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# Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of *katG*: a decade of experience in The Netherlands

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## ABSTRACT

A previous limited study demonstrated that *Mycobacterium tuberculosis* isolates with a mutation at amino-acid position 315 of *katG* (Δ315) exhibited high-level resistance to isoniazid and were more frequently resistant to streptomycin. In the present study, isoniazid-resistant *M. tuberculosis* isolates from 8332 patients in The Netherlands (1993–2002) were screened for the Δ315 mutation. Isoniazid resistance was found in 592 (7%) isolates, of which 323 (55%) carried Δ315. IS*6110* restriction fragment length polymorphism analysis showed that Δ315 isolates occurred in clusters, suggesting recent transmission, at the same frequency as isoniazid-susceptible isolates. In contrast, other isoniazid-resistant isolates clustered significantly less frequently. Δ315 isolates were high-level isoniazid-resistant, streptomycin-resistant and multidrug-resistant significantly more often, and may have a greater impact on public health, than other isoniazid-resistant isolates.

**Keywords** IS*6110*, isoniazid resistance, *katG* (Δ315 mutation), *Mycobacterium tuberculosis*, public health impact, restriction fragment length polymorphism analysis, The Netherlands

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## INTRODUCTION

Despite the availability of various drugs with activity against *Mycobacterium tuberculosis* and worldwide bacille Calmette–Guérrin vaccination, tuberculosis (TB) is the second most frequent infectious cause of death worldwide [1]. The emergence of drug resistance, and especially multidrug resistance (i.e., resistance to at least isoniazid and rifampicin), among strains of *M. tuberculosis* has become a major health threat in various parts of the world [2]. One of the mainstay drugs for the treatment of TB is isoniazid. Its effectiveness against *M. tuberculosis* was discovered simultaneously by three groups in 1952 [3–5], but resistant strains were reported shortly thereafter [6]. In The Netherlands, an isoniazid resistance level of 7% was reached in 1993–1997 [7], but resistance levels of up to 30–40% have been reached in several high-incidence countries [8].

Resistance against isoniazid is associated mostly with mutations or deletions in *katG*. This gene encodes the enzyme catalase peroxidase, which converts isoniazid into an active compound (isoniazid itself has no mycobactericidal activity) [9–11]. Other resistance mutations occur in the *inhA* gene (or its promoter), which encodes an enoyl acyl carrier protein reductase involved in fatty acid synthesis, which is the

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target of the active derivative of isoniazid [12]. Mutations in several other genes have been reported to be associated with isoniazid resistance, but occur less frequently, and their association with isoniazid resistance is less clear [13]. The most frequent mutation occurs at amino-acid position 315 of *katG* (Δ315), and this mutation accounts for 53–96% of resistance mutations among isoniazid-resistant isolates [7,14–18].

Part of the success of the Δ315 isolates is probably caused by the fact that catalase peroxidase is still active in these mutants; indeed, 30–40% of the initial catalase activity remains when this mutation is introduced into *katG* with site-directed mutagenesis [19]. A previous study in The Netherlands revealed that Δ315 isolates were found in clusters as frequently as isoniazid-susceptible isolates, whereas isoniazid-resistant isolates with another mechanism of resistance appeared to be transmitted less frequently (reflected by a lower percentage of clustered isolates). However, these differences were not significant. Furthermore, Δ315 isolates were reported to be associated significantly with high-level resistance to isoniazid and with additional streptomycin resistance [7].

In the present study, the prevalence of the Δ315 mutation was determined among 8332 *M. tuberculosis* isolates sent to the National Institute of Public Health and the Environment (RIVM, Bilthoven, The Netherlands) between 1993 and 2002. The associations between the presence of this mutation and other laboratory and clinical data were examined to determine the significance of the basis of isoniazid resistance in relation to the potential impact on public health.

## MATERIALS AND METHODS

Between 1993 and 2002, *c.*10 000 isolates of *M. tuberculosis* from TB patients in The Netherlands were submitted to the RIVM for species identification, drug susceptibility testing and IS*6110* restriction fragment length polymorphism (RFLP) typing [20,21]. Patient information was obtained from The Netherlands Tuberculosis Register, maintained by the KNCV Tuberculosis Foundation, which has been in place since 1993. The Netherlands Tuberculosis Register lists patient information anonymously; therefore, patient information was matched with laboratory information, using gender, date of birth, postal area code and year of diagnosis to identify matches.

The susceptibility of all isolates to isoniazid, streptomycin and rifampicin was determined with the MIC method [22], testing concentrations of 0.1, 0.5, 1, 2, 5, 10, 20 and 50 mg/L in 7H10 medium (Difco, Detroit, MI, USA). The isolates were considered to be resistant if >1% of the original inoculum grew on concentrations of at least 0.5, 10 and 2 mg/L for isoniazid, streptomycin and rifampicin, respectively. Multidrug resistance was defined, according to the definition of the WHO, as resistance to at least isoniazid and rifampicin.

Isoniazid-resistant isolates were investigated for the presence of the Δ315 mutation by PCR restriction endonuclease analysis with either *Aci*I [17] or *Msp*A1I [23] for the isolates obtained in 1993–1997, or by DNA sequencing of a 127-bp fragment of *katG*, using primers 315MGB-s and 315MGB-as [24], for the isolates obtained in 1998–2002. On the basis of these assays, the isoniazid-resistant isolates were divided into two groups: those with the Δ315 mutation, and other isoniazid-resistant isolates.

Standard RFLP typing was performed with IS*6110* as a probe [21]. If fewer than five bands were present in the RFLP pattern, polymorphic GC-rich sequence RFLP typing was also performed [20]. The term 'cluster' was used for two or more *M. tuberculosis* isolates with completely identical RFLP patterns, or for the respective patients. Based on IS*6110* RFLP analysis or spoligotyping, isolates were assigned to the Beijing genotype according to international guidelines [25].

ORs were calculated with the Epi6 program (CDC, Atlanta, GA, USA). Binary logistic regression to adjust for possible confounders (all variables were taken into account) was performed with SPSS v.11.5.2 software (SPSS Inc., Chicago, IL, USA).

## RESULTS

During the period 1993–2002, *c.*15 000 cases of TB were recorded in The Netherlands. Bacteria belonging to the *M. tuberculosis* complex were cultured from *c.*10 000 cases and were submitted to the RIVM [26]. Laboratory data from the RIVM were matched successfully with clinical data from the Netherlands Tuberculosis Register for 8332 patients. For these patients, all variables were known, except the results of microscopy (3495; 42%) and data concerning previous episodes of TB (7305; 88%). Of the 8332 patients, 592 (7.1%) yielded isoniazid-resistant *M. tuberculosis*, and 74 (0.89%) yielded multidrug-resistant (MDR) *M. tuberculosis*. PCR analysis showed that 323 isoniazid-resistant isolates (55%) had the Δ315 mutation, and of these, 95% had an isoniazid MIC >2 mg/L. In contrast, only 14% of isoniazid-resistant isolates without this mutation had an MIC >2 mg/L. Among all isoniazid-resistant isolates with an MIC >2 mg/L, 89% had the Δ315 mutation (Fig. 1).

Because of the different characteristics of isolates from Dutch patients and immigrants, the dataset was split into Dutch ($n$ = 3437; 41%) and non-Dutch ($n$ = 4895; 59%) patients. The countries

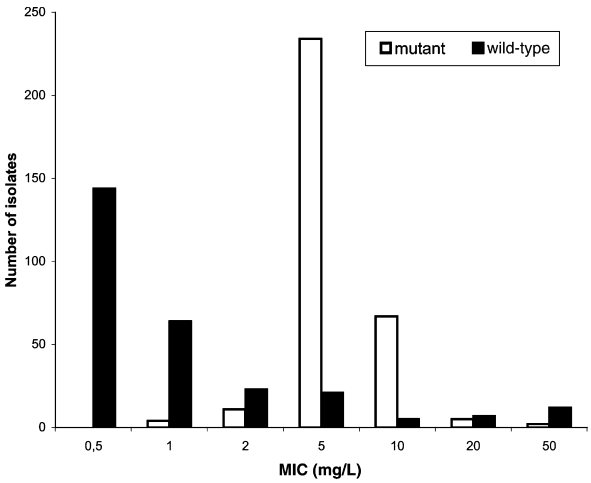


**Fig. 1.** Association between the MIC of isoniazid and a mutation at amino-acid 315 of *katG* in *Mycobacterium tuberculosis* isolates.

of origin of the non-Dutch patients were central and eastern Europe ($n$ = 283; 6%), Turkey ($n$ = 398; 8%), Morocco ($n$ = 752; 15%), Somalia ($n$ = 1156; 24%), other African countries ($n$ = 887; 18%), Asia ($n$ = 1009; 21%) and other countries ($n$ = 410; 8%). Similarities among these groups were observed in terms of age group composition and resistance to all tested anti-TB agents (the highest resistance rates were found among central and eastern European patients), in contrast to Dutch patients. Striking differences were: the rate of clustering was higher among Somalian and Moroccan patients (62%), but lower among Asian patients (24%); the prevalence of the Beijing strain was highest among Asian patients (18% vs. 3%); and a large proportion of Somalian patients presented with non-pulmonary TB (59% vs. 31%).

In general, as a group, non-Dutch patients suffered less frequently from pulmonary TB, had fewer positive smears, and were less frequently clustered; they yielded more isoniazid-resistant, rifampicin-resistant, streptomycin-resistant or MDR *M. tuberculosis* isolates, and were infected more frequently with a Δ315 isolate (Table 1). Furthermore, the age distribution differed between Dutch and non-Dutch patients (Fig. 2). Differences in age group, smear positivity, clustering and resistance to rifampicin, isoniazid and streptomycin were associated independently with Dutch or non-Dutch nationality (Table 1).

Among Dutch patients, isoniazid-resistant *M. tuberculosis* was associated with a different age distribution (with a higher prevalence among patients aged <45 years; data not shown) and with co-resistance to streptomycin and rifampicin. Isoniazid-resistant *M. tuberculosis* among non-Dutch patients was associated with a previous episode of TB, a positive Ziehl–Neelsen-stained smear, infection with an isolate belonging to the Beijing lineage, and resistance to streptomycin and to rifampicin. Independent co-variates of isoniazid resistance among non-Dutch patients were a pulmonary localisation, clustering, and streptomycin and rifampicin resistance (Table 2).

Δ315 isolates were obtained from 40 (36%) of 111 isoniazid-resistant Dutch cases, and from 283 (59%) of 481 isoniazid-resistant non-Dutch cases (OR 0.39, 95% CI 0.25–0.62). Among Dutch patients with isoniazid-resistant isolates, the presence of Δ315 isolates was associated with a pulmonary localisation and streptomycin resistance; among non-Dutch patients, the presence of Δ315 isolates was associated with clustering, streptomycin resistance and MDR (Table 3).

Associations between the different mutations that were found, namely AGC (Ser) to ACC (Thr), ACA (Thr), ACG (Thr), ATC (Ile) or AAC (Asn), and the clinical and laboratory data could not be assessed. More than 95% of all mutations were AGC to ACC. Therefore, the numbers of isolates with other mutations were too small to allow conclusions.

## DISCUSSION

This study shows that more than half of all isoniazid-resistant *M. tuberculosis* isolates from patients in The Netherlands have a mutation at amino-acid position 315 of *katG*. This mutation appeared to be associated with three important factors: first, high levels of drug resistance, with 89% of all isolates with an isoniazid MIC ≥5 mg/L carrying this mutation; second, Δ315 isolates from Dutch and non-Dutch patients were more often co-resistant to streptomycin, and Δ315 isolates from non-Dutch patients to rifampicin (i.e., MDR), than non-Δ315 isoniazid-resistant isolates (Table 3); third, among non-Dutch patients, isoniazid-resistant Δ315 isolates were associated with an increased transmission risk (or an increased progression from infection to disease), as reflected by a greater percentage of clustered Δ315 isolates compared to non-Δ315 isoniazid-resistant isolates. This association was strongest among Somalian patients (OR 3.67,

**Table 1.** Associations of *Mycobacterium tuberculosis* isolates from Dutch and non-Dutch patients with clinical and laboratory variables

| Variable | All ($n$ = 8332) | | Crude OR (95% CI) | Adjusted OR (95% CI) |
|---|---|---|---|---|
| | Dutch ($n$ = 3437) $n$ (%) | Non-Dutch ($n$ = 4895) $n$ (%) | | |
| Previous TB | | | 0.80 (0.63–1.02) | |
| Yes | 138 (4.4) | 150 (3.6) | | |
| No | 2979 (95.6) | 4038 (96) | | |
| Pulmonary TB | | | 0.56 (0.51–0.62) | |
| Yes | 2604 (83) | 3122 (64) | | |
| No | 833 (17) | 1773 (36) | | |
| ZN smear | | | 0.71 (0.62–0.81) | 0.69 (0.59–0.81) |
| Positive | 804 (57) | 1023 (49) | | |
| Negative | 597 (43) | 1071 (51) | | |
| Cluster | | | 0.80 (0.73–0.88) | 0.42 (0.35–0.49) |
| Yes | 1843 (54) | 2354 (48) | | |
| No | 1594 (46) | 2541 (52) | | |
| Beijing strain | | | 1.12 (0.92–1.35) | |
| Yes | 199 (5.8) | 314 (6.4) | | |
| No | 3238 (94.2) | 4581 (93.6) | | |
| Rifampicin-resistant | | | 6.85 (3.19–15.3) | 4.63 (1.06–20.0) |
| Yes | 8 (0.23) | 77 (1.6) | | |
| No | 3429 (99.8) | 4818 (98.4) | | |
| Isoniazid-resistant | | | 3.27 (2.63–4.06) | 1.66 (1.13–2.43) |
| Yes | 111 (3.2) | 481 (9.8) | | |
| No | 3326 (96.8) | 4414 (90) | | |
| Δ315 | | | 2.54 (1.62–3.98) | |
| Yes | 40 (36) | 283 (59) | | |
| No | 71 (64) | 198 (41) | | |
| Streptomycin-resistant | | | 3.26 (2.68–3.96) | 1.51 (1.10–2.09) |
| Yes | 139 (4) | 591 (12) | | |
| No | 3298 (96) | 4304 (88) | | |
| Multidrug-resistant | | | 8.06 (3.36–20.6) | |
| Yes | 6 (0.17) | 68 (1.4) | | |
| No | 3431 (99.8) | 4827 (98.6) | | |

TB, tuberculosis; ZN, Ziehl–Neelsen; Δ315, mutation at amino-acid position 315 of *katG*.

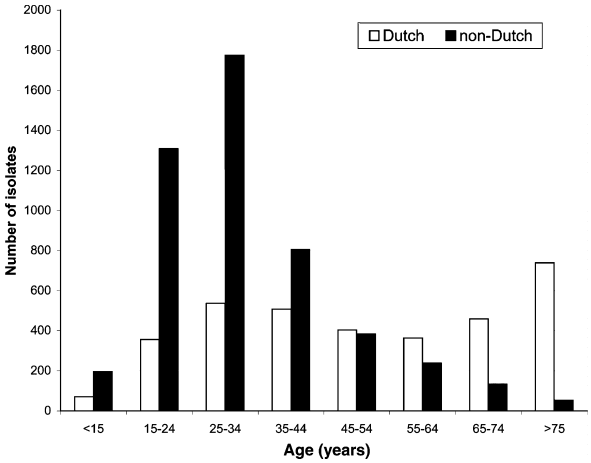


**Fig. 2.** Age group distribution for Dutch and non-Dutch tuberculosis patients.

95% CI 1.57–8.65) and other African patients (OR 2.45, not significant), but was absent among Moroccan and Asian patients (not significant). Whereas isoniazid-resistant isolates in general were less clustered than isoniazid-susceptible isolates, Δ315 isolates were clustered as frequently as isoniazid-susceptible isolates (Table 3). A possible relationship between this mutation and transmissibility was also suggested in an earlier study [7], but the differences in clustering were not significant. Among Dutch patients in the present study, increased clustering was also observed, but this was again not significant (Table 2). These three observations indicate that the Δ315 mutation may be an interesting target for diagnostics and further research.

Resistant isolates were reported shortly after the introduction of isoniazid in the 1950s. These isolates had a significantly reduced level of virulence in guinea-pigs [27], and were less likely to result in secondary cases (i.e., transmission) in population studies [28,29]. The ability of *M. tuberculosis* to persist in mice and guinea-pigs was shown to depend on the presence of *katG* or certain mutations therein [30]; isolates with a deleted or a mutated and dysfunctional *katG* that lacked catalase activity were less virulent and did not persist in laboratory animals. In contrast, Δ315 isoniazid-resistant isolates retained 30–40% of their original catalase activity after site-directed mutagenesis [19], and it has been shown in mice that isogenic *M. tuberculosis* strains with and

**Table 2.** Associations of isoniazid-sensitive and -resistant *Mycobacterium tuberculosis* isolates from Dutch and non-Dutch patients with clinical and laboratory variables

| | Dutch ($n$ = 3437) | | | | Non-Dutch ($n$ = 4895) | | | |
|---|---|---|---|---|---|---|---|---|
| Variable | INH-s ($n$ = 3326) $n$ (%) | INH-r ($n$ = 111) $n$(%) | Crude OR (95% CI) | Adjusted OR (95% CI) | INH-s ($n$ = 4414) $n$ (%) | INH-r ($n$ = 481) $n$(%) | Crude OR (95% CI) | Adjusted OR (95% CI) |
| Previous TB | | | 1.35 (0.48–3.13) | | | | 3.24 (2.15–4.86) | |
| Yes | 132 (4.4) | 6 (5.8) | | | 114 (3.0) | 36 (9.1) | | |
| No | 2882 (95.6) | 97 (94.2) | | | 3679 (97.0) | 359 (90.9) | | |
| Pulmonary TB | | | 1.05 (0.66–1.68) | | | | 1.19 (0.97–1.46) | |
| Yes | 2519 (76) | 85 (77) | | | 2798 (63) | 324 (67) | | |
| No | 807 (24) | 26 (23) | | | 1616 (37) | 157 (33) | | |
| ZN smear | | | 1.03 (0.56–1.89) | | | | 1.55 (1.15–2.09) | 1.47 (1.05–2.06) |
| Positive | 775 (57) | 29 (58) | | | 900 (48) | 123 (59) | | |
| Negative | 576 (43) | 21 (42) | | | 984 (52) | 87 (41) | | |
| Cluster | | | 1.23 (0.82–1.84) | | | | 0.82 (0.68–1.00) | |
| Yes | 1778 (53) | 65 (59) | | | 2144 (49) | 210 (44) | | |
| No | 1548 (47) | 46 (41) | | | 2270 (51) | 271 (56) | | |
| Beijing strain | | | 1.10 (0.43–2.39) | | | | 1.82 (1.31–2.53) | |
| Yes | 192 (5.8) | 7 (6.3) | | | 264 (6.0) | 50 (10) | | |
| No | 3134 (94.2) | 104 (93.7) | | | 4150 (94.0) | 431 (90) | | |
| RIF-resistant | | | 95.0 (16.6–966) | 94.7 (13.4–669) | | | 80.6 (38.6–174) | 78.7 (28.7–215) |
| Yes | 2 (0.06) | 6 (5.4) | | | 9 (0.2) | 68 (14) | | |
| No | 3324 (99.94) | 105 (94.6) | | | 4405 (99.8) | 413 (86) | | |
| STR-resistant | | | 24.6 (15.7–38.7) | 19.3 (12.3–30.3) | | | 12.5 (10.1–15.5) | 14.2 (10.1–20.1) |
| Yes | 93 (2.8) | 46 (41) | | | 344 (7.8) | 247 (51) | | |
| No | 3233 (97.2) | 65 (59) | | | 4070 (92.2) | 234 (49) | | |

TB, tuberculosis; ZN, Ziehl-Neelsen; RIF, rifampicin; INH-s, isoniazid-sensitive; INH-r, isoniazid-resistant; STR, streptomycin.

**Table 3.** Associations of isoniazid-resistant *Mycobacterium tuberculosis* isolates, with and without a mutation at amino-acid 315 of *katG*, from Dutch and non-Dutch patients with clinical and laboratory variables

| | Dutch INH-r | | | | Non-Dutch | | | |
|---|---|---|---|---|---|---|---|---|
| Variable | Δ315 ($n$ = 40) $n$ | Wild-type ($n$ = 71) $n$ | Crude OR (95% CI) | Adjusted OR (95% CI) | Δ315 ($n$ = 283) $n$ (%) | Wild-type ($n$ = 198) $n$ (%) | Crude OR (95% CI) | Adjusted OR (95% CI) |
| Previous TB | | | 0.35 (0.01–3.38) | | | | 1.04 (0.49–2.22) | |
| Yes | 1 | 5 | | | 22 (9.2) | 14 (8.9) | | |
| No | 35 | 62 | | | 216 (90.8) | 143 (91.1) | | |
| Pulmonary TB | | | 4.04 (1.21–17.3) | 4.81 (1.46–15.9) | | | 1.10 (0.73–1.64) | |
| Yes | 36 | 49 | | | 193 (68) | 131 (67) | | |
| No | 4 | 22 | | | 90 (32) | 67 (33) | | |
| ZN smear | | | 0.78 (0.21–2.79) | | | | 1.10 (0.61–2.01) | |
| Positive | 12 | 17 | | | 75 (60) | 48 (57) | | |
| Negative | 10 | 11 | | | 51 (40) | 36 (43) | | |
| Cluster | | | 1.29 (0.54–3.09) | | | | 1.67 (1.13–2.46) | 1.71 (1.15–2.54) |
| Yes | 25 | 40 | | | 138 (49) | 72 (36) | | |
| No | 15 | 31 | | | 145 (51) | 126 (64) | | |
| Beijing strain | | | 2.52 (0.40–18.0) | | | | 1.55 (0.80–3.04) | |
| Yes | 4 | 3 | | | 34 (12) | 16 (8.1) | | |
| No | 36 | 68 | | | 249 (88) | 182 (91.9) | | |
| RIF-resistant/ multidrug-resistant | | | 3.83 (0.51–43.7) | | | | 1.97 (1.09–3.61) | 1.92 (1.05–3.50) |
| Yes | 4 | 2 | | | 49 (17) | 19 (9.6) | | |
| No | 36 | 69 | | | 234 (83) | 179 (90.4) | | |
| STR-resistant | | | 2.82 (1.18–6.82) | 3.26 (1.40–7.56) | | | 4.31 (2.87–6.48) | 4.27 (2.88–6.33) |
| Yes | 23 | 23 | | | 186 (66) | 61 (31) | | |
| No | 17 | 48 | | | 97 (34) | 137 (69) | | |

TB, tuberculosis; ZN, Ziehl-Neelsen; RIF, rifampicin; INH-r, isoniazid-resistant; Δ315, mutation at amino-acid 315 of *katG*; STR, streptomycin.

without the Δ315 mutation are equally virulent and persistent [31]. The Δ315 mutation appears to interfere less with the catalase and peroxidase activities of the enzyme than with the isoniazid-converting activity. The combination of a residual catalase activity and isoniazid resistance might explain the success of Δ315 isolates. This hypothesis is supported further by the reduced transmissibility of non-Δ315 isoniazid-resistant isolates and the maintained transmissibility of Δ315 isolates detected in the present, much extended study.

Although increased clustering among non-Dutch patients could also be caused by (e.g.) the

prevalence of clustered strains in the countries of origin, the duration of stay in The Netherlands, or the number of people per household, the presence of a biological explanation and the findings of the present study suggest that a causative relationship between the Δ315 mutation and increased clustering among isoniazid-resistant isolates is plausible. The use of a shorter period of 2–3 years to define clusters would have allowed a more specific definition of recent transmission, but could not be used with the data that were available for analysis.

Interestingly, a study in Spain, in which 180 MDR isolates were characterised, revealed that the Δ315 mutation was present in only 25% of clustered MDR isolates. No data were available for the non-clustered isolates. In studies in eastern Europe or southeast Asia with MDR isolates, the percentage of Δ315 isolates is usually very high [32,33]. These differences might be explained by the lower percentage of immigrants with TB in Spain (i.e., a higher prevalence of Δ315 among immigrants in The Netherlands), and by the presence of an indigenous MDR strain without the Δ315 mutation that is responsible for 29% of clustered MDR cases [34].

The acquisition of resistance to anti-TB drugs by mycobacteria is a stepwise process in which resistant bacteria have an advantage over non-resistant bacteria in gaining additional resistance. In the present study, isoniazid-resistant isolates were more likely to be streptomycin- or rifampicin-resistant, and vice versa (data not shown). An explanation for the observation that Δ315 isolates were more likely to be co-resistant to streptomycin, or to be MDR, than other isoniazid-resistant isolates may be that mono-resistant unattenuated mycobacteria (Δ315 isolates) are, under sub-optimal therapy, more likely to persist and to become resistant to other agents.

An alternative explanation could be that the Δ315 mutation and the additional resistance are both caused by a common underlying mechanism. One such mechanism might be an increased tendency to mutation, e.g., caused by a defect in the DNA repair machinery, as has been described for the hyper-virulent Beijing lineage [35]. The observation that certain mutations in *rpoB* that are associated with resistance to rifampicin are much more frequent among Δ315 isolates than among other rifampicin-resistant isolates (data not shown) might also be indicative of the presence of a common underlying mechanism.

In conclusion, the present work suggests that screening of mycobacteria for the presence of the Δ315 mutation may be a useful tool in developed countries, both for therapy guidance for individual patients, and for the surveillance of resistance. The question as to whether the therapy of individual patients should be adjusted immediately after detection of the Δ315 mutation in *M. tuberculosis* (preferably directly in clinical material) remains to be answered.

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