JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Sanoussi *et al.*, *Journal of Medical Microbiology* 2018;67:1718–1727

DOI 10.1099/jmm.0.000846

MICROBIOLOGY SOCIETY

OPEN MICROBIOLOGY

Low sensitivity of the MPT64 identification test to detect lineage 5 of the *Mycobacterium tuberculosis* complex

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Abstract

Purpose. Differentiation of the *Mycobacterium tuberculosis* complex (MTBc) from non-tuberculous mycobacteria (NTM) is important for tuberculosis diagnosis and is a prerequisite for reliable phenotypic drug-resistance testing. We evaluated the performance of the rapid MPT64 antigen identification test for the detection of *Mycobacterium africanum* lineage 5 (MAF L5).

Methodology. Smear-positive tuberculosis patients' sputa were included prospectively. Culture was performed on Löwenstein–Jensen medium and, when positive, the MPT64 test and the classical para-nitro benzoic acid susceptibility and heat-labile catalase (PNB/catalase) identification tests were performed. The MPT64 test was repeated 14 days after an initially negative first testing. Direct spoligotyping was performed for MTBc lineage determination.

Results. In total, 333 isolates were tested for all of the methods. Three hundred and twenty-two (96.7 %) were pure MTBc, by agreement between spoligotyping and PNB/catalase, and 11 were NTM or a mixture of MTBc/NTM. The MPT64 test conducted on day zero of culture-positivity correctly identified most of the pure MTBc isolates (93.2 %, 300/322), but it failed to detect 24 % of the L5 isolates (18/75) versus 2 % (4/202) of the L4 ones [OR=15.6 (5.3–45.8), P<0.0001], with improved sensitivity for L5 detection on repeat testing after 14 days. The L5-wide non-synonymous single-nucleotide polymorphism in the *mpt64* gene may explain the poor performance of the MPT64 test for L5.

Conclusion. The MPT64 test has a lower sensitivity for detecting L5 isolates of the MTBc, and can be considered as a first-screening test that should be confirmed by another identification method when it produces negative results in countries with L5. Given the microbiological bias in both the isolation and identification of MAF lineages, diagnostics with high sensitivity for direct testing on clinical material are preferable.

INTRODUCTION

Tuberculosis (TB) remains a public health problem, especially in low-resource countries, where 95 % of global tuberculosis is detected [1]. Microscopic detection of acid-fast bacilli (AFB) has been the main TB diagnostic tool for more than a century, yet it cannot distinguish MTBc (*Mycobacterium tuberculosis* complex) from non-tuberculous mycobacteria (NTM) [2]. The advent of the molecular Cepheid GeneXpert MTB/RIF test partially resolved this problem, as it can confirm the presence of MTBc but not NTM, and is also more sensitive than AFB microscopy for TB diagnosis [2]. Nevertheless, compared to culture, GeneXpert MTB/RIF still has a lower sensitivity for the diagnosis of

Received 25 May 2018; Accepted 7 September 2018

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Keywords: Rapid MPT64 identification test; *Mycobacterium tuberculosis* complex lineages; *Mycobacterium africanum* West African 1 (Lineage 5); non-tuberculous mycobacteria; *mpt64* gene (Rv1980c); non-synonymous SNP.

Abbreviations: AFB, acid-fast bacilli; CI, confidence interval; DST, drug susceptibility testing; D0, date when primary colonies were large enough that they could be scraped from the culture medium slant; D14, 14 days after D0; HCl, hydrochloric acid; IUATLD, International Union Against Tuberculosis and Lung diseases; L1, lineage 1; L2, lineage 2; L3, lineage 3; L4, lineage 4; L5, lineage 5; L6, lineage 6; LJ, Löwenstein–Jensen; LR−, negative likelihood ratio; LR+, positive likelihood ratio; MAF, *Mycobacterium africanum*; MGIT, mycobacterial growth indicator tube; MTBc, *Mycobacterium tuberculosis* complex; NaOH, sodium hydroxide; nSNP, non-synonymous single-nucleotide polymorphism; NTM, non-tuberculous mycobacteria; OR, odds ratio; PNB, para-nitro-benzoic acid; Sen, sensitivity; Spe, specificity; TB, tuberculosis.

Two supplementary tables are available with the online version of this article.

TB in smear-negative pulmonary and extra-pulmonary specimens [3, 4], as well as for TB in children [4]. Culture thus remains the most sensitive laboratory test for the diagnosis of TB. The isolation of strains in culture is also a prerequisite for conventional phenotypic drug susceptibility testing (DST) techniques. When a culture is positive, the rapid differentiation of MTBc from NTM is necessary for prompt TB treatment initiation to ensure a better outcome. Moreover, the differentiation of MTBc from NTM is crucial for a valid interpretation of resistance patterns in phenotypic DST assays, as NTM can be intrinsically resistant to anti-TB drugs and can be mistaken for multidrug-resistant MTBc.

Many techniques have been described for the differentiation of MTBc from NTM, such as growth in the presence of para-nitro benzoic acid (PNB), the heat-labile catalase test, cord-formation ability, hybridization with specific molecular probes and high-performance liquid chromatography [5, 6]. Due to their speed and simplicity, the most popular tests are the rapid and simple immuno-chromatographic methods, which yield results in 15 min [2]. The MPT64 antigen test was reported to be highly sensitive and specific for the identification of MTBc in a systematic review [7]. Few studies have included strains isolated from solid medium [7, 8] or measured the performance of the test for different MTBc lineages [9].

The human-adapted MTBc members comprise *M. tuberculosis sensu stricto* and *Mycobacterium africanum* (MAF). The latter is subdivided into MAF West African 1 and 2, also called lineages 5 (L5) and 6 (L6), respectively, and is mostly restricted to the West African region, where it causes up to 40 % of TB [10]. Recently, a study in The Gambia, where L6 is prevalent, found that the MPT64 antigen test has low sensitivity for the detection of L6 isolates grown in automated liquid mycobacterial growth indicator tubes (MGITs) [9], while the sensitivity of the test for the detection of L5 isolates remains unknown. Nevertheless, a recent study found a substitution (I43N) in the *mpt64* gene of L5 isolates in Ghana [11], while Gagneux *et al.* [12] suggested that L5 has a non-synonymous single-nucleotide polymorphism (nSNP; not specified) in the *mpt64* gene that could impact negatively on the performance of the MPT64 antigen test for L5 detection, affecting countries where this lineage is endemic.

We evaluated the performance of the MPT64 antigen rapid identification test for L5 detection on isolates from solid culture medium in Benin, where L5 causes up to one-third of human TB [13, 14], and compared the available L5 and L6 genomes for nSNPs in the *mpt64* gene.

METHODS

Patient selection and specimens

Presumptive TB patients were systematically screened with-AFB microscopy nationwide through the 24 largest TB clinics in Benin from April to December 2016. TB patients with smear-positive sputa were prospectively recruited. In each TB clinic, all retreatment TB patients and a sample of new TB patients (the next four diagnosed after a retreatment TB patient) were included. Their sputa were collected before the initiation of TB treatment and then shipped to the National Reference Laboratory for Mycobacteria (Laboratoire de Référence des Mycobactéries) in Cotonou, Benin, where further laboratory analyses were performed.

Specimen preparation: sputum decontamination and culture of mycobacteria

The sputa were decontaminated using the Petroff method [5] (2 % NaOH final for 15 min and neutralization using 1 N HCl containing phenol red), followed by centrifugation at 3000 ***g*** and 4 °C for 20 min. The pellet was resuspended in 1.5 ml of phosphate-buffered saline (PBS). Two standard Löwenstein–Jensen (LJ) slants and one LJ slant without glycerol but supplemented with 0.5 % sodium pyruvate were inoculated per patient's sputum and then incubated at 37 °C and read weekly for 13 weeks (90 days) before a negative culture was reported. An aliquot of the sediment was stored at −20 °C for direct spoligotype analysis.

MPT64 antigen rapid identification test

The SD Bioline TB Ag MPT 64 Rapid assay (Standard Diagnostics, Republic of Korea) was used for the identification of isolates from culture-positive specimens following the manufacturer's instructions [15]. The test was performed at day 0 (D0) and, if negative, was repeated 14 days later (D14). D0 was defined as the date when primary colonies were large enough that they could be scraped from the slant. The test was repeated at D14 on colonies remaining on the primary slant after it had been reincubated at 37 °C. A few colonies were suspended in 200 µl of the extraction buffer provided in the kit or in the condensation fluid (if any) of the slant [15]. Then, 100 µl of this suspension was deposited in the sample well of the MPT64 device [15]. After 15 min incubation, the MPT64 was reported as negative if a colour signal line only appeared in the control band and not in the test band, and as positive if a colour signal line appeared in the test band as well[15]. For MPT64-positive isolates, the intensity of the positive colour signal line was also recorded as 'strong' (strong intensity) or 'faint' (weaker intensity). MPT64 devices were double-read by another person who was blinded for the results of the first reading, mainly because of faint positivity signals. The MPT64 antigen test was performed without prior knowledge of the spoligotype analysis results (spoligotype analysis for all specimens was performed in batches after the availability of the MPT64 results). The MPT64 test, spoligotype analysis and conventional identification were performed blindly by different persons.

Conventional identification with PNB/catalase along with DST

Identification with PNB on LJ medium was performed along with first-line DST. Mycobacterial suspensions were inoculated on LJ medium with a final concentration of 500 µg ml^{-1} PNB and on LJ control slants, as described by

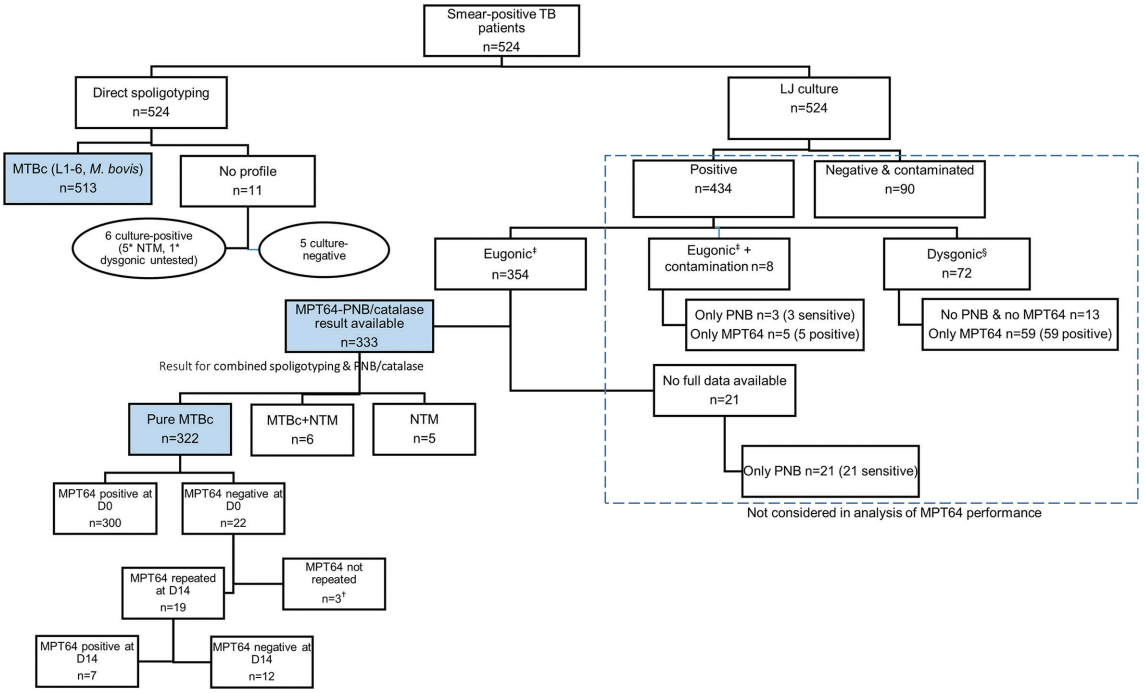

Fig. 1. Specimen flow chart. *, using PNB/catalase and GenoType CM. †, MPT64 could not be repeated at D14 as very few colonies remained on the LJ slants, which were used for DST and PNB/catalase. ‡, eugonic isolate: isolate with easily scrapable colonies that grow easily in subculture. §, dysgonic isolate: isolate with very small dry flat or convex [27], hardly scrapable colonies, not improved by subculture. Dysgonic colonies can be hardly visible.

the International Union Against Tuberculosis and Lung Diseases (IUATLD) [5]. The first-line DST included, as usual, rifampicin, isoniazid, streptomycin and ethambutol, respectively, at the critical concentrations of 40 μg ml^{-1}, 0.2 μg ml^{-1}, 4 μg ml^{-1} and 2 μg ml^{-1} using the proportion method on LJ medium [5, 16]. As recommended by the IUATLD [5], when an isolate was PNB-susceptible and resistant to at least one of the first-line drugs, it was tested for the production of 68 °C-labile catalase to rule out NTM.

DNA extraction and spoligotyping

For each specimen, 200 μl of decontaminated sputum from the aliquot that had been stored at −20 °C was heat-inactivated at 100 °C for 5 min [17], followed by DNA extraction in 300 μl elution buffer using the Promega Maxwell16 Tissue DNA Purification kit (AS1030)[18] with the Promega Maxwell 16 machine model AS2000 version 4.9, as described previously [19]. A mycobacterial sediment known to be PCR-positive for MTBc was included as a positive extraction control and distilled water was used as a negative extraction control. Spoligotyping (PCR followed by hybridization) was then performed as previously described [19, 20]. The *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG reference strains were included as positive PCR controls and distilled water was used as a negative control. The MTBc lineage was assigned using the online tool, the TB-Lineage database [21].

Determination of NTM species and prevalence in the study population

The Genotype *Mycobacterium* CM version 1 (Hain Lifescience) [16], molecular species identification test was used to identify presumed NTM isolates or MTBc/NTM mixtures based on PNB, catalase and spoligotyping results. DNA was extracted from the isolates by heat inactivation (100 °C for 20 min)[17, 19]. PCR followed by hybridization of the PCR products on the Genotype Hain CM strip were performed as described by the manufacturer [22]. The H37Rv reference strain was included as a positive control and distilled water was used as a negative control. After hybridization, the strips were fixed on an interpretation sheet and interpreted following the species profiles provided by the manufacturer.

Single-nucleotide polymorphism (SNP) analysis of *mpt64* gene in L5

To better understand the MPT64 test performance obtained for the L5 strains in our study, we investigated whether mutations are present in their *mpt64* gene (Rv1980c) using Snippy 3.1 [23] with H37Rv (NC_000962.3) as the reference MTBc genome. The available whole-genome sequences (FastQ files, File S1) from 25 strains belonging to L5 previously isolated in different countries were used (21 from Benin, 2 from Ivory Coast and 2 from Guinea). As a control group, we also checked for the presence of mutations in the *mpt64* gene of the 18 L6 strains (2 from Benin, 4 from

Table 1. Overall performance of the MPT64 antigen test at day 0 (D0) and at day 14 (D14)

MPT64	**Spoligotyping**			**PNB/catalase**			**Combined PNB/catalase + spoligotyping**				**Sensitivity (95 % CI)** **Specificity (95 % CI)** **LR+ (95 % CI)** **LR− (95 % CI)**
MPT64 at D0	MTBc	No profile (NTM or test failure)	Total	MTBc	NTM	Total	**Pure MTBc**	NTM	**Mixture spol MTBc + pnb NTM**	Total	**Sen: 93.2** (89.8–95.7)
+ (MTBc)	366	1	367	300	3	303	300	1*	2†	303	**Spe: 80** (**28.4–**99.5)
− (NTM)	26	4	30	22	8	30	22	4	4‡	30	**LR+: 4.7** (0.8–26.9)
Total	392	5	397	322	11	333	322	5	6	333	**LR−: 0.09** (0.05–0.16)
MPT64 at D0/D14							MTBc	NTM	**Mixture spol MTBc + pnb NTM**	Total	**Sen: 96.2** (93.5–98)
											Spe: 80 (**28.4–**99.5)
+ (MTBc)							307	1*	2†	310	**LR+: 4.8** (0.8–27.8)
− (NTM)							12	4	4‡	20	
Total							319§	5	6	330§	**LR−: 0.05** (0.02–0.1)

*Identified as MTBc by MPT64 but NTM by PNB/catalase and no spoligo bands or MTBc found using Genotype *Mycobacterium* CM, which identified this isolate as *Mycobacterium scrofulaceum* (or parafunicum or parasrofulaceum).

†MTBc/NTM mixtures confirmed using Genotype *Mycobacterium* CM on the isolates.

‡Identified as NTM by MPT64 and PNB/catalase (agreement). Spoligotyping of specimens detected the presence of MTBc (two L6, one L4 and one *M. bovis*), yet no mixture (MTBc/NTM) found using Genotype CM on the isolates.

§Three isolates that were MPT64-negative at D0 did not have MPT64 repeated at D14 and so are not included in the table.

Burkina-Faso, 3 from Guinea, 5 from Ivory Coast and 4 from Senegal) for which genomes were available. For genome sequencing, multiplexed Illumina libraries were prepared following manufacturer's guidelines using custom multiplex tags. Pooled samples were sequenced on an Illumina HiSeq2000 using the V4 kit to produce paired-end reads that were 100 bp in length. We aimed to attain an average depth of coverage of ~100-fold for each sample.

Statistical analyses

We used Stata12.0 (StataCorp, USA) for statistical analyses. The McNemar exact Chi-squared test was used to compare paired data, and Fisher's exact test was used for independent data.

The odds ratios (OR), sensitivity, specificity, positive likelihood ratio (LR+) and negative likelihood ratio (LR−) were all calculated, along with the 95 % confidence interval (CI). The difference was considered significant when the two-sided *P*-value was below 0.05.

RESULTS

Specimens testing flow

The specimen flow chart is presented in Fig. 1.

Overall performance of MPT64 test

MPT64 at D0

In total, 397/434 (91.5 %) positive cultures had MPT64 results available (Fig. 1, Table 1). Using either spoligotyping [19, 20] or PNB/catalase [5] as the reference standard for MTBc identification, 93.4 % (366/392; 95 % CI: 90.4–95.6) and 93.2 % (300/322; 95 % CI: 89.8–95.7) of MTBc isolates were correctly classified as MTBc by the MPT64 test, respectively (Table 1). The PNB/catalase results showed that six isolates (2 MPT64-positive and 4 MPT64-negative) identified as MTBc using spoligotyping were identified phenotypically as NTM (PNB/catalase), suggesting the presence of MTBc and NTM in the same specimen. This could be due to a mixed infection of MTBc/NTM (Table 1). Excluding these isolates from the analysis, 93.2 % (300/322; 95 % CI: 89.8–95.7) of MTBc isolates were correctly classified as MTBc by the MPT64 test on D0, with a specificity of 80 % (4/5; 95 % CI:28.4–99.5 (Table 1).

MPT64 at D14

The MPT64 test was repeated at D14 for 19 of the 22 isolates with a negative MPT64 test at D0 (Fig. 1). Seven of the 19 isolates became MPT64-positive, increasing the sensitivity of the MPT64 test to 96.2 % (307/319; 95 % CI: 93.5–98), considering PNB/catalase and spoligotyping agreement as the reference standard (Table 1).

Table 2. Performance of the MPT64 test across MTBc lineages

Only pure MTBc isolates (detected using PNB/catalase and spoligotyping) are included in this comparison

PNB/catalase (All MTBc)				
MTBc lineages	**MPT64 at D0**			**OR with 95 % CI, *P****
	Positive (MTBc)	**Negative**	**Total**	
L1	15 (100 %)	0	15	–
L2	14 (100 %)	0	14	–
L3	3 (100 %)	0	3	–
L4	198 (98 %)	4	202	• OR=0.06 (0.02 to 0.19)
L5	57 (76 %)	18	75	• *P*<0.0001
L6	8 (100 %)	0	8	–
M. bovis	5 (100 %)	0	5	–
Total	300	22	322	Overall *P*<0.001
MPT64 at D0-14				
L1	15 (100 %)	0	15	–
L2	14 (100 %)	0	14	–
L3	3 (100 %)	0	3	–
L4	198 (98.5 %)	3†	201	• OR=0.11 (0.03 to 0.38)
L5	64 (87.7 %)	9‡	73	• *P*=0.0005
L6	8 (100 %)	0	8	–
M. bovis	5 (100 %)	0	5	–
Total	307	12	319	Overall *P*=0.018

**P*-values were calculated using Fisher's exact test.

†MPT64 was not performed at D14 for one specimen that was MPT64-negative at D0 and so it was excluded.

‡MPT64 was not performed at D14 for two specimens that were MPT64-negative at D0 and so they were excluded; MPT64 became positive for seven specimens.

Species of NTM identified

Of the 11 isolates phenotypically identified as not being pure MTBc, 6 were considered to be mixtures (MTBc/NTM) based on the double banding profile on the Genotype *Mycobacterium* CM strip and/or a spoligotyping profile from the sputum specimen combined with growth of the isolate on the PNB tube. The five pure NTM isolates included one *M. kansasii*, one *M. abscessus* (or *M. immunogenum*), one *M. malmoense* (or *M. haemophilum*, *M. palustre* or *M. nebraskense*), one *M. scrofulaceum* (or *M. paraffunicum* or *M. parascrofulaceum*) and one *M. species*. Among the MTBc/NTM mixtures, two isolates were positive in the MPT64 assay (Table 1). They were confirmed as mixtures by Genotype CM and the spoligotype banding profiles, revealing L4 + *M. fortuitum* and L6 + *M. species*. For the four remaining presumed mixtures, spoligotyping of the sputum suggested the co-existence of MTBc, whereas no MTBc was detected in the isolates with only the NTM probe of the Genotype CM reacting and growth on PNB. The combined results suggested the following mixtures: *M. bovis* + *M. intracellulare*, L4 + *M. fortuitum* and 2× L6 + *M. intracellulare*. The overall proportion of NTM identified was 3.3 % (11/333). *M. intracellulare* followed by *M. fortuitum* were the most observed.

Performance of MPT64 test across MTBc lineages

For the following comparisons, NTMs and mixtures were excluded; only pure MTBc (PNB/catalase and spoligotyping-confirmed) isolates (322/333, Table 1) were included.

MPT64 at D0 in MTBc isolates

At D0, MPT64 positivity varied significantly across lineages (*P*<0.001, Table 2). MPT64 was positive for all isolates from lineages 1, 2, 3, 6 and *M. bovis*, whereas almost one-quarter (24 %) of the MAF L5 isolates and 2 % of the L4 isolates were MPT64-negative. MPT64-positive isolates were significantly under-represented among L5 versus L4 isolates, the most prevalent lineage (OR=0.06, 95 % CI: 0.02–0.19, *P*<0.0001; Table 2), corresponding to 15.6-fold odds (95 % CI: 5.3–45.8) of MPT64 false-negativity in L5 isolates (*P*<0.0001).

MPT64 at D14 in MTBc isolates

The 19 isolates that were MPT64-negative at D0 and repeat tested at D14 included 16 L5 and 3 L4 isolates. MPT64 became positive for an additional seven (43.8 %) L5 isolates, significantly increasing the positivity of MPT64 from 76 % at D0 to 87.7 % at D14 (RR=1.1, 95%CI: 1.0–1.2, *P*=0.016, exact McNemar). None of the three L4 isolates that were MPT64-negative at D0 became positive. Despite the positivity at D14 of some previously MPT64-negative isolates,

Table 3. Variation of the intensity of the positivity signal line of the MPT64 strip across MTBc lineages

Intensity of MPT64 cartridge positivity signal band (MPT64 positive specimens)				
	MPT64 positive at D0			OR (95 % CI), P^*
MTBc Lineages	**Strong** signal	**Faint** signal	**Total**	
L1	14	1 (6.7 %)	15	-
L2	14	0	14	-
L3	3	0	3	-
L4	195	3 (1.5 %)	198	•OR=21.2 (6.2–71.5)
L5	43	14 (24.6 %)	57	•P<0.0001
L6	7	1 (12.5 %)	8	-
M. bovis	5	0	5	-
Total	281	19	300	
Modern MTBc (L2+L3+L4)	212	3 (1.4 %)	215	•OR=17.7 (5.3–58.4)
Ancestral MTBc (L1 +L5+L6)	64	16 (20 %)	80	•P<0.0001
Other than MAF (L1+L2+L3+L4)	226	4 (1.7 %)	230	•OR=16.9 (5.6–50.6)
MAF (L5+L6)	50	15 (23.1 %)	55	•P<0.0001
	MPT64 positive at D0-14			
L1	14	1 (6.7 %)	15	-
L2	14	0	14	-
L3	3	0	3	-
L4	195	3 (1.5 %)	198	•OR=21.7 (6.4–72.2)
L5	48	16 (25 %)	64	•P<0.0001
L6	7	1 (12.5 %)	8	-
M. bovis	5	0	5	-
Total	286	21	307	
Modern MTBc (L2+L3+L4)	212	3 (1.4 %)	215	•OR=18.4 (5.6–60.3)
Ancestral MTBc (L1 +L5+L6)	69	18 (20.7 %)	87	•P<0.0001
Other than MAF (L1+L2+L3+L4)	226	4 (1.7 %)	230	•OR=17.5 (5.9–51.4)
MAF (L5+L6)	55	17 (23.6 %)	72	•P<0.0001

*P-values were calculated using Fisher's exact test.

MPT64 positivity still varied across lineages (Table 2). The difference in MPT64 false-negativity between L5 versus L4 isolates was still strongly significant, with 12.3 % (9/73) of L5 isolates versus 1.5 % (3/201) from L4 remaining negative at D14 (false-negativity OR=9.3, 95 % CI: 2.6–32.6, P=0.0005).

Variation of the intensity of the positivity signal (colour) line of the MPT64 cartridge

At D0, a total of 19 (6.3 %) of the 300 MPT64-positive isolates had a faint signal. Most of them were from L5 (24.6 %, 14/57), followed by L6 (12.5 %, 1/8), versus only 1.5 % (3/198) from L4 (Table 3). The odds of obtaining a faint signal in L5 isolates was 21.2-fold (95 % CI: 6.2–71.5) the odds of that for L4 isolates (P<0.0001), which did not decrease after the D14 repeat (OR=21.7, 95 % CI: 6.5–72.2, P<0.0001) (Table 3). The proportion of isolates with a faint signal was also higher in the MAF group (L5+L6) versus the non-MAF group (P<0.0001) or the ancestral lineages group (L1+L5 +L6) compared to the modern lineages group (P<0.0001, Table 3).

Variation of the time from inoculation (start of incubation) to the realization of the MPT64 test (Δt)

The median Δt at D0 was shortest for L1, L3 and L4 (3 weeks) followed by L2 and *M. bovis* (4 weeks), L5 (6 weeks) and L6 (8 weeks). Including repeated testing, the median Δt increased to 8 weeks for L5, while it remained similar for the other lineages (Table S2).

SNP analysis of *mpt64* gene in L5

All 25 of the L5 isolate genomes shared the same nSNP 128T>A (I43N) in the *mpt64* gene (File S1). One of the L5 genomes had in addition, a synonymous SNP 519G>A (V197V). Among the L6 isolate genomes, there was no mutation in the *mpt*64 gene for 17 of the 18 isolates analysed. One genome harboured a synonymous SNP 81C>T (T27T) in the gene.

Lineage distribution among population of isolates included versus excluded in the MPT64 comparison

The lineage distribution in the isolates (pure MTBc) included in the MPT64 vs combined spoligotyping and PNB/catalase comparison differed significantly to the

Table 4. MTBc isolate culture outcome across lineages: lineage distribution among the population of isolates that was included versus that among the population that was excluded (dysgonic/partially contaminated) in the MPT64/PNB comparison. *P*-values were calculated using Fischer's exact test

MTBc lineages	All specimens (culture positive, negative and contaminated) % (*n*)	All culture-positive specimens % (*n*)	Included isolates (pure MTBc) % (*n*)	Included vs excluded for quality (dysgonic, partial contamination)				Included (eugonic) vs excluded (dysgonic only)			
				Excluded isolates (dysgonic/partially contaminated) % (*n*)	$OR_{excluded/included}$, 95%CI	$\%Difference_{excluded - included}$, 95%CI	*P*	Dysgonic among excluded % (*n*)	$OR_{dysgonic\ among\ excluded/\ eugonic\ (included)}$, 95%CI	$\%Difference_{dysgonic\ among\ excluded - eugonic\ (included)}$, 95%CI	*P*
Total	***n*=513***	***n*=428†**	***n*=322**	***n*=79‡**				***n*=72**			
L1	3.9 (20)	4.2 (18)	4.7 (15)	3.8 (3)	0.8 (0.2 to 2.7)	−0.9 (−5.7 to 3.9)	1	2.8 (2)	0.6 (0 to 2.4)	−1.9 (−6.3 to 2.6)	0.749
L2	4.3 (22)	4.7 (20)	4.3 (14)	3.8 (3)	0.9 (0.3 to 2.9)	−0.6 (−5.3 to 4.2)	1	2.8 (2)	0.6 (0 to 2.5)	−1.6 (−6 to 2.8)	0.747
L3	0.8 (4)	0.7 (3)	0.9 (3)	0 (0)	0 (0 to 5.3)	−0.9 (−2 to 0.1)	1	0 (0)	0 (0 to 5.8)	−0.9 (−2 to 0.1)	1
L4	52.4 (269)	56.1 (240)	**62.7** (202)	**29.1** (23)	**0.2** (0.1 to 0.4)	**−33.6** (−44.9 to −22.3)	**<0.0001**	**25** (18)	**0.2** (0.1 to 0.4)	**−37.7** (−49 to −26.4)	**<0.0001**
L5	27.9 (143)	26.4 (113)	**23.3** (75)	**43.0** (34)	**2.5** (1.5 to 4.2)	**19.7** (7.9 to 31.6)	**0.0007**	**47.2** (34)	**3** (1.7 to 5)	**23.9** (11.5 to 36.4)	**0.0001**
L6	8.4 (43)	6.1 (26)	**2.5** (8)	**17.7** (14)	**8.5** (3.5 to 20.5)	**15.2** (6.6 to 23.8)	**<0.0001**	**19.4** (14)	**9.5** (3.9 to 23.1)	**17** (7.7 to 26.3)	**<0.0001**
M. bovis	2.3 (12)	1.9 (8)	1.6 (5)	2.5 (2)	1.7 (0 to 7.5)	1 (−2.7 to 4.7)	0.628	2.8 (2)	1.8 (0 to 8.3)	1.2 (−2.8 to 5.3)	0.616
Other than MAF	63.7 (327)	67.5 (289)	**74.2** (239)	**39.2** (31)	**0.2** (0.1 to 0.4)	**−35** (−46.8 to −23.2)	**<0.0001**	**33.3** (24)	**0.2** (0.1 to 0.3)	**−40.9** (−52.8 to −29)	**<0.0001**
MAF	36.3 (186)	32.5 (139)	**25.8** (83)	**60.8** (48)	**4.5** (2.7 to 7.5)	**35** (23.2 to 46.8)		**66.7** (48)	**5.8** (3.3 to 9.9)	**40.9** (29 to 52.8)	

*There were 11 specimens that did not yield a spoligotype profile among the 524 specimens.

†There were 6 specimens that did not yield a spoligotype profile among the 434 culture-positive specimens.

‡There was 1 specimen with failed spoligotyping among the 80 dysgonic/partially contaminated specimens (101 excluded in total – 21 with PNB available and MPT64 missing).

distribution in isolates excluded for the unavailability of MPT64 and/or PNB/catalase results (poor quality for subsequent tests) (Table 4). MAF L5 and L6 were significantly over-represented in that excluded group relative to the included group (OR=4.5, 95 % CI: 2.7–7.5, P<0.0001). The excluded group of isolates (n=80) included 72 dysgonic and 8 partially contaminated isolates. When comparing the included group solely to the dysgonic isolates among the excluded group, we found that the dysgonic nature of the isolates was strongly associated with the lineage, with L5 and L6 being over-represented among dysgonic isolates (OR=5.7, 95 % CI: 3.3–9.9, P<0.0001), especially L6 (OR=9.5, 95 % CI: 3.9–23.1, P<0.0001) (Table 4).

DISCUSSION

Our evaluation of LJ-based primary cultures from smear-positive TB patients in Benin in a nationwide prospective study suggests that the SD Bioline MPT64 antigen test offers relatively low performance for the rapid identification of the L5 of MTBc. Its lower sensitivity for the confirmation of L5 as MTBc leads to the misclassification of L5 as NTM. Repeating the test 14 days after a first negative result improved the sensitivity for L5 detection significantly, but not completely. The few L4 isolates that tested negative at D0 (1.5 %) remained negative at D14 testing, which could mean that those L4 isolates and the L5 isolates that were MPT64-negative at D14 need an incubation time beyond 14 days after D0, or could point to mutations in the *mpt64* gene, as found in L4 (including a 63 bp deletion [24]) in other studies [24, 25]. The MPT64 test positivity at D0 for L5 isolates cultured on LJ (solid) medium in our study (76 %) was similar to that for L6 isolates cultured in MGIT (liquid) medium (78.4 %) at the same D0 testing time-point in the study by Ofori-Anyinam *et al.*, while there was a similar increase in positivity to that found for L5 at D14 in our study (87.7 %) for L6 at D15 (90.2 %)[9]. In our study, however, all L6 isolates (n=8) were positive in the MPT64 test, even at D0. This observed difference – albeit for a small number – may be explained by the lower performance of MPT64 for L6 cultured in MGIT than for isolates cultured on LJ, as mycobacterial growth is more rapid in MGIT (liquid) than on LJ (solid) medium, allowing the production of a higher quantity of MPT64 protein on LJ compared to in MGIT. Indeed, a shorter incubation time posed an independent risk for a false-negative MPT64 test in the MGIT-based study [9].

SNP analysis of the *mpt64* gene in L5 genomes confirmed that L5 isolates harbour an nSNP in this gene [11] (also confirmed in all the 367 L5 genomes available in another genome collection from various countries; M. Coscolla, personal communication), leading to a modification of the amino acid chain of the MPT64 produced by L5 (I43N), probably impacting on the protein structure. Jiang *et al.* [24] found that nSNPs in the *mpt64* gene rarely changed the structure and function of the protein, in contrast to a 63 bp deletion (amino acids 66–86) that is mostly observed in L4, but also in some L1 isolates [24–26].

Oettinger *et al.* created five monoclonal antibodies (C24b1-3, L24b4-5) that reacted with four epitopes of the MPT64 antigen [26]. The MPT64 antigen's epitope for the C24b3 antibody consists of two structural domains found in the sequences Ala1-Leu43 and Ala108-Ser152 [26]. The Ile43Asn mutation (I43N) found in all L5 isolates genomes coincides with the final amino- acid of the first part of this epitope. This could lead to partial binding and lower adherence or prevent the binding of the C24b3 antibody to its partially modified epitope in the MPT64 antigen, resulting in a faint positivity signal band or negative MPT64 test if that antibody was used in the development of the MPT64 test. The mouse antibodies (at least three) [15] used in the development of the MPT64 test are not specified, but are likely included among the five detected by Oettinger *et al.* [26], as this paper is cited in the SD Bioline MPT64 sheet [15]. This could explain why, despite the non-synonymous mutation in the *mpt64* gene of L5, there was a significant proportion of isolates with faint positivity signal bands and isolates with strong positivity signal bands, and a significant proportion of MPT64-negative isolates (even after a D14 repeat). Nevertheless, changing the mouse antibody from which the corresponding MPT64 epitope is mutated in L5 isolates may improve the sensitivity for L5. It was reported that the MPT64 test has lower sensitivity for L6 strains [9], although no missense *mpt64* mutations were identified in L6 genomes. So another mechanism may account for the lower sensitivity in MAF L5 like for example in MAF L6 strains, in which the expression of the *mpt64* gene was lower than in *M. tuberculosis sensu stricto* [9]. More extended gene expression and regulation studies should be conducted in order to confirm these possible causes for the lower performance of MPT64 tests for the L5 and L6 MAF lineages.

Importantly, in West Africa where MAF is common, MPT64-negative tests should be confirmed by another identification method (such as Genotype Hain CM, IS6110 PCR, spoligotyping, Cepheid GeneXpert, PNB/catalase) before being classified as NTM. If available, molecular analyses [Genotype *Mycobacterium* CM, IS6110-PCR, spoligotyping, GeneXpert MTB/RIF (on a diluted bacterial suspension)] should be prioritized for this confirmation, as these are more rapid and sensitive (they can identify MTBc in mixtures of MTBc/NTM isolates) than PNB/catalase. If only PNB/catalase is available, further MPT64 testing can be done at at least day 14 after the first testing (while PNB/catalase is underway) as the result (if positive) can be obtained more rapidly than that for PNB/catalase.

In our study, MAF isolates were more likely to be dysgonic, as previously reported [27, 28], particularly for L6. Gehre *et al.* found that L6 has non-synonymous mutations in genes related to growth in culture (*aceE, recA*, *Rv2112* and *Rv0862*) that may explain its attenuated growth in culture [28]. A previous study also indicated that L5 is less likely to grow in culture compared to *M. tuberculosis sensu stricto* [19]. Our findings suggest that, in addition to the lower

ability of MAF strains to yield a positive culture, the MAF isolates that are successfully grown are more likely to be dysgonic, jeopardizing phenotypic post-culture tests. These findings highlight the need for simple phenotypic or genotypic diagnostic tests that can be applied directly to patient sputa/uncultured specimens to detect all species of the MTBc, distinguish MTBc strains from NTM, and detect possible mixed MTBc/NTM infections and drug resistance against multiple antibiotics.

One strength of our study is that it was conducted in a setting where L5 is common, allowing the prospective assessment of the performance of the MPT64 test for identifying this lineage as an MTBc member. Testing on primary isolation reflects how the MPT64 test is used in routine practice, typically on cultures from smear-negative or GeneXpert MTB/Rif-negative specimens, or extra-pulmonary specimens, and for rapid screening identification before phenotypic DST. Our testing was essentially blinded to the strain lineage, which was determined later and by different technicians. Possible limitations of our study include the fact that we only used one of the four commercially available MPT64 assays (Capillia, SD Bioline, MGIT TBc and TB Check), although a study on L6 did not find a difference between the MPT64 tests provided by different manufacturers [9]. We expect that our findings will therefore be generalizable to other settings, especially in West and Central Africa, where L5 is prevalent.

We therefore strongly recommend that the differences in MTBc lineage characteristics should be considered in diagnostics development, so tests will perform equally well in ancestral lineages such as L5 and L6. Furthermore, their performance should be evaluated in different settings, especially where such ancestral lineages are common.

Funding information
This work was supported by funds from the Directorate General for Development (DGD), Belgium (FA4) to C. N. S., B. C. d. J. and D. A., and the European Research Council-INTERRUPTB starting grant no. 311725 to B. C. d. J., L. R. and B. O. The funders had no role in study design, data collection and interpretation or the decision to submit the work for publication.

Acknowledgements
The MAF genomes analysed in this study were sequenced by the Wellcome Trust Sanger Institute. We thank Julian Parkhill (Wellcome Trust Sanger Institute, United Kingdom) for contributing to the genome sequencing and Conor Meehan (Institute of Tropical Medicine, Antwerp, Belgium) for advising on genome analysis and providing feedback on the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest

Ethical statement
This study is part of the BeniDiT study that has been approved by the Ethics Committee of Benin, and those of the Institute of Tropical Medicine and the University of Antwerp, Belgium. Its registration number on ClinicalTrials.gov is NCT02744469. All included patients provided written informed consent. Moreover, all sputa were anonymized before laboratory analyses.

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