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Original Research Article

MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX STRAINS IN KINSHASA, DR CONGO

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Abstract: Tuberculosis (TB) is an infectious and communicable granulomatous disease caused by the acid fast bacilli bacteria of the genus *Mycobacterium*. The disease is mainly spread through inhalation of infected droplets expelled from infected lungs. Transmission, pathogenicity, geographical spread and host ranges differ among the *Mycobacterium tuberculosis* complex (MTBC) subspecies. We typed *Mycobacterium* spp. from pulmonary secretion samples collected from suspected TB patients, selected in different health centres in Kinshasa, the capital city of DR Congo, where TB is highly endemic. Analysis was conducted using Ziehl-Neelsen, Genotype® MTBDR *plus* and PCR techniques. The results showed that out of 155 samples collected from suspected TB patients, 95 (61.2%) were positive using the bacteriological Ziehl-Neelsen test. Among these 95 positive samples, the Genotype® MTBDR *plus* test (Hain® test) characterized 93 samples (97.9%) as belonging to the MTBC. The remaining two samples (2.1%) that could not be amplified were classified as *Mycobacteria* other than tuberculosis (MOTT). These 93 Genotype® MTBDR *plus* positive samples were further characterized using the PCR technique based on genomic Regions of Difference whereby 74 samples (79.5%) were classified as *Mycobacterium tuberculosis*, 16 (17.2%) as *M. africanum*, 1 (1%) as *M. bovis* and the remaining 2 samples could not be classified. This study reveals the co-circulation of various MTB species in the human population of Kinshasa where most TB patients are infected with *M. tuberculosis* or *M. africanum*. However the presence of the zoonotic *M. bovis* in the human population highlights the possible existence of the animal-human transmission.

Key words: *Mycobacterium tuberculosis* complex strains, Regions of Difference, Ziehl-Neelsen, Genotype® MTBDR *plus*, Kinshasa

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Introduction: The global distribution of Tuberculosis (TB) cases is skewed heavily toward low-income and emerging economies [1]. The highest number of cases is in Asia, where China, India, Bangladesh, Indonesia, and Pakistan collectively make up over 50% of the global burden [2]. Africa, and more specifically

sub-Saharan Africa, has the highest incidence rate of TB cases, with approximately 83 and 290 per 100,000, respectively [1]. Approximately 6 million of the 8 million of TB cases occur predominantly in the economically most productive 15 to 49 year-old age group [3]. Currently our understanding of TB epidemiology and the efficacy of control activities are complicated by the emergence of drug-resistant bacilli and by the co-infection of TB with the human immunodeficiency virus (HIV/AIDS) [1-2]. In 2000, an estimated 8 to 9 million incident cases and approximately 3 million deaths due to TB occurred worldwide [2]. After HIV/AIDS, TB is the second most common cause of death related to infectious diseases, and current trends suggest that TB will still be among the 10 leading causes of global disease burden in the year 2020 [4-5].

Tuberculosis is caused by several agents belonging to the *Mycobacterium tuberculosis* complex (MTBC), which includes *Mycobacterium tuberculosis* (*M. tuberculosis*) and *M. africanum* as the main causative agents of TB in humans; the animal TB agent *M. bovis*; and other species such as *M. canettii* [6-7]. Although not clinically important the characterization of these subspecies is crucial for epidemiological purposes. While some of these agents can circulate between humans others co-circulate between humans and animals. In such case disease interruption can only be achieved if the agent is fully characterised leading to the sound determination of transmission route including its natural reservoir.

For a very long time, the diagnosis of TB in DR Congo was achieved using the Ziehl-Neelsen technique, which cannot distinguish the mycobacteria subspecies in infected patients. The molecular genetics assay *Genotype*® MTDR *plus* test (Hain® test), was developed to discriminate between MTBC and "Mycobacterium Other Than Tuberculosis complex" (MOTT), as well as to determine the resistance profile of the isolates belonging to MTBC. However, it cannot distinguish subspecies belonging to the MTBC [8-9-10].

Previous studies from comparative genome sequencing of MTBC subspecies (*M. tuberculosis*, *M. africanum*, *M. canettii*, *M. microti*, *M. bovis*, and *M. bovisbacillus Calmette-Guérin* (BCG)) strains revealed that they have a highly conserved genome (98% similarities) [8-9].Unfortunately, the high degree of sequence conservation among the MTBC subspecies has resulted in difficulties for the diagnosis using molecular techniques since commercial DNA probe and amplification assays based on 16S rDNA sequences, identical for all MTBC subspecies, cannot be used to differentiate members of the complex. Despite the difficulties described above, advances in molecular methods and the accumulating knowledge of the MTBC genome have resulted in methods designed to rapidly identify the MTBC subspecies [10]. One of the methods uses the genomic Regions of Difference (RD) techniques which are based on the loss of genetic material in certain specific regions of MTBC subspecies [9-10-11]. In previous studies RD techniques have successfully been used to differentiate MTBC subspecies from various areas [9-10].

Based on previous findings using the RD techniques it was shown that *M. tuberculosis* is the most widespread etiological agent of human tuberculosis [11-12]. However the existence or reemergence of other species in human tuberculosis including the BCG vaccine strain causing *M. bovis* BCG tuberculosis has also been acknowledged [10-13]. Such diversity, when it occurs in a specific area, can complicate the transmission patterns of the disease and have negative impact on the application of effective integrated control measures.

The DR Congo is classified among the most TB affected countries in Africa [1].Althoughthe disease is widely spread throughout the country, in humans as well as in animals, information on the diversity of MTBC subspecies circulating in various areas is scanty. Therefore, the objective

of this study was to investigate the diversity of MTBC isolates circulating in TB patients in Kinshasa, the capital of DR Congo.

Materials and Methods

Study area and sample collection: This study was carried out at the "Institut National de Recherche Biomedicale" (INRB) in Kinshasa/DR Congo. Kinshasa is a large and densely populated town (area: 9,965 km^2, population: 9,463,749) where TB remains an important public health concern with an increasing number of new cases (20,000 in 2007). During 2011 and 2012, 155 pulmonary secretions of suspected TB patients living in Kinshasa were collected from selected heath centers through the TB network of the national program against tuberculosis and sent to the INRB for identification as shown in Figure 1.

Ziehl-Neelsen stain: Immediately after collection, samples were decontamined using NaOH according to the manufacturer's instructions (Genotype® MTBDR *plus*, 2011). Samples were then screened using the Ziehl–Neelsen stain which is a special bacteriological stain for acid-fastorganisms' identification, mainly Mycobacteria. These organisms contain large amounts of lipid substances within their cell walls called mycolic acid that resist staining by ordinary methods such as a gram stain[14]. Ziehl–Neelsencarbolfuchsin, acid alcohol, and methylene blue were used as reagents. Using this technique acid-fast bacilli turn bright red after staining.

DNA extraction: All positive Ziehl–Neelsen stained samples were subjected to DNA extraction that was carried out according to the manufacturer's instructions (Genotype® MTBDR *plus*, 2011). Briefly, a volume of 500 µl of decontamined patient samples was subjected to centrifugation for 15 min at 10,000xg. The supernatant was discarded and the pellet was re-suspended in 100 µl Lysis buffer by vortexing. The mixture was incubated at 95ºC for 5 minutes then centrifuged at 13000xg for 5 minutes and re-suspended in 100 µl of Neutralization buffer by vortexing for 5 min. After spinning down for 5 minutes at full speed in a table top centrifuge, 2.5 µl of the supernatant was directly used as template for Geno Type® MTBDR *plus* (Hain® test) and PCR.

GenoType® MTBDRplus (Hain® test): All DNA samples were subjected to the GenoType® MTBDR *plus* test following manufacturer's instructions. The Geno Type® MTBDR *plus* VER 2.0 is a qualitative in vitro test with high sensitivity and specificity for identification of the MTBC. Following DNA isolation, nucleic acids are selectively replicated in an amplification reaction. In the next step, amplicons are chemically denatured, since detection on the DNA-STRIP is done using single-stranded DNA. The DNA- STRIP is coated with highly specific probes which are complementary to selectively amplified nucleic acid sequences. The single-stranded amplicon binds specifically to the complementary probes during hybridization, while non-specifically bound amplicons are removed in subsequent washing steps. During the conjugate reaction, the specifically bound amplicon is marked with the enzyme alkaline phosphatase and is then made visible in a colorimetric detection reaction. In this way, a specific banding pattern develops on the DNA-STRIP. Using a test-specific evaluation template, the test result can be read out quickly and clearly. This test allows discrimination of MTBC from MOTT samples (Genotype® MTBDR *plus*, 2011).

PCR amplification using RD primers: All positive samples from the Genotype® MTBDR *plus* test were subjected to PCR amplification, in order to discriminate MTBC members. Three genomic regions (RD 1, RD 5, and RD 9) were targeted for amplification. These genomic regions were previously reported as being all deleted in the genomes of BCG isolates relative to the sequence of *M. tuberculosis* H37Rv [10-11-15-16]. As shown in Table 1, three primers were used to detect RD1 and RD9 while only two primers were needed for RD 5 analysis.

The first analysis involved RD1 and RD9 in separate tubes. For these assays, three primers were used, two primers complementary to sequences flanking the deleted region and a third primer complementary to internal sequences. The primer sequences are listed in Table 1 as described by Gaudrat *et al*. [10]. Using these techniques, PCR products are of different sizes depending on the presence or absence of the regions. A band of 150bp for RD1 and 306bp for RD9 are yielded when the region is present while bands of 200bp and 206bp are produced for RD1 and RD9 respectively when the region is absent [10].

PCR amplification was carried out using a 25 µl reaction mixture that contained 25 pmol of each primer, 100 mMKCl, 20 mMTris-HCl (pH 8.6), 3.3 mM $MgCl_2$, 0.2% Triton X-100, 200 µM of each dNTP, 0.2 U of Hot Star Taq DNA polymerase (Perkin Elmer), and 2.5 µl of template. The mixture was denatured for 15 min at 95ºC, and cycled 40 times for 1 min at 94ºC of denaturation, 1 min at 65ºC of annealing and 30 sec at 72ºC for extension, followed by a final extension for 10 min at 72ºC. A volume of 12 µl of each sample was analyzed on a 2% agarose gel. The presence and size of each PCR product were determined by comparison to a 100 bp plus DNA ladder (Gibco BRL, Life Technologies, Gaithersburg, Md.).

In a second analysis only samples where the region was present for RD1 and not present for RD9 were subjected to the RD5 analysis. In this assay only two primers were used to amplify a specific region of 152bp, when the RD5 was present. In samples were the RD5 region was not present no amplification was expected. The reaction mixture, PCR conditions and gel analysis were used as above, except for the annealing temperature that was set to 64ºC [10]. Results were interpreted based on Table 2 [10].

Results: Analysis of the 155 samples collected from suspected TB patients revealed that only 95 (61.2%) were positive using the bacteriological Ziehl-Neelsentest. Among these 95 positive samples, GenoType® MTBDR *plus* (Hain® test) test identified 93 samples (97, 9%) as members of the MTBC and 2 samples (2.1%) were classified as MOTT since they were only positive in the Ziehl-Neelsen stain, but they remained negative to the GenoType® MTBDR *plus* test. When PCR based on the RD1 technique was used the region was present in 91 samples (97.9%). In two samples (2.1%) no band could be produced. When the 91 RD1+ samples were analyzed using the RD9 technique, the region was present (RD1+RD9+) in 74 (79.5%) and absent (RD1+RD9-) in 17 (18%) of them. Samples with RD1+RD9+ were classified as *M. tuberculosis*. The RD1+RD9- samples were later subjected to the RD5 technique that classified 16 (17.2%) samples in which the band was present as *M. africanum* while the remaining sample (1%) that didn't produce any band was classified as *M. bovis*. PCR profiles of the RD1, RD9 and RD5 techniques are shown in Figure 1.

Discussion: Differentiation of MTBC subspecies based on phenotypic methods is time consuming, making surveillance of species-specific disease difficult, and does not give an unambiguous result in every case. In most endemic settings in sub-Saharan Africa, tuberculosis is mostly diagnosed using the Ziehl-Neelsen stain technique [1-18] and positive cases are admitted for treatment. Results of this study showed that about 61% of suspected TB patient samples analyzed using Ziehl–Neelsen stains were indeed positive. This finding was later confirmed using the GenoType® MTBDR *plus* test since all but two (98%) of the Ziehl–Neelsen positive samples were correctly identified as MTBC subspecies showing that the Ziehl–Neelsen technique is reliable for diagnostic purposes. From the current finding 39% of the TB suspected patients were not infected with tuberculosis. This study was conducted in Kinshasa where most health centers strictly use microbiological techniques to diagnose tuberculosis. However in other areas where above techniques for TB diagnosis are not available 39% of TB

suspected patients can be misdiagnosed and in case of hospitalization they run the risk of contracting tuberculosis at the hospital.

Based on TB control strategies sound characterization of MTBC subspecies is not required for treatment implementation. However for a best understanding of the occurrence and spread of tuberculosis in the human population subspecies characterization of MTBC is highly required. Whether control measures should be restricted to humans or other hosts species such as cattle or other animals depends on the subspecies circulating in the human population. In case only *M. tuberculosis* and *M. africanum*are present in the area it can be assumed that the disease is circulating specifically among humans; in such situation control measures should target the interruption of human-to-human transmission. However in a situation where *M. bovis* is present in humans the "One Health" approach should be applied whereby control measures targeting the animal-to-human transmission are implemented[15-16-19].

Through our results the presence of RD1 and RD9 that characterize the *M. tuberculosis* subspecies was found in 79.5% of the isolates tested(Figure 2A and 2B). All these isolates produced the expected bands of 150bp for RD1 and 306bp for RD9 techniques asdescribed by previous authors [10]. Despite the presence of the RD1 region (Figure 2A) the remaining 17 samples produced a 206bp band for the RD9 technique (Figure 2B) suggesting that the RD9 region was absent. Based on Gaudrat*et al*. [10] interpretation, these samples could be classified either as *M. africanum*or *M. bovis*. To discriminate these strains the 17 samples were further subjected to the RD5 technique. As in previous studies, the absence of RD5 was revealed by the absence of amplification in 1 (1%) isolate that were further classified as *M. bovis*. The remaining 16 (17.2%) isolates were classified as *M. africanum*since they produced a band with expected size of 152bp (Figure 2C) as reported previously [10].

To the best of our knowledge this is the first time molecular techniques based on RD are used to characterize MTBC in Kinshasa. Our finding revealed that most isolates circulating in humans are *M. tuberculosis* or *M. africanum*. Since these strains are known to be restricted to humans their high prevalence indicates the human-to-human transmission as the principal source of infection [10-20-21]. For the control purposes this finding is very crucial in a way that prevention measures should focus on factors underlying the transmission of tuberculosis between humans rather than other transmission routes such as the animal-human transmission. In DR Congo care of patients who are hospitalized is generally under the supervision of family members who share the same space and goods during the hospitalization of their relatives. Such practices can indeed constitute major risk factors for the transmission of human tuberculosis. The presence of several people suffering from tuberculosis in the same family can also support this transmission route as principal source of tuberculosis infection in DR Congo. Sound understanding of the transmission of tuberculosis in this context can be realised through the analysis of the genetic relatedness of isolates collected from patients and their contacts that further developed the disease in the hospital or in the community [20-22].

Although the current study revealed human-to-human transmission as the main source of tuberculosis infection, the presence of *M. bovis* isolate reveals, at the same time, the possible existence of an animal-to-human transmission [19]. In our study *T. bovis* represented only 1% of cases in humans. This sample was characterized as such following the interpretation described by Gaudrat *et al.* [10] despite the fact that lack of amplification of the PCR product can also result from several factors including possible inhibitions (see following paragraph). Compared to the Eastern part of the country where a 40% prevalence of *M. bovis* was found in humans [19] the prevalence of this

TB species found in Kinshasa is very low. In the Eastern part of the DR Congo such prevalence of *M. bovis* in humans can be explained by the fact that people drink unpasteurized milk collected directly from the cow while in the Western part of the country and particularly in Kinshasa most people use pasteurized powdered milk. Although the origin of the *M. bovis* isolate found in the current study was not assessed epidemiologically, the introduction of such strain from the Eastern Congo cannot be excluded considering the movement of people inside the country. Meat and unpasteurized milk from the East and sold in Kinshasa can also represent a possible source of human contamination even though such risk is rather minimal since the market of cattle meat and milk from Eastern Congo is very restricted in Kinshasa[19]. It's not impossible that the origin of this transmission could come from cattle slaughtered at the abattoirs of Kinshasa. Indeed internal reports from the two abattoirs of cattle in Kinshasa mention the existence of TB in animals originated from Kwango, Kwilu, Maï-Ndombe and Kongo-Central provinces located in the Western part of the country (Unpublished data). Further studies using phylogenetic relatedness of *M. bovis* circulating in the Eastern and Western parts of the country are needed to clarify the origin of this *T. bovis* isolate.

Two of the isolates used in our study could not be amplified using the RD1 technique. Based on the previous reports [9-10-11-15] RD1 region is totally absent in *T. bovis* BCG isolates. However the absence of this region is indicated by the presence of a 200bp band when these isolates are amplified with the primers used in our study [11]. Lack of amplification of a PCR product can be related to several factors including a low concentration of DNA or the presence of inhibitors in the PCR mix. Nucleotide mutations at the primer binding site of the template can also lead to absence of PCR amplification. In the previous analysis using the GenoType® MTBDR *plus*test all expected bands were obtained in all the isolates including the two that did not amplify with the RD1 technique. The expected band was also produced with the same isolates when subjected to the 16S analysis (data not shown). Further analyses are needed to elucidate factors underlying the lack of amplification of the PCR product of these isolates by the RD1 technique.

Conclusion: In conclusion our study revealed occurrence of various members of MTBC in TB patients in Kinshasa which suggests a genetic diversity of human TB in the area. The study further highlights the existence of transmission risk of *M. bovis* to human possibly through consumption of infected cattle products. Results of the current study can be used to enhance primary care and public health services in DR Congo.

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Competing interest : The authors declare they have no competing interest

Author contributions: HKM participated in design, implementation of study protocol and writing of the manuscript, **JM** supervised the design, implementation of the study protocol, supervised the laboratory work and contributed to manuscript writing, **MA** and **LN** contributed in sample analysis and data analysis, **MK** contributed to the sample collection, data analysis and manuscript writing, **DM** participated to the proposal implementation and manuscript writing, **VAG** contributed to data analysis and manuscript writing, **GT** contributed in sample analysis and data analysis, **JJM** supervised the implementation of study protocol and the manuscript writing.

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Table 1: Primer sequences used in this study (Gaudrat*et al*, 2006)

Primer sequences
RD1ff : 5’-AAG-CGG-TTG-CCG-CCG-ACC-GAC-C-3’
RD1Int: 5’-CTG-GCT-ATA-TTC-CTG-GGC-CCG-G3’
RD1fr : 5’-GAG-GCG-ATC-TGG-CGG-TTT-GGG-G-3’
RD9ff: 5’-GTG-TAG-GTC-AGC-CCC-ATC-C-3’
RD9Int: 5’-CAA-TGT-TTG-TTG-CGC-TGC-3’
RD9fr: 5’-GCT-ACC-CTC-GAC-CAA-GTG-TT-3’
RD5intf: 5’-CTG-GTC-GAA-TTC-GGT-GGA-GT-3’
RD5intr:5’-ATG-GTC-TCC-GAC-ACG-TTC-GAC-3’

Table 2: Classification of MTBCmembers based on the results of primers combination

(Gaudrat*et al.*, 2006)

	M. tuberculosis	*M. africanum*	*M. bovis*	*M. bovis BCG*
RD1 assay[a]	+	+	+	-
RD9 assay[b]	+	-	-	-
RD5 assay[c]	ND	+	-	ND

+: Region present, - : Region absent, ND: not done

[a]Expected PCR fragment is 150bp for RD1+ and 200bp for RD1-

[b]Expected PCR fragment is 306bp for RD9+ and 206bp for RD9-

[c]Expected PCR fragment is 152bp for RD5+ and no amplification for RD5-

Figure 1: Flow showing all the process for identification of MTBC species

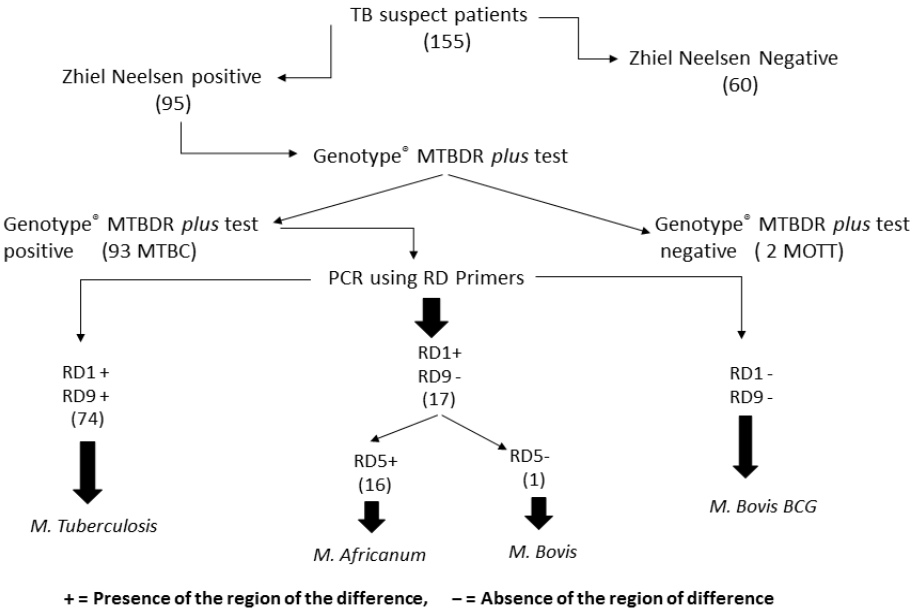

Figure 2: A 2% agarose gel electrophoresis showing DNA amplification of MTBC samples using RD1 (A), RD 9 (B) and RD5 (C) techniques

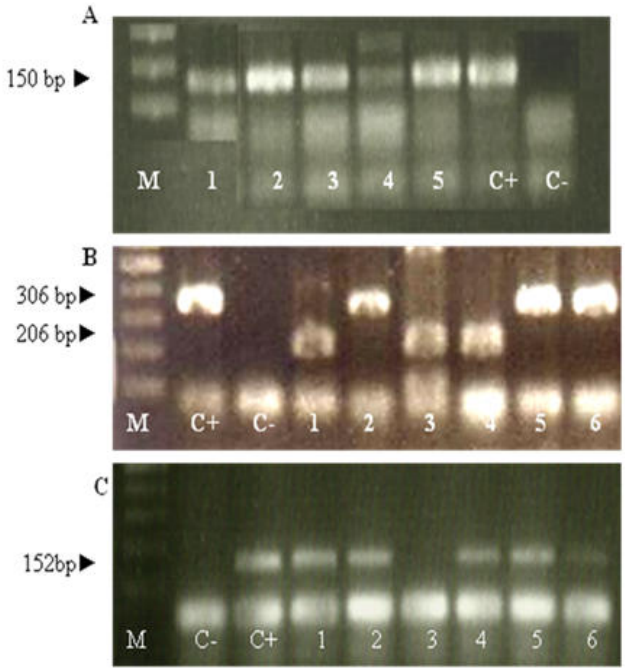

Figure A : A 2% agorose gel electrophoresis showing DNA amplification of MTBC using RD1 primers. Lane 1,2,3,4,5 represent positive isolate, Lane C+ represents positive control , Lane C- negative control and Lane M molecular weight marker 100bp plus DNA ladder

Figure B : A 2% agorose gel electrophoresis showing DNA amplification of MTBC using RD 9 primers. Lane 2,5,6 represent positive isolates primers, Lane 1,3,4 represent negative isolates, Lane C+ represents positive control , Lane C- negative control and Lane Mmolecular weight marker 100bp plus DNA ladder

Figure C : A 2% agorose gel electrophoresis showing DNA amplification of MTBC using RD 5 primers. Lane 1,2,4,5,6 represent positive isolates primers, Lane 3 represent negative isolates, Lane C+ represents positive control , Lane C- negative control and Lane M molecular weight marker 100bp plus DNA ladder