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Unequal distribution of resistance-conferring mutations among *Mycobacterium tuberculosis* and *Mycobacterium africanum* strains from Ghana

Susanne Homolka[a,*], Christian G. Meyer[b], Doris Hillemann[a], Ellis Owusu-Dabo[c,d], Ohene Adjei[c], Rolf D. Horstmann[b], Edmund N.L. Browne[d], Amanua Chinbuah[e], Ivy Osei[e], John Gyapong[e], Tanja Kubica[a], Sabine Ruesch-Gerdes[a], Stefan Niemann[a]

[a] *Research Centre Borstel, National Reference Centre for Mycobacteria, Borstel, Germany*
[b] *Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany*
[c] *Kumasi Centre for Collaborative Research in Tropical Medicine, Kwame Nkrumah University of Science and Technology, Ghana*
[d] *Dept. of Community Health, School of Medical Sciences, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Ghana*
[e] *Health Research Unit, Ghana Health Service, Accra, Ghana*

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ABSTRACT

Isoniazid (INH) and rifampicin (RMP) resistance in *Mycobacterium tuberculosis* complex (MTC) isolates are mainly based on mutations in a limited number of genes. However, mutation frequencies vary in different mycobacterial populations. In this work, we analyzed the distribution of resistance-associated mutations in *M. tuberculosis* and *M. africanum* strains from Ghana, West Africa. The distribution of mutations in *katG*, *fabG1-inhA*, *ahpC*, and *rpoB* was determined by DNA sequencing in 217 INH-resistant (INH^r) and 45 multidrug-resistant (MDR) MTC strains isolated in Ghana from 2001 to 2004. A total of 247 out of 262 strains investigated (94.3%) carried a mutation in *katG* (72.5%), *fabG1-inhA* (25.1%), or *ahpC* (6.5%), respectively. *M. tuberculosis* strains mainly had *katG* 315 mutations (80.1%), whereas this proportion was significantly lower in *M. africanum* West-African 1 (WA1) strains (43.1%; $p < 0.05$). In contrast, WA1 strains showed more mutations in the *fabG1-inhA* region (39.2%, $p < 0.05$) compared to *M. tuberculosis* strains (20.9%). In 44 of 45 MDR strains (97.8%) mutations in the 81-bp core region of the *rpoB* gene could be verified. Additionally, DNA sequencing revealed that 5 RMP-susceptible strains also showed mutations in the *rpoB* hotspot region. In conclusion, although principally the same genes were affected in INH^r *M. tuberculosis* and *M. africanum* strains, disequilibrium in the distribution of mutations conferring resistance was verified that might influence the efficiency of molecular tests for determination of resistance.

Introduction

Mycobacterium tuberculosis and other members of the *M. tuberculosis* complex (MTC) such as *M. africanum* belong to the most harmful pathogens worldwide (Corbett et al., 2003; World Health Organization, 2009). It is estimated that approximately one-third of the world's population is infected with the pathogen and there are an estimated 2 million tuberculosis deaths annually (World Health Organization, 2009). As a result of the increase in the incidence of tuberculosis, there has been a corresponding rise of drug-resistant and multidrug-resistant strains [MDR TB; resistant to at least isoniazid (INH) and rifampicin (RMP)] of *M. tuberculosis* especially in Eastern Europe and Asia (World Health Organization, 2007). In the recent report of the World Health Organization, it is estimated that 5.3% of all TB cases are multidrug-resistant which are the highest rates ever recorded (World Health Organization, 2007). Additionally, the situation is exacerbated by the existence of XDR-TB (MDR plus resistance to any fluoroquinolone and any of the second-line anti-TB injectable drugs). Recent studies also indicate an increasing rate of drug-resistant strains in some regions of Africa (World Health Organization, 2007) and also a higher rate of initial drug resistance in Ghana, West Africa (Lawn et al., 2001; Owusu-Dabo et al., 2006; van der Werf et al., 1989). Drug resistance in *M. tuberculosis* is the result of genetic mutations in certain genes conferring resistance, which occur spontaneously and independent of exposure to drugs. Resistant bacilli may be selected if large numbers of tubercle bacilli are present in a patient treated with only one effective drug (Musser, 1995). Resistance to INH is based on a complex genetic system. INH is a pro-drug that requires activation for its active form (Slayden and Barry, 2000). This step is catalyzed by the catalase-peroxidase enzyme encoded by *katG* (Slayden and Barry, 2000). Several studies have shown that resistance to INH is most frequently associated with substitutions in codon 315 of *katG* gene

* Corresponding author at: Research Centre Borstel, Molecular Mycobacteriology, Parkallee 1, 23845 Borstel, Germany. Tel.: +49 4537 188274; fax: +49 4537 188311.
E-mail address: shomolka@fz-borstel.de (S. Homolka).

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(*katG* 315) (Musser, 1995). Furthermore, mutations in the regulatory region of the *fabG1-inhA* encoding for enoyl-ACP-reductase and in the promoter region of the *ahpC* gene encoding for alkyl-hydroperoxide-reductase were described to lead to INH resistance (Ramaswamy and Musser, 1998).

In contrast to INH resistance, RMP resistance is due to the genetic alteration of a small hotspot region of only 81 bp of the *rpoB* gene (codon 507–533) in more than 95%, which encodes the beta subunit of the DNA-dependent RNA polymerase (Musser, 1995; Zhang and Telenti, 2000). Mutations in this region lead to different levels of RMP resistance depending on a specific genetic background of the host strain (Zaczek et al., 2009). Although in general the same genes are involved in the development of resistance in clinical isolates of different phylogenetic branches of the MTC, the frequency of particular mutations can vary significantly in different geographical regions or among strains from different genotypes (Dalla Costa et al., 2009; Doustdar et al., 2008; Evans et al., 2009; Lipin et al., 2007). This disequilibrium in the distribution of resistance mutations has the potential to affect the sensitivity of molecular diagnostic tests in different geographical areas. Thus, the identification of certain mutations with regard to genotypes is desperate for developing efficient and economic tools for the rapid detection of drug resistance and for an effective TB treatment (Ali et al., 2009; Warren et al., 2009).

West Africa is presumably the region with the most diverse MTC population structure ranging from 2 *M. africanum* variants (West-African 1 and 2, WA1 and WA2) to a variety of *M. tuberculosis* lineages (de Jong et al., 2009; Homolka et al., 2008). This directly addresses the question whether this high degree of population diversity has an effect on the genetic basis of resistance found in clinical isolates and the performance of molecular resistance tests, however, such data are only sparely available (Haas et al., 1997a).

Therefore, DNA sequencing of established "resistance genes" in a large sample of well-defined INH and/or RMP-resistant *M. tuberculosis* complex strains from Ghana was carried out. Based on the sequence information, we obtained a detailed picture of genetic variation possibly associated with the development of resistance in this high-incidence region. The data obtained were stratified according to strain classification in *M. tuberculosis* and *M. africanum* WA1 and WA2.

Materials and methods

Bacterial strains

Patients with pulmonary TB were recruited at the 2 Ghanaian University Teaching Hospitals (Korle Bu Teaching Hospital, Accra; Komfo Anokye Teaching Hospital, Kumasi) and additionally from peri-urban hospitals from both the Greater Accra and the Ashanti Regions. Furthermore, patients were recruited from regional and district hospitals in the eastern, central and northern regions of Ghana. From 2001 to 2004, mycobacterial cultures were grown from 1972 patients with newly diagnosed, smear-positive pulmonary TB and no history of previous TB treatment and consecutively enrolled in a prospective case–control study.

Primary isolation and culture was performed at the Kumasi Centre for Collaborative Research (KCCR) in Kumasi, Ghana. Briefly, sputum samples were taken from each patient for microscopy to identify acid-fast-bacilli. After decontamination with N-acetyl-L-cysteine/NaOH and centrifugation, the sediment of the sputum sample was inoculated onto Löwenstein–Jensen (L/J) media. Cultures were incubated at 37 °C and read weekly for visible growth of mycobacteria for a maximum duration of 10 weeks. Subsequently, for further analysis, cultures were sent to the German National Reference Centre for Mycobacteria in Borstel, Germany. Identification of strains was based on molecular and biochemical tests as described previously (Kent and Kubica, 1985; Niemann et al., 2002). Drug-sensitivity testing (DST) to the first-line anti-tuberculosis drugs INH, RMP, pyrazinamide (PZA), and ethambutol (EMB) was performed using the proportion method on L/J media according to the German "Deutsches Institut für Normung" guidelines (Deutsches Institut für Normung; DIN, 1996). Alternatively, the modified proportion method in the BACTEC 460TB (Becton Dickinson Microbiology Systems, Cockeysville, USA) was performed according to the manufacturer's instructions.

Out of all strains, a total of 262 was resistant to INH and included in this investigation. These comprise 65 INH-resistant *M. africanum*, 1 INH-resistant *M. bovis*, and 196 INH-resistant *M. tuberculosis* strains.

Furthermore, 45 (13 *M. africanum*, 32 *M. tuberculosis*) of these 262 strains were also resistant to RMP. Sixty-three randomly chosen, fully susceptible isolates were included as controls (29 *M. africanum*, 34 *M. tuberculosis*).

Biochemical tests

Biochemical analyses for differentiation included colony morphology, nitrate reduction on modified Dubos broth, niacin accumulation test (INH test strips; Difco, Detroit, USA), semi-quantitative catalase test, and growth in presence of thiophen-2-carboxylic acid hydrazide (TCH, 2 μg/ml). Growth characteristics on Lebek media and on bromcresolpurple media were performed as described previously (Meissner and Schröder, 1969).

Strain classification based on IS6110 RFLP DNA fingerprinting and spoligotyping analysis

Extraction of DNA from mycobacterial strains was performed according to a standardized protocol (van Embden et al., 1993). All strains were genotyped by spoligotyping and IS*6110* DNA fingerprinting as described elsewhere (Kamerbeek et al., 1997; van Embden et al., 1993). The stepwise procedure for strain classification included an initial cluster analysis of IS*6110* fingerprinting data and lineage identification according to specific spoligotype signatures. Assignment of lineages was based on the MIRU-VNTRplus webpage (www.miru-vntrplus.org; Allix-Beguec et al., 2008) and a reference strain collection using the Bionumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium).

DNA sequencing

Hotspot regions of the *katG* gene (210 bp), the promoter region of the *fabG1-inhA* gene (248 bp), and of the *ahpC* gene (237 bp) as well as the RMP resistance-determining region of the *rpoB* gene (RRDR; codon 507–533 according to *E. coli* numbering system; 158 bp) were amplified. Primer sequences are summarized in Table 1. Direct sequencing of PCR fragments was carried out using a commercially available sequencing kit (BigDye terminator v1.1, Applied Biosystems, Foster City, USA) and the ABI 3130XL sequencer according to the manufacturer's instructions (Applied Biosystems).

Computer analysis

SeqScape v2.6 software (Applied Biosystems) was used for DNA sequence comparisons. Statistical analysis was performed by the χ^2-test. *P*-Values of less than 0.05 were considered statistically significant.

Table 1
Primers used in this study.

	Oligonucleotides	Position[a]
katG-F[b]	5′-GAAACAGCGGCGCTGATCGT-3′	2155331–2155312
katG-R[b]	5′-GTTGTCCCATTTCGTCGGGG-3′	2155122–2155141
fabG1-inhA-F[b]	5′-CCTCGCTGCCCAGAAAGGGA-3′	1673272–1673291
fabG1-inhA-R[b]	5′-ATCCCCCGGTTTCCTCCGGT-3′	1673519–1673500
ahpC-F[b]	5′-ACCACTGCTTTGCCGCCACC-3′	2726015–2726034
ahpC-R[b]	5′-CCGATGAGAGCGGTGAGCTG-3′	2726232–2726251
rpoB-F[b]	5′-TCGCCGCGATCAAGGAGT-3′	761059–761075
rpoB-R[b]	5′-GTGCACGTCGCGGACCTCCA-3′	761197–761216

[a] The positions of the primer correspond to GenBank ref. numbers NC_000962.2.
[b] Primer: TibMolBiol, Berlin, Germany.

Results

Strain classification

According to biochemical test and molecular typing results, the 325 strains investigated (262 INH-resistant strains, 63 INH-susceptible strains) were classified in 94 *M. africanum*, 1 *M. bovis*, and 230 *M. tuberculosis* strains. Main criteria for species differentiation were described in detail in previous publications (Kent and Kubica, 1985; Niemann et al., 2002, 2004). Among INH-resistant *M. africanum* strains, 51 isolates were identified as West-African 1 (WA1) strains (78.5%) and 14 as West-African 2 (WA2) strains (21.5%). All WA2 strains investigated were INH-resistant only. In contrast, 13 of 51 INH-resistant WA1 isolates were MDR strains (25.5%).

Mutations in INH- and RMP-resistant strains

Overall, 247 of 262 INH-resistant strains (94.3%) carried resistance-associated mutations in *katG, fabG1-inhA*, or *ahpC* genes (Table 2 stratified for 261 *M. tuberculosis* and *M. africanum* strains). The *M. bovis* isolate showed no mutation in all genes investigated. Additionally, 44 of 45 RMP-resistant strains (97.8%) had mutations in the 81-bp hotspot region of the *rpoB* gene. However, 5 INH-resistant, but RMP-susceptible strains also had mutations in the *rpoB* gene [2 *M. africanum*; 3 *M. tuberculosis*; L511P, D516Y, H526N, L533P (2 strains)]. To further analyze these discrepancies between phenotypic and sequencing test results, the minimum inhibitory concentration (MIC) was determined by the proportion method on L/J media (5, 10, 20, and 40 μg/ml). Low-level resistance (MIC = 10–20 μg/ml) could be detected in one strain (*M. africanum* WA2, L533P). Mutations were confirmed by repeated sequencing analyses of new isolated DNA and by GenoType® MTBDRplus assay according to the manufacturer's instruction (Hain LifeScience GmbH, Nehren, Germany). In contrast, no resistance-associated mutations were found in 63 INH- and RMP-susceptible control strains.

Mutations in the katG gene

Out of 262 INH-resistant strains analyzed, 190 (72.5%) carried a mutation in *katG* 315 (Table 2). Further mutations were detected in codon 291 and codon 293 (one strain each). In three strains (1.5%), codon 315 showed the wt sequence and an ACC substitution.

If *M. africanum* and *M. tuberculosis* strains were considered separately, it becomes obvious that *M. tuberculosis* strains showed a higher rate of mutations in *katG* 315 [157 out of 196 (80.1%)] than *M. africanum* strains [33 out of 65 (50.8%)]. In particular, 49.2% of the INH-resistant *M. africanum* strains had a *katG* 315 ACC mutation, and one strain showed an ACA substitution [1.5% (Table 2)]. Further stratification for WA1 and WA2 revealed that these differences were mainly due to a significantly lower number ($p < 0.05$) of *katG* 315 mutations in the WA1 genotype (43.1%). *M. africanum* WA2 strains showed an equal amount of *katG* 315 mutations (78.6%) to *M. tuberculsosis* strains (Fig. 1a).

Among the 196 INH-resistant *M. tuberculosis* strains, 144 strains carried a *katG* 315 ACC substitution (73.5%). Furthermore, substitutions into ATC (1%), ACA (1%), AAC (2%), or GGC (1%) could be detected. Three isolates (1.5%) had a mixture of wt and *katG* 315 ACC mutations and must be considered as heteroresistant. Codon 291 GCT was altered into ACT in one strain.

Mutations in the fabG1-inhA gene

In total, 66 of 262 strains (25.2%) carried a mutation in the regulatory region of the *fabG1-inhA* gene (Table 2). The most common point mutation was a C-to-T variation at position −15 upstream of the translation start site of the *fabG1-inhA* gene (74.2%). Eight of these strains were also altered in *katG* 315. All strains with substitutions at positions −8, −9, and −34 showed also a *katG* 315 mutation. Besides, we could detect variations at positions −47, −34, or −9 which are not described yet.

If the results were stratified for *M. africanum* and *M. tuberculosis*, 23 of 65 *M. africanum* strains showed a C-to-T substitution at position −15 (35.4%). Three of these strains carried also an alteration in *katG* 315. WA1 strains had higher numbers of mutations at position −15 in comparison to WA2 strains (WA1 37.3%; WA2 28.6%), however, they were not statistically significant (Fig. 1b). In addition, we could detect a G-to-A variation at position −102 in all WA1 strains, but not in WA2 strains. Therefore, we assume that this is a WA1 genotype-specific marker and not associated with resistance.

The proportion of strains with mutations in the *fabG1-inhA* region was significantly lower among *M. tuberculosis* as only 41 of 196 (20.9%) strains had a mutation in *fabG1-inhA* (Fig. 1b). Twenty-six of these had a C-to-T substitution at position −15 (Table 2), whereas 5 strains also showed a mutation in *katG* 315. Furthermore, we also observed the 3 newly described mutations at positions −47, −34, and −9 in *M. tuberculosis*.

Mutations in the ahpC gene

In total, 17 of 262 INH-resistant strains investigated (6.5%) showed a mutation in the promoter region of the *ahpC* gene (Table 2). Five of these strains were also altered in *katG* (4 strains: 1× L293P and C-to-T −57 *ahpC*, 3× *katG* 315 and G-to-A −48 *ahpC*) or *fabG1-inhA* (one strain: G-to-C −47 *fabG1-inhA* and G-to-A −48 *ahpC*). One strain showed mutation in all genes investigated (*katG* 291, C-to-T −34 and G-to-A −48). Besides, we could detect substitutions at positions −93, −81, −57, −49, and −43 which have not been described yet and may contribute to INH resistance. Overall, there were no significant differences in the appearance of mutations in the *ahpC* gene between *M. africanum* and *M. tuberculosis* as well as between WA1 and WA2 strains of *M. africanum* (Fig. 1c).

Mutations in the rpoB gene

Mutations in the 81-bp hotspot region of the *rpoB* gene were detected in 44 of 45 MDR strains (13 *M. africanum*, 32 *M. tuberculosis* [97.7%]). Ten different variants of single mutations in codon *rpoB* 513, 516, 522, 526, 531 and 533 as well as a G insertion in codon 526 were found (Table 2). One strain carried a point mutation in two separated codons (S531L and L533P). All substitutions analyzed were non-synonymous. The most prevalent alteration could be detected in *rpoB* 531 (25 strains [55.6%]), while codon 526 was shown to be the most variable one.

If *M. africanum* and *M. tuberculosis* strains were considered separately, *M. africanum* strains had a significant higher number of mutations in *rpoB* 531 (76.9%) than *M. tuberculosis* (46.9%). As all *M.*

Table 2
DNA sequencing results for INH-resistant and MDR strains from Ghana stratified for *M. africanum* and *M. tuberculosis*[a].

Gene	Codon (SNP)	No. of *M. africanum* (%) (65 INH-resistant, 13 MDR)	No. of *M. tuberculosis* (%) (196 INH-resistant, 32 MDR)
katG	Wild-type	31 (47.7)	38 (19.4)
	Mutation (total)	34 (52.3)	158 (80.6)
	S315T aGc>aCc	32 (49.2)	144 (73.4)
	S315T aGC>aCA	1 (1.5)	2 (1)
	S315N aGc>aAc	0	4 (2)
	S315G Agc>Ggc	0	2 (1)
	S315I aGc>aTc	0	2 (1)
	S315T/wt aGc>aCc/aGc	0	3 (1.5)
	A291T Gct>Act	0	1 (1)
	L293P cTg>cCg	1 (1.5)	0
fabG1-inhA	Wild-type	40 (61.5)	155 (79.1)
	Mutation (total without[b])	25 (38.5)	41 (20.9)
	−8 T>A	0	3 (1.5)
	−8 T>C	0	4 (2)
	−9 G>A	0	1 (0.5)
	−15 C>T	23 (35.4)	26 (13.3)
	−17 G>T	1 (1.5)	0
	−19 G>A/G[c]	0	1 (0.5)
	−34 C>T	1 (1.5)	1 (0.5)
	−47 G>C	0	6 (3)
	−102 G>A[b]	51 (78.5)	0
ahpC	Wild-type	61 (93.8)	183 (93.4)
	Mutation (total)	4 (6.2)	13 (6.6)
	−43 C-insertion[d]	0	1 (0.5)
	−48 G>A	0	9 (4.6)
	−48 G>A/G[e]	0	1 (0.5)
	−49 T-insertion	0	1 (0.5)
	−52 C>T	2 (3.1)	0
	−52 C>T/C[e]	0	1 (0.5)
	−54 T-insertion	0	1 (0.5)
	−57 C>T	1 (1.5)	0
	−81 C>T	0	1 (0.5)
	−93 G>T	1 (1.5)	0
rpoB	Wild-type	0	1 (3.1)
	Mutation (total)	13 (100%)	31 (96.9)
	Q513P cAa>cCa	0	1 (3.1)
	D516V gAc>gTc	1 (7.7)	4 (12.5)
	D516Y Gac>Tac	1 (7.7)	0
	S522L cTg>tTg	0	1 (3.1)
	H526D Cac>Gac	0	2 (6.3)
	H526Y Cac>Tac	1 (7.7)	5 (15.6)
	H526C CAc>TGc	0	1 (3.1)
	526 G insertion	0	1 (3.1)
	S531W tCg>tGg	1 (7.7)	1 (3.1)
	S531L tCg>tTg	9 (69.2)	14 (43.8)
	L533P cTg>cCg[f]	0	2 (6.3)

[a] The *M. bovis* isolate is not included in the table. Therefore, percentage values refer to 261 INH-resistant *M. tuberculosis* and *M. africanum* strains.
[b] Mutation was *M. africanum* genotype West-African 1-specific and not resistance-associated. Nineteen of these strains had a −15 C>T mutation and one strain a −17 G>T variation.
[c] Strain carried also a −15 C>T mutation.
[d] Strain carried also a −48 G>A mutation.
[e] Strain showed 2 sequences: −48 G>A/G and −52 C>T/C.
[f] One strain carried also a mutation in codon 531 (S531L).

africanum strains investigated belong to the WA1 genotype, differences just apply to WA1 strains and not to *M. africanum* strains in general.

Overall, *M. tuberculosis* strains showed a higher variability in the 81-bp hotspot region (Table 2). Nine different variants could be detected in *M. tuberculosis*.

Discussion

In this study, we addressed the question whether INH mono-resistance and MDR *M. africanum* strains carry the same distribution of mutations in the key genes involved in the development of resistance when compared with strains of *M. tuberculosis*. Our results clearly show that most of the strains investigated have mutations in already described regions of *katG*, *fabG1-inhA*, *ahpC*, and *rpoB* irrespective of whether the isolates were classified as *M. africanum* or *M. tuberculosis*. However, we could identify a disequilibrium in the distribution of mutations between these two species and between different *M. africanum* genotypes. Our findings are in agreement with several studies of *M. tuberculosis* where significant differences concerning *katG* 315 and *rpoB* mutations between Beijing and non-Beijing strains as well as between Beijing and CAS1 strains could be detected (Ali et al., 2009; Hillemann et al., 2005; Park et al., 2005). However, to our knowledge, disequilibrium in the distribution of mutation between *M. africanum* and *M. tuberculosis* are not described yet. These differences may affect the efficiency of molecular tests for the prediction of INH and RMP resistance especially in West Africa (Evans et al., 2009).

The observed discrepancy between negative or weakly positive test results in the semi-quantitative catalase test (Frothingham et al., 1999; Haas et al., 1997a) and normal rates of INH resistance

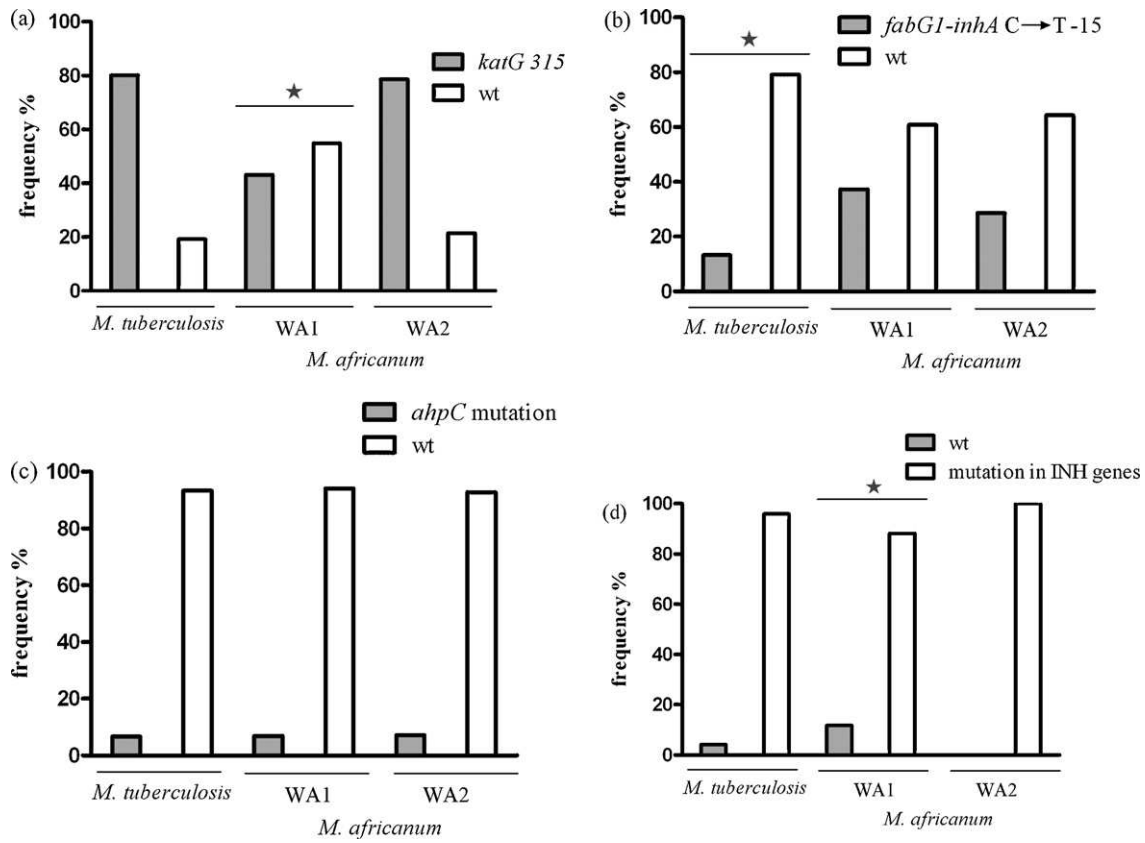

Fig. 1. Distribution of mutation in *katG* 315 (a), *fabG1-inhA* C-to-T −15 (b), *ahpC* (c), and wt sequences in all INH-related genes (d) among the INH-resistant strains. Results were stratified according to strain classification in *M. tuberculosis*, *M. africanum* West-African 1 (WA1) and West-African 2 (WA2). *Differences are statistically significant.

in clinical isolates (Bercion and Kuaban, 1997; Haas et al., 1997a) in the case of *M. africanum* has brought our attention to the question whether another mechanism for activation of INH is present in *M. africanum* strains, which should also result in completely other genetic alterations in INH-resistant *M. africanum* isolates. It is therefore noteworthy that in this large-scale investigation of INH-resistant *M. africanum* strains both, *M. africanum* and *M. tuberculosis* isolates, mainly had mutations in *katG* 315 encoding for the catalase-peroxidase gene (Slayden and Barry, 2000). Therefore, it is very likely that the principle of INH activation by the catalase-peroxidase encoded by *katG* seems to be the same for *M. africanum* and *M. tuberculosis*.

These findings are in agreement with two earlier investigations of smaller samples of INH-resistant *M. africanum* strains, which also detected an alteration in *katG* as the mutation occurring most frequently (Haas et al., 1997b). However, the interpretation of these results is even more complex as it seems that in spite of a lack of measurable catalase activity in *M. africanum*, KatG is able to catalyze the activation of INH. This finding is in accordance with a recent report analyzing the influence of different *katG* mutations on the catalase activity and the level of resistance (DeVito and Morris, 2003). In this study, the mutations tested generally resulted in a reduction of catalase activity and an increased resistance to INH. However, in the case of one mutation (N138S), the catalase activity was significantly decreased while the sensitivity to INH was retained. Whether this effect is the reason for our findings in *M. africanum*, is not clear yet and needs further investigation.

The occurrence of certain *katG* mutations in clinical *M. tuberculosis* complex isolates (especially S315T) varies between 40% and 100% in different geographical regions (Mokrousov et al., 2002; Riska et al., 2000). In this study, we could confirm the importance of *katG* 315 mutations in INH-resistant isolates from Ghana, which was detected in 72.5% of all strains investigated. A similarly high frequency of this mutation was also found in Latvia (91.0%) (Tracevska et al., 2002), in northwestern Russia (93.6%) (Mokrousov et al., 2002), in Lithuania (85.7%) (Bakonyte et al., 2003) and in South America (80.8%) (Dalla Costa et al., 2009). Furthermore, a high prevalence of *katG* S315T substitutions was also reported in some studies from Sierra Leone, South Africa, and Kenya (Githui et al., 2004; Haas et al., 1997b; Kiepiela et al., 2000). However, a strikingly different example is a study from Equatorial Guinea in which mutations in *inhA* were determined in 80% of all INH-resistant strains, whereas no alteration in *katG* could be detected (Tudo et al., 2004).

The reasons why different distributions of mutations have been documented in different geographical settings are not well understood.

It might be the case that the predominant spread of clones with distinct mutations may lead to a shift in the distribution of mutations observed among strains analyzed in short-term studies. However, another reason may be the difference in the population structure of *M. tuberculosis* complex isolates in different geographical areas.

Our study revealed an interesting finding concerning the disequilibrium in the distribution of the mutations in *katG* and *fabG1-inhA* between *M. africanum* WA1 and *M. tuberculosis*. INH-resistant *M. tuberculosis* strains showed a substitution in codon 315 in 80.1%, whereas *M. africanum* WA1 carried significantly less mutations (43.1%) in this region ($p < 0.05$). On the other hand, *M. africanum* WA1 strains showed significantly ($p < 0.05$) more mutations in the *fabG1-inhA* gene (39.2%) than *M. tuberculosis* strains (20.9%). With regard to the fact that the presence of mutations in the *fabG1-inhA* gene or promoter region generally confers low-level INH resistance that can be overcome by high-dose INH (Warren et al., 2009), our observation may have an impact on the deci-

sion whether to include high-dose INH for the treatment of INH mono-resistant-, MDR- or XDR-TB in West Africa.

Additionally, it is interesting to note that *M. africanum* WA1 strains showed a significantly higher number ($p < 0.05$) of wild-type (wt) sequences in all INH-related genes investigated (11.8%) in comparison to *M. tuberculosis* or *M. africanum* WA2 strains (Fig. 1d). All INH-resistant WA2 strains carried mutations in at least one of the INH-related genes. Our findings suggest that additional mechanisms are involved in developing INH resistance in *M. africanum* WA1 strains which may warrant further investigation.

An unequal distribution of mutations was also detected in the 81-bp hotspot region of the *rpoB* gene. Previous studies described that certain mutation profiles result from strain variations circulating in different geographical regions (Afanas'ev et al., 2007). Additionally, different mutations are associated with certain fitness levels (Gagneux et al., 2006; Mariam et al., 2004). In our study, WA1 strains showed significantly higher numbers of *rpoB* 531 (76.9%) mutations in comparison to *M. tuberculosis* strains (46.9%).

In this context, it is noteworthy that S531L substitution confers a relatively low fitness cost, which leads to a more likely spread in human population (Billington et al., 1999; Gagneux et al., 2006). The discrepancies observed between a RMP-susceptible phenotype and mutations in the *rpoB* gene observed in our study, confirm the previously described existence of low-level RMP resistance associated with distinct variations (Taniguchi et al., 1996; Zaczek et al., 2009). Zaczek et al. (2009) showed that specific substitutions in different codons lead to different resistance levels. Mutations in *rpoB* 516 are connected with low- or high-level resistance depending on the amino acid exchange (D516V: high; D516Y: low). Similar results were found by Taniguchi et al. (1996) where RMP-susceptible strains carried a mutation in *rpoB* L533P. In our study, we could confirm these observations. Besides, we showed that variations in *rpoB* L511P and *rpoB* H526N can also be associated with low-level resistance. As the same mutations appear in clinical isolates which are high-level resistant (MIC > 40 μg/ml), it seems to be that the level of RMP resistance by these mutations is also depending on the genetic background of the given clinical isolate. The consequences of these results for the clinical situation are unclear and require further investigation. For some mutations, DNA sequencing might not be enough to classify clinical MTC strains as RMP-resistant.

In conclusion, our results demonstrate disequilibrium in resistance-conferring mutations in *M. tuberculosis* and *M. africanum* strains. These findings confirm geographical differences in resistance mutations that are likely to be caused by the phylogeographical population structure of the MTC. Such differences should be considered when molecular tests are established in certain regions. The phenomenon of low-level RMP resistance needs special attention concerning clinical consequences and possible implications for conventional susceptibility testing.

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