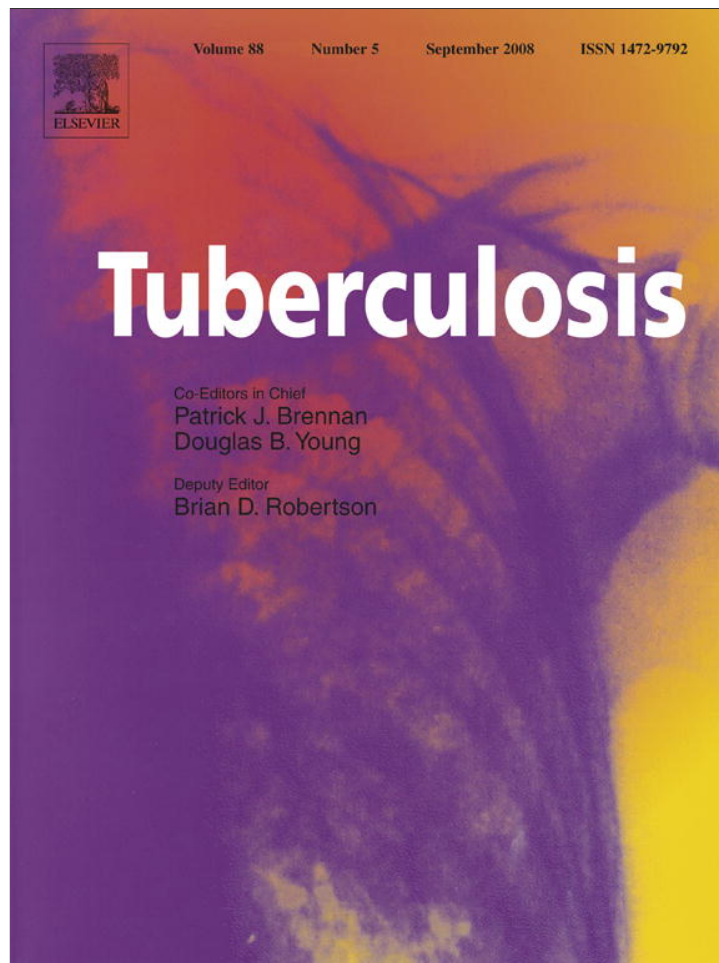


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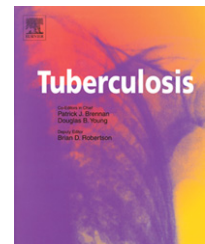
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Mixed infections of *Mycobacterium tuberculosis* in tuberculosis patients in Shanghai, China

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Summary

We applied a 7 loci Variable-Number-Tandem-Repeats (VNTR-7) analysis method to identify mixed infections of *Mycobacterium tuberculosis* and to estimate the rate of mixed infections among pulmonary tuberculosis patients in Shanghai, China. We validated the VNTR-7 method and used it to genotype an isolate from each of the 249 from pulmonary tuberculosis patients reported from the Songjiang and Chongming districts in Shanghai during 2006. We identified 14 patients with mixed infections, and the estimated rate of mixed infections was 5.6% (14/249) (95% CI 3.1%–9.2%). Mixed infections were observed more frequently among tuberculosis patients undergoing retreatment (15.6%) than among new cases (4.1%) ($p < 0.05$), and among tuberculosis patients whose disease was caused by non-Beijing genotype strains (12.5%) versus Beijing genotype strains (3.5%) ($p < 0.05$). The VNTR-7 method is a highly sensitive, practical tool with relatively high discriminatory power, making it useful for studying mixed infections. © 2008 Elsevier Ltd. All rights reserved.

Introduction

For many years, it was believed that tuberculosis is caused by infection with a single strain of *Mycobacterium tuberculosis*.¹ Recently, molecular epidemiologic research has shown that *M. tuberculosis* infection is more complicated than previously thought. For example, exogenous reinfection can occur,

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especially in high incidence populations and poor control programs.²⁻⁴ Infection with multiple strains of *M. tuberculosis*, distinguishable by their different genotypes, within the same patient before, during or after successful treatment is defined as a mixed infection. Mixed infections were studied by different methods, and found that mixed infections occur in different geographic settings, among both HIV positive and negative patients.⁵⁻¹¹ Mixed infections are one of the mechanisms that can explain the change in drug resistance patterns during the same treatment episode or during retreatment,^{12,13} but the frequency of mixed infections is often unknown and needs further study.

Information about the number and rate of mixed infections are important for several reasons. First, a high frequency of mixed infections could indicate there is a lot of transmission of *M. tuberculosis*. Mixed infections could explain conflicting laboratory drug susceptibility test results on specimens from the same patient. In clinical practice, mixed infections could explain heterogeneous clinical responses to anti-tuberculosis therapy. Previous studies of mixed infections have been performed in very specific locations and populations.^{14,15} However, studies of mixed infections remain relatively difficult because a specimen repository is needed and confirmation of mixed infections depends on studies of single colonies, which is time-consuming, tedious and labor intensive. More rapid genotyping methods, such as PCR-based methods, have recently been used in mixed infection studies and showed great promise.^{14,16} We previously reported that the PCR-based VNTR-7 set of loci is a useful genotyping method to differentiate *M. tuberculosis* in clinical isolates in Shanghai.¹⁷ In the present study, we applied the VNTR-7 method to identify mixed infections and to estimate the rate of mixed infections among pulmonary tuberculosis patients in Shanghai, China.

Materials and methods

Patients and isolates

All of the isolates in our study were from the strain collection of the tuberculosis reference laboratory, Shanghai Municipal Center for Disease Control and Prevention (SCDC). The incidence of pulmonary tuberculosis among residents in Shanghai was approximately 38 cases per 100,000 populations. To validate the VNTR-7 method for identifying mixed infections, we selected tuberculosis patients from January 1999 through September 2004 whose drug resistance pattern changed during the same treatment episode. Because previous studies reported that mixed infections were the underlying reason for changes in drug susceptibility patterns during treatment,^{12,13} we selected tuberculosis patients who had a pair of isolates with discordant drug resistant patterns in order to maximize the likelihood of detecting isolates with mixed infections. From the first isolate of these patients, single colonies were harvested according to Warren et al.¹⁴ To estimate the rate of mixed infections, we collected the isolates from all of the pulmonary tuberculosis patients in the Songjiang and Chongming districts of Shanghai during 2006. Demographic characteristics, treatment regimens, adherence and outcomes, and the results of drug-susceptibility testing and

species identification for each patient were obtained from the SCDC tuberculosis registry's database.

Drug susceptibility testing (DST)

DST was performed on all *M. tuberculosis* isolates at the SCDC tuberculosis reference laboratory using the proportion method.¹⁸

VNTR genotyping

VNTR-7 genotyping method was used according to Zhang et al.¹⁷ The results of VNTR genotyping were analyzed using Bionumerics 4.6 software (Applied Maths NV, Belgium).

Deletion-Targeted Multiplex-PCR (DTM-PCR) genotyping

DTM-PCR was used to differentiate between Beijing and non-Beijing genotype strains, following the methods of Chen et al.¹⁹

Definition of mixed infections

If different alleles were found in one or more loci of the VNTR genotype, we recorded that different genotype was present. If two or more alleles were found simultaneously in a specific locus from one isolate, we defined it as a mixed infection.

Steps to minimize laboratory cross contamination

To avoid laboratory cross contamination, only a limited number of samples were processed at the same time, and the preparation of samples was performed in a laminar flow cabinet. For each PCR reaction, DNA from *M. tuberculosis* strain H37Rv and deionized water were added as positive and negative controls, respectively.

Statistical methods

We used the χ^2 test of proportions and $p < 0.05$ for statistical significance, to compare mixed infections in different groups of patients. The data were analyzed using Stata statistical software (version 8, Stata Corporation, College Town, TX, USA).

Results

Patients and isolates

To validate the VNTR-7 method for identifying mixed infections, we identified 31 patients who had a pair of isolates with different drug resistance patterns during the same treatment episode from 1999 to 2004. For 9 patients, the isolates were missing or could not be recovered. We recultured the isolates from the remaining 22 patients. From each of the 22 isolates, 30 single colonies were selected and cultured for further study.

Table 1 Characterization of patients from Songjiang and Chongming districts of Shanghai, 2006.

Isolates	Number, n (%)	Male, n (%)	Female, n (%)	Median age, (years)	DST*		Case definition		DTM-PCR†	
					Sensitive, n (%)	Resistant, n (%)	New, n (%)	Retreatment, n (%)	Beijing, n (%)	Non-Beijing, n (%)
All	249 (100)	180 (72.3)	69 (38.3)	51	199 (79.9)	50 (20.1)	217 (87.1)	32 (12.9)	200 (80.3)	48 (19.3)
Infection with a single strain of <i>M. tuberculosis</i>	235 (94.4)	168 (93.3)	67 (97.1)	51	188 (94.5)	47 (94.0)	208 (95.9)	27 (84.4)	193 (96.5)	42 (87.5)
Mixed infections	14 (5.6)	12 (6.7)	2 (2.9)	49	11 (5.5)	3 (6.0)	9 (4.1)	5 (15.6)	7 (3.5)	6 (12.5)

* DST = drug susceptibility test results.

† DTM-PCR = Deletion-Targeted Multiplex PCR genotyping, one of the 249 isolates was mixed infection with both Beijing and non-Beijing genotype strain and excluded in the table.

In 2006, 561 pulmonary tuberculosis patients were diagnosed in the Songjiang and Chongming districts of Shanghai. Among them, 263 (46.9%, 263/561) tuberculosis patients were smear and culture positive. However, 14 patients were infected by mycobacterium other than *M. tuberculosis* and were excluded. The remaining 249 pulmonary tuberculosis patients were included in the study to estimate the rate of mixed infections among pulmonary tuberculosis patients. The median age of these patients was 51 years and 72.3% (180/249) were males. Most of the patients (87.2%, 217/249) were new cases of tuberculosis but 12.8% (32/249) had previously received anti-tuberculosis treatment and were being retreated. Of the 249 patients, 79.9% (199/249) had an initial isolate that was pan-susceptible to the first-line anti-tuberculosis drugs and 20.1% (50/249) had an isolate that was resistant to one or more first-line anti-tuberculosis drugs (Table 1). In addition, 7.6% (19/249) tuberculosis patients had an isolate that was resistant to at least isoniazid and rifampin, or multidrug-resistant (MDR).

Evaluation of the VNTR genotyping method

The VNTR-7 genotyping method was used to genotype each initial isolate directly and to genotype the 30 single colonies generated from the 22 patients with discordant drug resistance patterns. By directly genotyping the initial isolates from each of the patient, we identified two isolates that had different alleles in one or more locus. We obtained the same results when we genotyped the 30 single colonies from each of the two initial isolates, and the proportion of two different genotypes among the colonies were 24:6 and 29:1, respectively. In the remaining 20 patients, only one genotype was detected among all 30 colonies generated from their initial isolate, respectively. To minimize the possibility of laboratory cross contamination, we analyzed all of the 24 different genotypes that we detected from the 22 patients and found out that none of them were identical (data not shown).

Rate of mixed infections among pulmonary tuberculosis patients

To estimate the rate of mixed infections among pulmonary tuberculosis patients in Shanghai, we used the

VNTR-7 method to genotype one isolate from each of the total 249 pulmonary tuberculosis patients in Songjiang and Chongming in 2006. Fourteen isolates had two alleles in a specific locus. Eight of the 14 isolates had two alleles in the QUB-11a locus, and two isolates had two alleles in more than one locus (Table 2). Among the 14 mixed infections that we detected, 13 were caused by strains with unique genotype patterns and only one isolate in a mixed infection was in a genotype cluster. We rechecked and determined that the two isolates in the genotype cluster were processed on different dates (data not shown). It is therefore unlikely that this specific mixed infection was due to laboratory contamination (data not shown). Therefore, the isolates from 14 of 249 pulmonary tuberculosis patients had mixed infections and the estimated rate of mixed infections was 5.6% (95% CI 3.1%–9.2%).

We investigated the mixed infections in new and retreated cases, and found that 4.1% (9/217) of the mixed infections occurred among new cases and 15.6% (5/32) occurred in isolates from patients who were retreated cases ($p < 0.05$). Of the 14 patients with mixed infections, the median age was 49 years and 12 (85.7%) were males. When comparing the demographics of the 14 tuberculosis patients with mixed infections and the remaining 235 tuberculosis patients without mixed infections, no significant differences were detected in the sex, age, drug susceptibility test results, treatment regimens and treatment outcomes of the two groups (Table 1).

Beijing genotype strains and mixed infections

Using DTM-PCR to genotype the 249 initial isolates from Songjiang and Chongming districts, 80.3% (200/249) of the isolates were Beijing genotype strains, 19.3% (48/249) of the isolates were non-Beijing genotype strains, and one isolate had both Beijing and non-Beijing genotype strains. The estimated rate of mixed infections was 3.5% (7/200) (95% CI 1.4%–7.1%) within Beijing genotype strains and 12.5% (6/48) (95% CI 4.7%–25.2%) within non-Beijing genotype strains ($p < 0.05$) (Table 1). Our results suggest that non-Beijing genotype strains are more likely than Beijing genotype strains to occur in mixed infections.

Table 2 Results of VNTR-7 genotyping on 14 isolates with mixed infections, Songjiang and Chongming districts in Shanghai, 2006.

Isolate number	Repeat numbers of VNTR locus							Number of polymorphic alleles per isolate	Strain of <i>M. tuberculosis</i> by DTM-PCR*
	Mtub21	QUB-18	QUB-11a	QUB-11b	MIRU26	VNTR3820	QUB-26		
1	5	9	27, 29	6	7	13	8	1	Beijing
2	4	12	27, 5	6	8	9	8	1	Beijing
3	5	11	5, 8	5	7	16	9	1	Beijing
4	1	8	5, 10	7	7	5	3	1	Beijing
5	5	9	5	5	6, 8	16	5	1	Beijing
6	5	7	8	6	8	16, 14	8	1	Beijing
7	5	4	1, 4	6	7	13	9	1	Beijing
8	1	1	no alleles	6	3	4	9, 6	1	Non-Beijing
9	2	6	25, 2	3	3	4	3	1	Non-Beijing
10	3	6	5	3	5, 3	6	7	1	Non-Beijing
11	2	1	7, 9	5	8	4	5	1	Non-Beijing
12	1	1	5, 4	5	5	4	8	1	Non-Beijing
13	17, 1	1	5	4	4	5, 7	9	2	Non-Beijing
14	3, 4	6	7	3	6	5	8, 10	2	Beijing and non-Beijing
Number of isolates with polymorphic alleles	2	0	8	0	2	2	2		

* DTM-PCR = Deletion-Targeted Multiplex-PCR.

Discussion

Several studies have reported the occurrence of mixed infections of *M. tuberculosis*, mostly among tuberculosis patients in jails, hospitals and geographical areas with a high incidence of tuberculosis. In the present study, we estimated that approximately 5.6% of pulmonary tuberculosis patients in the general population had mixed infections.

Our study and previous reports showed that mixed infections occurred more frequently among retreated cases.¹⁴ This finding is quite plausible if we assume that retreated cases have been infectious and symptomatic for longer periods of time and are therefore more likely to be reinfected with another strain of *M. tuberculosis*. We previously showed that exogenous reinfection is a major cause of recurrent tuberculosis,² and transmission of drug-resistant strains of *M. tuberculosis* was the main cause of drug-resistant tuberculosis in Shanghai.²⁰ Taken together, our results indicate there is an appreciable amount of ongoing transmission of *M. tuberculosis* in Shanghai and it is likely that mixed infections are occurring.

Beijing genotype strains have attracted much attention in recent years, with studies claiming they are more virulent, grow faster in animal or cell models, and may be hypermutable.^{21–24} In Shanghai, more than 80% of tuberculosis patients were infected with the Beijing genotype strains.²⁵ Therefore, we had expected that most of the mixed infections would have two different Beijing genotype strains. Surprisingly, we found the opposite: 43% of the mixed infections had non-Beijing genotype strains, and Beijing genotype strains were relatively seldom found in mixed infections. It remains to be determined if there is a biological mechanism that would explain this observation. For example, differences

in the relative fitness of different strains of *M. tuberculosis* and differential host immune responses to strains of *M. tuberculosis* from different phylogenetic lineages may play a role in mixed infections.

Several factors, such as the study population, sampling methods and the discriminatory power of the genotyping methods used, can influence the estimated rate of mixed infections. One study reported that mixed infections were more likely to be detected if two sputum specimens from each tuberculosis patient were analyzed, instead of just one sputum specimen.¹⁵ Had we used two sputum specimens from each tuberculosis patients, we might have detected a higher rate of mixed infections in Shanghai. We consider our estimate is conservative and the prevalence of mixed infections among pulmonary tuberculosis patients in Shanghai may well be higher than the 5.6% detected in this study.

To our knowledge, this is the first study of mixed infections among pulmonary tuberculosis patients in China, a country with the second highest number of tuberculosis cases reported worldwide and the highest number of MDR tuberculosis patients.²⁶ With increasing concern about drug-resistant tuberculosis in the world, mixed infections are a plausible explanation for conflicting drug susceptibility test results on specimens obtained from the same patient during anti-tuberculosis therapy, particularly when drug resistance is suspected. Our results provide new insight into mycobacterial infections and have important implications for tuberculosis control strategies.

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