, depending upon primer design. Each PCR product was sequenced and analyzed for homology with the TubercuList and BoviList Blast analysis.

3. Results

3.1. Molecular typing of *M. bovis* isolates

The five *M. bovis* strains from human sources contained three to five copies of IS6110 according to RFLP typing (Fig. 1a). Two strains isolated from cows harboured a single copy of IS6110 (Table 1) (Fig. 1a).

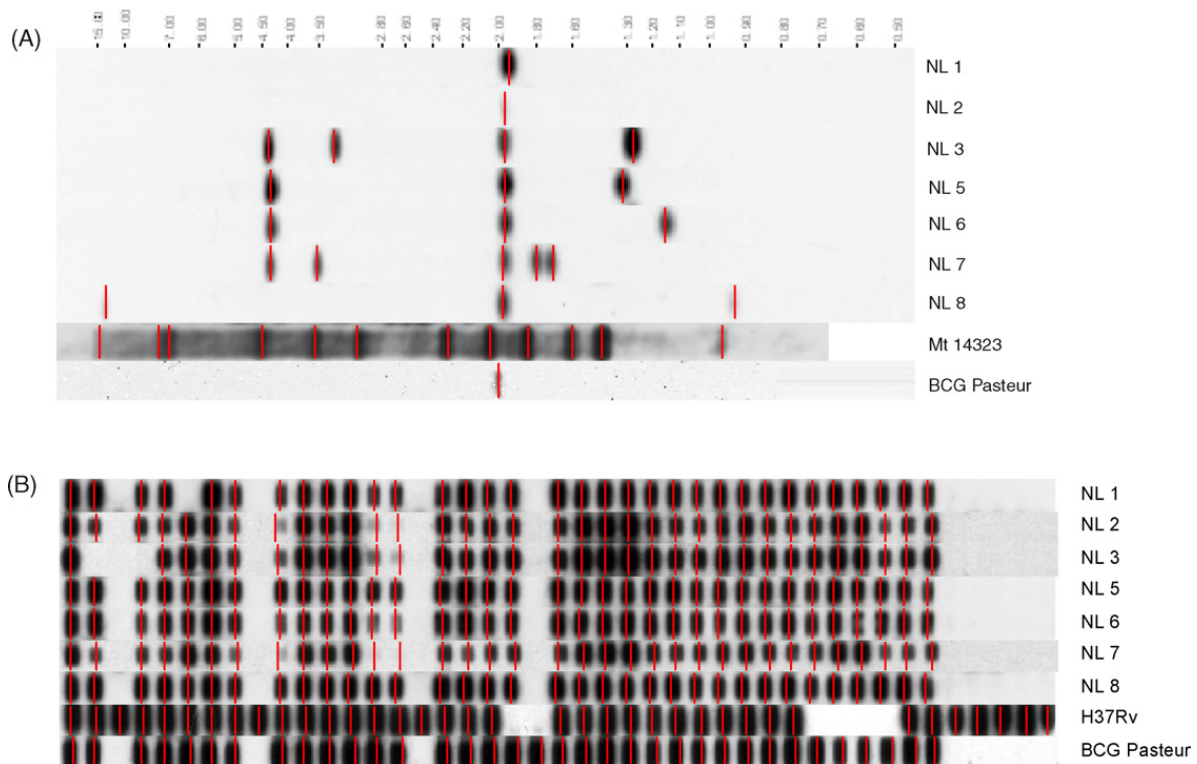


Fig. 1. IS6110 fingerprinting (a) and spoligotyping (b) of DNA from the seven *M. bovis* isolates of this study and the control Mt14323, H37Rv and BCG Pasteur.

In order to study genetic homogeneity of the isolates from human and animal sources, two other typing methods were applied; PGRS RFLP typing (Ross et al., 1992) and spoligotyping (Kamerbeek et al., 1997). Using PGRS typing all seven *M. bovis* isolates were almost identical (not shown) besides, using spoligotyping the seven strains were also highly similar (Fig. 1b).

3.2. Sequencing of IS6110 flanking regions

In order to study IS6110 flanking regions, LMPCR technique was carried out on DNA as described in Section 2 from the seven *M. bovis* isolates by amplifying one or both ends of each copy of IS6110 and its flanking sequence.

The number of amplified fragments in most of the cases correlated with the number of RFLP fragments hybridizing with the IS6110 probe on the PvuII restriction fragment blots (Table 3). The size of two different PvuII DNA restriction fragments hybridizing with an IS6110 probe can be similar and result in one band in the RFLP pattern. In two cases (NL3 and NL5) there were more amplified fragments than hybridizing bands. In one isolate four insertion sites were detected, while five bands were visualized by RFLP typing.

The PCR bands obtained ranged from 130 to 1800 bp in size. The individual fragments were purified and bi-directionally sequenced. Up to 21 flanking regions were identified, corresponding to 12 unique IS6110 insertional sites (Table 4).

We confirmed the IS6110 location in the genome and detected three bp Direct Repeat (DR) duplication at the insertion site in all cases, except in one site that exhibited a four bp DR (Table 4).

Table 3
Copy number of IS6110 in the *M. bovis* strains of the study

<i>M. bovis</i> strain	Copy number (RFLP)	Insertion sites (LMPCR)
NL1	1	1
NL2	1	1
NL3	4	5
NL5	3	4
NL6	3	3
NL7	5	4
NL8	3	3

3.3. IS6110 insertion sites shared in the *M. bovis* isolates

The seven strains studied presented an identical IS6110 insertion in the Direct Repeat Locus (DRL) which is known to be a hot spot site of the IS6110 insertion in *M. tuberculosis* complex strains (Hermans et al., 1991; Beggs et al., 1996; Fomukong et al., 1997). IS6110 was found at an indistinguishable position within the DRL compared to *M. bovis* BCG and H37Rv. Identical DR, CCC sequences were found outside the IS in the seven strains analyzed.

Four *M. bovis* isolates shared identical IS6110 insertions located in the intergenic region between Mb1794c and Mb1795c in the *M. bovis* genome (BoviList <http://genolist.pasteur.fr/BoviList/>) corresponding to Rv1765c-Rv1765A in the *M. tuberculosis* genome (TubercuList <http://genolist.pasteur.fr/TubercuList/>). Insertion at this location involved a three bp DR (GGT) at the site of insertion (Table 4). The sequence of this insertion site places IS6110 at 299 bp upstream from Mb1794c (Fig. 2). This gene codes for a hypothetical protein of unknown function. The coding region shows 121–848 bp substitution compared to its homolog in *M. tuberculosis* strain H37Rv (Rv1765c). The rest of insertion loci were different between the strains.

3.4. IS6110 insertion sites unique in the *M. bovis* isolates

In six of the isolates we were able to sequence all the integration sites of IS6110. From 12 unique insertion sites, 6 loci were located at intergenic regions and another 6 occurred within coding regions (Table 4).

The genes disrupted by IS6110 in this study corresponded to five ORFs with no predicted function. Some of them are member of the PE and PPE family. The gene *rmlB2*, involved in the bacterial metabolism and respiration was also disrupted (Table 4).

3.5. Analysis of IS6110 as possible promoter-carrier

Different studies showed that IS6110 can up-regulate the expression of downstream genes (Soto et al., 2004; Safi et al., 2004). To test that, we

Table 4
Insertion loci of IS6110 in the *M. bovis* strains of the study

Integration sites			<i>M. bovis</i> strain	DR	Orientation
Rv	Mb	Function			
1765c-1765A	1794c-179Sc	Unknown/IS	NL3–7	GGT	c
1800(PPE28)	1828(PPE28)	PPE family	NL7	GGG	c
1883c	1915c	Unknown	NL6	ATC	d
1917c(PPE34)	1951c(PPE34)	PPE family	NL8	TCG	d
1947-1948c	1982-1983c	Unknown/unknown	NL8	TGA	d
2016	2039	Unknown	NL3	CCCT	c
2107(PE22)	2131(PE22)	PE family	NL5	TAG	c
2407-PE24	2430-PE24	Unknown/PE	NL7	TTT	c
2813-2816c	2837-2840C	DR region	NL1–3, 5–8	CCC	c
PPE49-3126c	PPE49-3149C	PPE/unknown	NL5	GCA	c
3189-3190c	3211-3212C	Unknown/unknown	NL3	CTC	c
3468c(rmlB3)	3497c(rmlB2)	Glucose 4,6 dehydratase	NL3	GTC	c

The positions of insertion loci mapped to H37Rv and *M. bovis* AF2122/97 genomes. Orientation: d and c, direct and inverse orientations, respectively, with regards to the genome sequence.

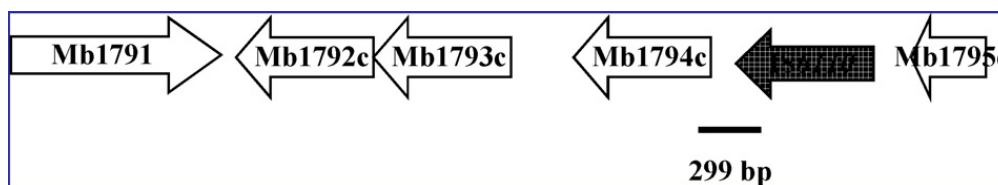


Fig. 2. Schematic representation of the Mb1794c-Mb1795c locus with IS6110 inserted 299 bp upstream from Mb1794c.

examined the location of IS6110 in the *M. bovis* strains when IS6110 was inserted in the same orientation as, and close enough to, a downstream gene thus IS6110 could potentially function as a promoter. Only the IS6110 element inserted 299 bp upstream of the Mb1794c gene, strains NL3–7 (Table 4 and Fig. 2) and that inserted 145 bp upstream of PPE49 gene (strain NL5, Table 4) agreed to those premises and are in a similar range as that described by Safi et al. (2004) concerning the up-regulation of genes in *M. tuberculosis*.

4. Discussion

In the *M. bovis* strains of the present study, 3 or 4 nucleotides DR were found in the flanking regions of all IS6110 copies studied. No consensus target sequence was detected (Table 4). The presence of these DR indicates that in all the cases the presence of IS6110 was due to transposition events and not rearrangements between IS6110 elements, described

for *M. tuberculosis* strains (Ho et al., 2000; Kato-Maeda et al., 2001).

The seven *M. bovis* strains in our study all presented an identical IS6110 insertion in the DRL known to be a hot spot integration site for IS6110 insertion (Mendiola et al., 1992; Hermans et al., 1991; Beggs et al., 1996; Fomukong et al., 1997). The insertion sequence was found at an identical position within the DRL in *M. bovis* AF2122/97, *M. bovis* BCG, *M. tuberculosis* H37Rv and CDC1551. Insertion of IS6110 into this specific site results in a three bp DR sequence (CCC) flanking IS6110, which is known to be a regular feature of transposition by IS3 elements (Mahillon and Chandler, 1998).

In the *M. bovis* strains from this study, IS6110 transposition into coding regions occurred on the 50% of 12 unique insertion sites. Our results agreed with other studies of *M. tuberculosis* isolates that found that 58% of discrete IS6110 insertion sites occurred within coding regions (Sampson et al., 2001).

In four of the five *M. bovis* strains isolated from humans (NL3–7) an IS6110 insertion was located at

identical position in the intergenic region between Mb1794c and Mb1795c (Rv1765c-Rv1765A) generating a DR sequence GGT. Insertions of IS6110 have been described in isolates of *M. tuberculosis* close to this position as a preferential site for IS6110 (Fomukong et al., 1997; Sampson et al., 2001). H37Rv has one copy of IS6110 upstream of the gene Rv1762c (Mb1793c). The integration of IS6110 at the same position in the four of the five *M. bovis* strains suggests, not only that these isolates could have shared a common ancestor, but also that the infection of this bacterium in a human host could be advantageous when IS6110 was inserted in that particular genomic region.

Previous studies have suggested that the variable expression levels of some PE genes in response to changing environmental conditions could have a role in the pathogenesis of tuberculosis (Dheenadhayalan et al., 2006). Near 50% of the unique insertion sites detected (5 of 12) corresponded to regions inside or upstream genes of members of the PE/PPE family (Table 4). This result supports the hypothesis that multiple copies of a gene (such as those members of PE/PPE family) could compensate any adaptative disadvantage of the bacteria due to the natural knockouts created by IS6110 insertion, mainly when the bacteria enter in a new environment.

Viana-Niero et al. have recently demonstrated that the *plcD* gene of *M. bovis* was interrupted by the insertion of one copy of IS6110 in 7.3% of isolates analyzed. All those isolates had four or more IS6110 copies (Viana-Niero et al., 2006). Contrary to that finding and despite our isolates also having multiple IS6110 copies, we have detected an intact *plcD* gene by PCR in our *M. bovis* isolates.

IS6110 can activate flanking genes by serving as outward directed, mobile promoter sequences. This activity has been recently demonstrated for up-regulation of the two-component system *phoP/R* (Soto et al., 2004). In addition it has been described that IS6110 promoter activity is increased under certain stress conditions like intracellular growth inside human monocytes and in late growth phases during *in vitro* growth in broth (Safi et al., 2004).

From the six IS6110 insertions within intergenic regions in our study only two were compatible with having possible influence on the expression of the neighbouring genes. The IS6110 copy inserted 299 bp

upstream of Mb1794c gene in *M. bovis* strains NL3–7 and the IS6110 inserted 145 bp upstream of PPE49 gene in NL5 strain are in a similar range as Safi et al. described for genes up-regulated in *M. tuberculosis*. Further analyses are required to investigate this phenomenon.

By studying *M. tuberculosis* strains with low and high-copy number of IS6110, Fomukong et al. (1997) found that some insertion sites were prevalent in the low-copy number strains and suggested a separate evolutionary lineage for the low and the high-copy number strains of *M. tuberculosis*. In contrast, the *M. bovis* isolates tested in this study do not have any copy of IS6110 inserted at the same genomic position described for low-copy *M. tuberculosis* strains. An interpretation of these results could be that the low-copy number *M. tuberculosis* strains and *M. bovis* strains originate from ancestors that evolved separately after the progenitor strain acquired IS6110 at the DRL.

We have not detected any insertion of IS6110 in genomic regions neighbouring cell cycle or dormancy genes in the *M. bovis* strains studied. *M. tuberculosis* complex strains containing a high-copy number of IS6110 are possibly better adapted to be transmitted, as is the case of Beijing genotype (van Soolingen et al., 1995), conferring the transposition of IS6110 an evolutionary advantage in comparison with strains with a low-copy number of IS6110, as is the case of *M. bovis*, less adapted to be transmitted between humans (Grange, 2001).

Genomic plasticity of members of the *M. tuberculosis* complex appears to be mediated by insertional changes of IS6110 at some stage (McEvoy et al., 2007). That plasticity is thought to drive bacterial evolution. It remains highly important to understand more about the direction *M. tuberculosis* complex bacteria are evolving. Taking into consideration the extremely short period in the evolutionary development of mycobacteria that can be subjected to studies, this is an enormous challenge.

Knowledge on the distribution of IS6110 insertion sites in various strains sheds more light on genetic and evolutionary processes involving members of the *M. tuberculosis* complex. Although several studies about IS6110 insertion sites in *M. tuberculosis* have been described thus far, this is the first study on insertion sites of IS6110 in *M. bovis* strains to our knowledge.

Almost all *M. bovis* strains, found in cattle still have just one copy of IS6110 at a fixed position. However, when *M. bovis* bacteria are incubated for longer period in human there is change as shown by IS6110 transposition detected in this work. We hypothesize that in *M. bovis* an evolutionary adaptation takes place during an incubation of decades in a human body and that transposition of IS6110 is involved in that adaptation. But more research on insertion sites of IS6110 in *M. bovis* is needed to confirm our hypothesis.

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