Mixed tuberculosis infections in rural South Vietnam

Running title: Mixed tuberculosis infections

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ABSTRACT

Tuberculosis patients may be infected or diseased by more than one *Mycobacterium tuberculosis* strain, usually referred to as ‘mixed infections’. These have mainly been observed in settings with a very high tuberculosis incidence and/or high HIV prevalence. We assessed the rate of mixed infections in a population-based study in rural Vietnam, where the prevalence of both HIV and tuberculosis are substantially lower.

In total, 1248 *M. tuberculosis* isolates from the same number of patients were subjected to IS6110 restriction fragment length polymorphism (RFLP), spoligotyping and variable numbers of tandem repeats (VNTR) typing. We compared mixed infections identified by 1) discrepant RFLP and spoligotype patterns in isolates from the same patient; 2) double alleles at ≥2 loci by VNTR typing, and assessed epidemiological characteristics of these infections.

RFLP/spoligotyping and VNTR identified 39 (3.1%) and 60 (4.8%) mixed infections respectively (Cohen’s Kappa statistic, 0.57). The number of loci with double alleles in the VNTR pattern was strongly associated with the proportion of isolates with mixed infections according to RFLP/spoligotyping (p<0.001). Mixed infections occurred more frequently in new than in re-treatment patients, were significantly associated with minor X-ray abnormalities and almost significantly associated with lower sputum smear grades.

Although the infection pressure in our area is lower than in previously studied populations, mixed *M. tuberculosis* infections do occur in rural South Vietnam in at least 3.1% of cases.
For a long time it was assumed that a tuberculosis (TB) infection protects against a subsequent infection. In fact, vaccination against infectious diseases is based on this principle. However, in 1976 there were already anecdotal indications that TB patients can be re-infected by another Mycobacterium tuberculosis strain and that infections with multiple strains exist. Using phage typing, Bates et al. (2) found different phage types of M. tuberculosis in single hosts. The occurrence of infections with multiple M. tuberculosis strains was confirmed by using DNA fingerprinting techniques, first at the turn of the century in selected patients by Yeh et al. (25) and by Braden et al. (3), and more recently in larger patient populations (14, 15, 22). The introduction of molecular techniques offered new possibilities for studying the natural history of TB infection more extensively. PCR assays targeting particular predominant M. tuberculosis genotype families were developed and by applying these methods to clinical material Warren et al. found that the rate of “mixed infections”, i.e. infections with multiple M. tuberculosis strains, amounted to 19% of examined patients in South Africa (22).

High rates of mixed infections have been found in populations living in crowded conditions, including a high-density urban community (22) and a hospital (7) in South Africa and a prison in Georgia (15). However, the frequency of mixed infections in human populations with a lower tuberculosis infection pressure (e.g. populations in less crowded conditions and with a lower HIV-prevalence) is unknown. Although the burden of TB is high in South Vietnam, with a prevalence of smear-positive TB of 219/100,000 (95% CI 145–294) (9), it is much lower than the prevalence in the studied areas in South Africa (1000/100,000) (22). Similarly, the prevalence of HIV among TB patients is lower in Vietnam than in South Africa (8.2% vs 50-80%) (18) (http://www.mediaclubsouthafrica.com/index.php?hivaids...south-africa.).

We studied the occurrence of mixed infections in a population-based study in a rural area in South Vietnam using IS6110 restriction fragment length polymorphism (RFLP) typing and spoligotyping (11, 20). The former method is used to distinguish M. tuberculosis isolates on strain level to study patient-to-patient transmission but also enables determination of the genotype to which the M. tuberculosis strain belongs, while the latter method can only be used for genotype determination (4). It is known from previous studies that about 35% of the M. tuberculosis isolates from South Vietnam are of the Beijing genotype, while about 49% of the isolates represent the East African Indian (EAI) genotype (previous known as the Vietnam genotype) (1, 5). The predominance of these two M. tuberculosis genotype families in South
Vietnam and their highly characteristic IS6110 RFLP and spoligotype patterns enabled us to detect possible mixed infections of strains of these different genotypes in a reliable way by comparing the results of both typing methods. In addition, mixed infections were detected by the visualization of double alleles in variable numbers of tandem repeats (VNTR) typing patterns (13, 15). In this study we were able to compare the sensitivity of the various typing approaches to detect mixed infections however, because much is unknown about the evolution of VNTR patterns, we used RFLP/spoligotype results to quantify the occurrence of mixed infections and investigate possible risk factors for mixed infections. This is the first report of mixed infections in rural Vietnam, where the population density and HIV infection prevalence is low and TB incidence is moderate, in contrast to settings in previous studies looking at mixed infections.
MATERIALS AND METHODS

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Patient population
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The study area consisted of three adjacent rural districts in Tien Giang Province, situated in the
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Mekong River Delta in Southern Vietnam. All patients aged ≥15 years, resident in the study
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area and registered for treatment of smear-positive pulmonary TB between 1 January 2003 and
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31 December 2005 at the participating District Tuberculosis Units, or at the provincial TB
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hospital, were eligible for inclusion in the study (10). By interviews using pre-structured
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questionnaires, we collected data on sex, age, BCG vaccination, X-ray abnormalities,
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educational level, marital status, occupation and previous history of treatment of all participants.
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HIV testing was not done routinely. Treatment outcomes were based on routine smear
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examination (24) described by Buu et al. (5). Buu et al. previously found that tuberculosis is
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usually transmitted outside the household in the study area (6), therefore, epidemiological links
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between patients were not assessed in in-depth interviews for the purpose of this study.

Mycobacterial isolates
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Sputum specimens were kept refrigerated and were transported to Pham Ngoc Thach Hospital
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in Ho Chi Minh City within 72 hrs after collection. Specimens were decontaminated and
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liquefied with 1% N-acetylcysteine, 2% NaOH, inoculated on modified Ogawa medium and
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incubated at 37°C. Cultures were examined for growth after 1, 2, 4, 6 and 8 weeks of
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incubation. Cultures with no growth after 8 weeks were reported as negative. M. tuberculosis
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was identified using the niacin and nitrate tests (10).

Drug susceptibility testing
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Drug susceptibility testing was performed by the proportion method following the guidelines of
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the World Health Organization and the International Union against Tuberculosis and Lung
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Disease (23). Criteria for drug resistance were ≥ 1% colony growth (10) at 28 or 40 days
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compared to the drug-free control medium at the following drug concentrations: isoniazid 0.2
136
μg/ml, rifampicin 40 μg/ml, streptomycin 4 μg/ml and ethambutol 2 μg/ml. Multidrug
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resistance (MDR) was defined as resistance to both rifampicin and isoniazid.

DNA typing
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We included all 1248 patients (66% of the eligible population) with isolates on which all 3 typing
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methods were applied (RFLP, spoligo and VNTR typing). Genomic DNA was extracted from
positive cultures by using an earlier described method (21). IS6110 RFLP typing and spoligotyping were performed according to the internationally standardized methods (11, 20) VNTR typing was done using 15 loci, as described by Supply et al. (17).

**Definition of mixed infections by RFLP, spoligo and VNTR typing**

All isolates that yielded discrepant results with regard to *M. tuberculosis* genotype family in RFLP and spoligotyping were subjected to both typing methods for a second time from the same DNA to ensure the reproducibility of the observation. Beijing and EAI genotypes were assigned as reported elsewhere based on IS6110 RFLP and spoligotyping (1, 4, 12). Isolates that repeatedly had a spoligotype characteristic of the EAI genotype and an IS6110 RFLP pattern characteristic of a Beijing genotype strain (12), or the other way around, or a spoligotype characteristic of the Beijing or EAI genotype and an IS6110 RFLP pattern characteristic of another genotype, or the other way around, were considered to represent a mixed infection.

VNTR typing has been shown to be sensitive in the detection of mixed infections by revealing double alleles, with mixed infections defined as double alleles in two or more VNTR loci (13, 15). Because mixtures of two strains of the same genotype are virtually impossible to detect by using only RFLP or spoligotyping, we used the VNTR typing results to check the number of potentially missed mixed infections. We defined occurrence of double alleles as at least two VNTR loci as potential mixed infections. But this definition was not used for final identification of mixed infections because at present, the sensitivity of VNTR typing for the detection of mixed infections is unknown and double alleles could also represent evolution of the bacterium. This is the reason why we did not report the mixed infection rate according to VNTR, but we typed all 1248 isolates by VNTR, RFLP and spoligotyping to compare their sensitivity for the detection of mixed *M. tuberculosis* infections as a first step for the studies in the future. Furthermore, VNTR was done since the data were also collected for other studies: to present an overview of genotypes in this part of Vietnam and to check for relapse vs new infections.

**Re-culture of sputum for analysis of single colonies**

We repeated RFLP and spoligotyping twice for all 39 mixed infections from their DNA (extracted from cultures) and we got the same results. Ideally, to exclude mixed infections occurred due to cross-contamination during culture processing, studies on mixed infections should be performed on original sputum samples. For two of the assumed mixed infection isolates we tried to confirm the observation, and exclude laboratory cross-contamination, by
using a bacteriological approach. Pre-treated sputum specimens, stored at -20°C were re-
cultured on 7H10 agar plates to grow single colonies. Spoligotyping was applied to 5-6 single
colonies of each of both isolates.

Data analysis
The Gene Marker software, version 1.5 (Softgenetics, PA, USA), was used for analysis and
automated allele calling of the VNTR patterns. The Bionumerics software, version 3.0 (Applied
Maths, Sint-Martens Latem, Belgium), was used for the analysis and comparison of IS6110
RFLP, spoligo and VNTR patterns. Data were entered in Epi Info version 6.04 (Centers for
Disease Control and Prevention, Atlanta, GA, USA). Double entry was done on a 20% random
sample (50% in 2003) of all records. Discrepancies were observed in <1% of all records, and in
<0.05% of all fields. Analyses were performed in Epi Info version 6.04 and Stata version 10SE
(Stata Corporation, College Station, TX, USA).

For comparison of categorical variables we used the chi-squared and 2-sided Fisher’s exact tests
as appropriate, with trends across ordered categories assessed by Cuzick’s test for trend. Results
were considered significant at P< 0.05. Associations between mixed infections and explanatory
variables were expressed as odds ratios; confounding effects were investigated by stratified
analysis using the Mantel-Haenszel test.
RESULTS

During the period January 2003 – December 2005, 1890 patients were eligible for inclusion, of whom 1248 (66%) patients whose isolates had complete IS6110 RFLP, spoligo and VNTR typing results were available for the analyses. Of these, 931 (74.6%) isolates were from male patients and 317 (25.4%) from female patients with a median age of 50 years (25th and 75th percentile, respectively, 37 and 66 years). Eleven hundred and seven (88.7%) were new patients, 139 (11.1%) were relapse patients, and the remaining two cases were of unknown status.

Identification of mixed infections identified by IS6110 RFLP, spoligotyping and VNTR typing

Thirty-nine (3.1%) of 1248 isolates had RFLP and spoligotype patterns that represented different Mycobacterium tuberculosis genotype families and were considered to be mixed infections. Repeated RFLP and spoligotype analysis from the same DNA confirmed the mixed infections for all 39 isolates. Twenty-eight isolates (71.8%) represented a mixture of Beijing and EAI strains, five isolates (12.8%) were a mixture of a Beijing strain and a strain of another genotype (not EAI), five isolates (12.8%) were a mixture of an EAI strain and a strain of another genotype (containing more than 4 IS6110 copies in RFLP and not belonging to the Beijing genotype), and one (2.6%) was a mixture of a Haarlem and another strain (Figure 1). Overall, the study population, including the mixed infection isolates, contained 549 (42.7%) EAI strains, 461 (35.8%) Beijing strains, and 277 (21.5%) strains of other genotypes. These rates of EAI and Beijing genotypes of M. tuberculosis were similar to those reported previously for the prevalence of these genotypes in rural areas of South Vietnam (49% for EAI and 35% for Beijing respectively (5)), but the prevalence of the Beijing genotype was lower than that observed in Hanoi and Ho Chi Minh City (1).

VNTR typing detected 122 (9.8%) of 1248 isolates with double alleles at at least one locus and 60 (4.8%) of these isolates revealed double alleles in two or more loci (Table 1). Of these 60 strains, 31 (51.7%) isolates had not been identified as being mixed infections by combining the results of RFLP and spoligotyping and 29 (48.3%) isolates represented mixed infections confirmed by RFLP and spoligotyping. Thus, of the 39 mixed infection isolates detected by RFLP and spoligotyping, 29 (74.4%) were confirmed by VNTR typing (Table 1). Comparing the RFLP/spoligotyping and VNTR results of the isolates revealed that the percentage of mixed
infections detected by RFLP and spoligotyping of isolates strongly increased with the number of loci at which double alleles were found (p<0.001, Figure 2). While only 3.2% (2/62) of isolates contained mixed infections (on basis of RFLP/spoligotyping) when only a single VNTR locus had double alleles, this was more than 80% for isolates that had double alleles in five more VNTR loci. Among isolates that had two, three or four loci with double alleles, the proportion of mixed infections was 12.5%, 20.0% and 44.4%, respectively. Of 1126 strains having single alleles, 0.7% (8/1126) were mixed infections according to combined RFLP and spoligotyping analysis (Table1). The agreement between the two definitions we used in this study (discrepant genotype results between RFLP and spoligotyping, and double alleles at at least 2 loci with VNTR typing) was 96.7% (Table 1) and Cohen’s Kappa statistic was 0.57. Of the 60 isolates that had two or more double alleles in VNTR typing, 29 represented a mixed infection based on the combination of the RFLP and spoligotype results; 25 of these were a mixture of Beijing and EAI genotypes, three were a mixture of EAI and another genotype and one was a mixture of a Beijing and another genotype. For 31 of the 60 isolates that had two or more double alleles in VNTR typing no mixed infection was detected with RFLP and spoligotyping.

Re-culturing of mixed infection isolates

Since identification of mixed infection by culture was not the primary purpose of this study, no attention was given to the phenotypic nature of the cultures. Only after we discovered several samples to contain mixed infections, we decided to re-culture these. Unfortunately, enough material to re-culture the sputum sample was available for only of two samples. For two of the assumed mixed infections of which there original sputum specimens were still available, pre-treated sputum specimens were re-cultured directly on 7H10 agar plates and spoligotyping was applied to 5-6 single colonies of each these two isolates. For one specimen, three colonies yielded Beijing specific spoligotype patterns, while two others colonies revealed an EAI spoligotype. For the other specimen only Beijing spoligotypes were obtained. Thus a mixed infection was confirmed microbiologically for one of two isolates.

In terms of microbiology, we observed that the colonies of Beijing genotype isolates were often smooth and large, with a diameter of more than 2 mm, while the colonies of EAI genotype strains were often dry and small, with diameter of less 1 mm.
Epidemiological and clinical characteristics of mixed infections

There were no significant associations between the probability of having a mixed infection and the district of residence, age, sex, treatment history, treatment delay, presence of systemic symptoms or treatment outcome, nor with the isolates’ resistance to isoniazid or streptomycin, or multi-drug resistance (Table 2). However, mixed infections were significantly less likely to occur in patients with extensive X-ray abnormalities (p<0.001) and there was a near-to-significant trend for lower sputum smear grades among mixed infections (p=0.054), both suggesting less extensive pathology in patients with mixed infections. Taking medium severity of X-ray abnormalities as the reference, the odds of mixed infection was 2.52 (95% confidence interval (CI) 1.08-5.87) times higher for minor X-ray abnormalities, and 0.42 (95% CI 0.19-0.92) times lower for major X-ray abnormalities. For sputum smear grading, taking a 1+ grade as the baseline, the odds of mixed infection was not higher for negative or scanty smears (1.10, 95% CI 0.48-2.52) but 0.48 times lower for 2+ or 3+ smears (95% CI 0.22-1.07). In stratified analyses, the odds ratios for the association of mixed infection with either severity of X-ray abnormalities or sputum smear grade were not affected by any of the variables in Table 2. Neither were X-ray abnormalities or sputum smear grade clearly associated with a specific genotype (EIA, Beijing or other) among the single infections (data not shown).

Based on demographic information, epidemiological links between patients with mixed infections were considered highly unlikely. Patients with mixed infections were distributed equally over the three different provinces, lived in different communities, and were from different sex, age classes and socio economic classes and had different types of jobs and did not occur within the same household (data not shown).
Our study shows that mixed tuberculosis infections occur also outside settings with extremely high TB incidence and population densities. The proportion of mixed tuberculosis infections found in rural South Vietnam in this study was 3.1% when identified by combined RFLP and spoligotyping results and 4.8% when identified by ≥2 loci with double alleles in VNTR typing, which is similar to that reported from South Africa by Richardson et al. (2.3%) (14), but lower than that found in Georgia (13.1%) or in South Africa by Warren et al. (19%) and Cohen et al. (9%) (7, 15, 22). These differences in the proportion of mixed infections could be explained by differences in the methodology of detection. The study from Georgia defined mixed infections based on RFLP as well as VNTR typing (15). If we would have used a definition for mixed infections adding RFLP/spoligotyping and VNTR results, our study would have identified (39+31)/1248 = 5.6% mixed infections. Moreover, a number of mixed infections in the Georgian study were only detected after typing multiple specimens from a single patient, and the study by Cohen et al. in South Africa focused on pooled multiple samples from autopsy patients, while we typed only one specimen per patient (7, 15). The study in South Africa by Warren et al. (22) used a methodology based on PCR probes distinguishing Beijing from non-Beijing strains which is more sensitive for identifying mixed infections than the RFLP typing-based method applied by Richardson et al. that we also used (14). In addition, differences in density of human populations and risk of TB infection are likely to have played a role. The study in Georgia took place in a crowded prison with a TB incidence of 5995/100,000, the study by Warren et al. was performed in an urban area with a TB incidence as high as 1000/100,000 (22), and the study by Cohen et al. in a highly HIV infected hospital population (7). In these settings, cross-infection could easily occur. In contrast, our study was performed in a rural area in Vietnam with a population density of only 837/km² and an observed TB incidence of new smear positive cases of 100/100,000 in 2005 (National Tuberculosis Program Vietnam, unpublished data), and cross-infection may be expected to be less common under these circumstances. Finally, HIV infection may increase the potential for acquiring mixed TB infections (16) and the prevalence of HIV infection among TB patients was higher in the South African studies (at least 10% (22) and 94% (7)) than in ours.

To identify mixed infections in our study we used RFLP and spoligotyping, in which strains of the EAI and Beijing genotype can be recognized easily. It is likely that mixed infections between a Beijing and an EAI strain are easier to detect by a Beijing RFLP and a EAI
spoligotype than the other way around because the Beijing RFLP pattern can mask the EAI RFLP and the EAI spoligotype can mask the Beijing spoligotype. Therefore the combination of methods is necessary to detect the mixture. The Beijing RFLP and EAI spoligotype type of mixture is also the most frequent mixture that we detected in this study. We did not find any mixtures of the reverse type; an EAI RFLP and a Beijing spoligo. This is because an EAI strain can only be visualized by RFLP when the Beijing strain is present in a very low concentration compared to the EAI strain in a sample. In such a case, in spoligotyping the EAI strain will also be amplified and visible in the spoligo pattern. The spoligotype of the Beijing strain that would be present in the sample would be masked by the EAI spoligotype. RFLP and VNTR typing have been used before to detect mixed infections by Shamputa et al. (15). Our study showed that RFLP and spoligotyping can also be used for detection of mixed infections.

A disadvantage of our approach is that we probably underestimated the true rate of mixed infections in Vietnam. The fact that we detected 60 strains with double alleles in at least two VNTR loci suggests that VNTR typing is a more sensitive method to detect mixed infections than RFLP/spoligotyping. Neither RFLP nor spoligotyping can independently detect a mixture of two strains of the same genotype very efficiently (8), whereas VNTR typing results can show double alleles at chromosomal loci and thus in principle also detect multiple strains of the same genotype. On the other hand, 8/39 (21%) of mixed infections were missed by VNTR (Table 1). We found that the more loci with double alleles an isolate showed in VNTR typing, the higher the probability was that these were also identified as mixtures by combined RFLP and spoligotyping (Figure 2). At present, the sensitivity of VNTR typing for the detection of mixed infections is unknown and double alleles could also represent evolution of the bacterium (13), and therefore we did not define the mixed infection rate according to VNTR results, but we type all 1248 isolates by VNTR, RFLP and spoligotyping to compare sensitivity of these methods to detect mixed M. tuberculosis infections as a first step for the studies in the future.

With 100% of the cases being cured, we do not have an indication that mixed infections lead to higher failure rates. In fact, they associated with less extensive pulmonary pathology than single infections. Although this is based on routine chest X-ray results that were not standardized as part of our study, we found this association across all three districts, suggesting that it does not reflect observer bias. Moreover, we found a similar pattern with regard to sputum smear grading (which had been done centrally); mixed infections were associated with a lower degree of smear positivity. Interestingly, neither of these associations was determined by underlying differences
in duration of cough or presence of systemic symptoms such as fever, night sweats and weight loss. This suggests that the observed associations do not reflect early diagnosis (i.e. with still limited pulmonary pathology), for example because of a higher incidence of systemic symptoms with mixed than with single infections. This finding requires further studies; one hypothesis could be that patients with multiple infections have an increased immunological tolerance to *M. tuberculosis* infections. This may be due to HIV infection, for which we did not test. However, other causes are likely to play a role as well, since mixed infections occurred equally among both sexes and all age groups, while HIV infection among TB patients in Vietnam is strongly associated with young age (<35 years) and male sex (18). It is also interesting that mixed infection cases were not related to a history of TB treatment, as they occurred in 3.4% of the new patients, compared to 0.72% of the patients with recurrent TB. This finding was in agreement with the report from Georgia (15). In contrast, Warren et al. (22) found that multiple infections were more frequent in relapse cases.

Although we cannot completely exclude that (part of the) mixed infections detected in our study were the result of errors or cross-contamination in the laboratory, various observations support our finding. The mixed infections were found in all three districts, with the highest frequency of mixed infections in the district with the highest TB rate, and at different points in time. Furthermore, repeated analysis with all three DNA typing methods invariably confirmed the initially obtained results. Finally, to check the possibility of cross-contamination, we re-cultured single colonies of the only two sputum specimens that were still available and could confirm mixed bacterial populations in one of these two with typing of only a very limited number of single colonies. This suggests that the observed discordant results between RFLP and spoligotyping results indeed represented mixed infections.

There were some limitations to our study. First, we did not perform HIV testing, so we could not study the relationship between HIV and mixed infections, although the HIV prevalence is still very low in rural Vietnam with 0.5% (19). Second, we did not store all sputum samples for re-culture to re-check for mixed infections. Third, the ability of RFLP and spoligotyping to detect mixtures of strains with the same genotype is limited. Fourth, VNTR typing is thought to be more sensitive than RFLP and spoligotyping to detect mixed infections but the exact sensitivity of VNTR typing for the detection of mixed infections is unknown. Finally, the number of mixed infections we identified was relatively small, thereby limiting the power of our study to detect significant associations with potential risk factors.
We believe that the observed 3.1% of mixed infections in the rural part of South Vietnam represents a minimum estimate, as the explored approach by combining RFLP and spoligotyping has a restricted detection limit. Most likely, due to the visual aspect involved in this approach many more mixed infections that have more uneven ratio’s between the number of bacteria of the respective strains will go unnoticed. With the application of VNTR typing, which may be more sensitive in the detection of mixed infection, the true magnitude of this phenomenon may be unravelled in the future.
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REFERENCES


Table 1: Comparison of the sensitivity of detection of mixed *M. tuberculosis* infections by VNTR typing and by combined typing with IS6110 RFLP and spoligotyping.

<table>
<thead>
<tr>
<th>Combined VNTR typing</th>
<th>15 loci VNTR typing</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>IS6110 RFLP and spoligotyping</td>
<td>Double alleles in two or more loci</td>
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<tr>
<td>MIXED INFECTION</td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>29</td>
<td>2</td>
</tr>
<tr>
<td>No</td>
<td>31</td>
<td>60</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>62</td>
</tr>
</tbody>
</table>
Table 2: Patient and TB disease characteristics for patients with mixed *M. tuberculosis* infections.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Mixed infections</th>
<th>P value*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td></td>
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<tr>
<td><strong>TOTAL</strong></td>
<td>1248</td>
<td>39 (3.1)</td>
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<td><strong>DISTRICT</strong></td>
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<tr>
<td>Cai Lay</td>
<td>575</td>
<td>22 (3.8)</td>
<td>0.299</td>
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<tr>
<td>Chau Thanh</td>
<td>392</td>
<td>8 (2.0)</td>
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<tr>
<td>Cai Be</td>
<td>281</td>
<td>9 (3.2)</td>
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<td><strong>SEX</strong></td>
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<tr>
<td>Male</td>
<td>931</td>
<td>29 (3.1)</td>
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</tr>
<tr>
<td>Female</td>
<td>317</td>
<td>10 (3.2)</td>
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<td><strong>AGE (YEARS)</strong></td>
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<tr>
<td>15-34</td>
<td>246</td>
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<td>657</td>
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<td>&gt;= 65</td>
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<td><strong>TREATMENT HISTORY</strong></td>
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<tr>
<td>New</td>
<td>1107</td>
<td>38 (3.4)</td>
<td>0.17</td>
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<tr>
<td>Previously treated</td>
<td>139</td>
<td>1 (0.7)</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>2</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><strong>TREATMENT OUTCOME</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favourable</td>
<td>1226</td>
<td>39 (3.2)</td>
<td>1.000</td>
</tr>
<tr>
<td>Unfavourable</td>
<td>23</td>
<td>0 (0)</td>
<td></td>
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<tr>
<td><strong>ABNORMALITIES ON X-RAY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minor</td>
<td>90</td>
<td>8 (8.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>Medium</td>
<td>590</td>
<td>22 (3.7)</td>
<td></td>
</tr>
<tr>
<td>Major</td>
<td>562</td>
<td>9 (1.6)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>6</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><strong>SMEAR GRADE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative or scanty</td>
<td>194</td>
<td>8 (4.1)</td>
<td>0.117</td>
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</table>

*Bold indicates statistical significance.*
<table>
<thead>
<tr>
<th></th>
<th>1+</th>
<th>2-3+</th>
<th>Missing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>557</td>
<td>484</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>21 (3.8)</td>
<td>9 (1.9)</td>
<td>1 (7.7)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Duration of Cough</th>
<th>&lt;4 weeks</th>
<th>4-7 weeks</th>
<th>≥8 weeks</th>
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<tbody>
<tr>
<td></td>
<td>324</td>
<td>469</td>
<td>455</td>
</tr>
<tr>
<td></td>
<td>10 (3.1)</td>
<td>14 (3.0)</td>
<td>15 (3.3)</td>
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<table>
<thead>
<tr>
<th>Fever</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1108</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>36 (3.2)</td>
<td>3 (2.1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Night Sweats</th>
<th>Present</th>
<th>Absent</th>
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<tr>
<td></td>
<td>748</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>27 (3.6)</td>
<td>12 (2.4)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Weight Loss</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1223</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>38 (3.1)</td>
<td>1 (4.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isoniazid</th>
<th>Resistant</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>246</td>
<td>1002</td>
</tr>
<tr>
<td></td>
<td>8 (3.3)</td>
<td>31 (3.1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Streptomycin</th>
<th>Resistant</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>341</td>
<td>907</td>
</tr>
<tr>
<td></td>
<td>15 (4.4)</td>
<td>24 (2.6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Multidrug Resistant</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>1198</td>
</tr>
<tr>
<td></td>
<td>3 (6.0)</td>
<td>36 (3.0)</td>
</tr>
</tbody>
</table>

Notes: Calculated by Fisher's exact test; \(^a\) the results in the reference lab; maximum grading of two smear examinations; \(^b\) Cuzick's test for trend: \(p=0.054\).
Figure 1: Mixed infections detected by IS6110 RFLP and spoligotyping. Spoligotype and IS6110 RFLP patterns of the M. tuberculosis isolates that were identified as mixed infections on the basis of discordant genotyping results. The dendrogram (shown on the left) shows the similarity of the spoligotype patterns, as determined by using the Dice coefficient and UPGMA for clustering. To the right of the IS6110 RFLP patterns the interpreted genotype families are indicated, as determined by spoligotyping and IS6110 RFLP typing, respectively.
Figure 2. Correlation between the heterogeneity in VNTR patterns and the detection of mixed infections by combined IS6110 RFLP and spoligotyping among 1248 M. tuberculosis isolates. The percentage of mixed infections by (IS6110 RFLP /Spoligotyping) is indicated for isolates showing VNTR patterns with no double alleles (n=1126), a double allele at 1 VNTR locus (n=62), or double alleles at 2 to 9 different VNTR loci (n=60).

Mixed Infection (%)

Number of VNTR loci showing double alleles

0 1 2 3 4 5 6 7 8 9
0 10 20 30 40 50 60 70 80 90 100
mixed infection
single infection

0.7 3.2 12.5 20 44.4 33.3 90.9 66.7 100 100
99.3 96.8 87.5 80 55.6 16.7 9.1 33.3 100 100

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