Table 3. Characteristics of MDR-TB patients.

No.	Gender, age	Residential area	HIV	DR pattern	MTB spoligotype	VNTR pattern	Clustered among MDR cases	Clustered among all cases
138	M, 40	Old urban	Neg.	IRS	Beijing	233643446844243	Yes (cluster I)	Yes (cluster I)
294	M, 22	Old urban	Neg.	IRSE	Beijing	233643446844243	Yes (cluster I)	Yes (cluster I)
166	M, 50	Old urban	Neg.	IRS	Beijing	233653446744243	Yes (cluster II)	Yes (cluster II)
347	F, 18	Suburban	Neg.	IRS	Beijing	233653446744243	Yes (cluster II)	Yes (cluster II)
356	M, 30	New urban	Neg.	IRSE	Beijing	233653446744243	Yes (cluster II)	Yes (cluster II)
239	M, 43	New urban	Neg.	IRSE	Unclassified	642245742652124	Yes (cluster III)	Yes (cluster III)
256	M, 34	New urban	Pos.	IRSE	Unclassified	642245742652124	Yes (cluster III)	Yes (cluster III)
48	F, 55	Old urban	Neg.	IRS	Beijing	233753447534443	Yes (cluster IV)	Yes (cluster IV)
449	M, 29	Old urban	Pos.	IRS	Beijing	233753447534443	Yes (cluster IV)	Yes (cluster IV)
205	M, 52	Suburban	Neg.	IRSE	Beijing	233751445854242	No	Yes (cluster V)
474	M, 26	New urban	Neg.	IR	Beijing	223753445854243	No	Yes (cluster VI)
36	M, 44	Old urban	Neg.	IRSE	Beijing	243753N42344335	No	No
69	M, 35	New urban	Neg.	IRS	Beijing	233753446754243	No	No
126	M, 26	New urban	Pos.	IRSE	Beijing	233751545854242	No	No
236	M, 34	Suburban	Pos.	IRS	EAI5	632253742692122	No	No
368	M, 40	New urban	Neg.	IRS	Beijing	232543443844443	No	No
409	M, 30	New urban	Pos.	IRSE	Beijing	233455444832423	No	No
489	M, 44	Old urban	Neg.	IRS	Beijing	223753445864243	No	No
528	M, 55	New urban	Neg.	IRSE	Unclassified	642245442652124	No	No
16	M, 62	Old urban	Neg.	IRSE	N/A	N/A	N/A	N/A
264	M, 36	New urban	Pos.	IRSE	EAI5	N/A	N/A	N/A
333	M, 31	New urban	Pos.	IRS	EAI5	N/A	N/A	N/A

HIV: human immunodeficiency virus; MDR-TB: multidrug-resistant tuberculosis; DR: drug-resistant; VNTR: variable numbers of tandem repeats; M: male; F: female; IR: resistant to isoniazid and rifampicin; IRS: resistant to isoniazid, rifampicin, and streptomycin; IRSE: resistant to isoniazid, rifampicin, streptomycin, and ethambutol; Neg: negative; Pos: positive; MTB: *Mycobacterium tuberculosis*; N (in "VNTR pattern" column): polymerase chain reaction negative; EAI: East African–Indian; N/A: not available.

Molecular genotyping

Spoligotyping was performed to confirm the presence of Beijing strains and to identify sublineages of non-Beijing strains using a spoligotyping kit (Ocimum Biosolutions LLC, Houston, TX, USA), according to the standard protocol [13]. Classification of the spoligotype family was based on the international database, SpolDB4 [14].

We analyzed a single-nucleotide polymorphism at the 3284855 position using real-time polymerase chain reaction to further confirm the presence of Beijing strains [15].

Variable numbers of tandem repeats (VNTR) analysis was conducted for all strains using the international standard 15 mycobacterial interspersed repetitive unit (MIRU)-VNTR proposed by Supply et al. [16], with the exception of DNA samples with ambiguous results. The copy number of each locus of the H37Rv strain was used as to confirm the different definition in VNTR analysis. The copy numbers in MIRUs-4, 10, 16, 26, 31, and 40; ETRs-A and C; and VNTRs-2163b, 4052, 1955, 2401, 4156, 0424, and 3690 were defined as 3-3-2-3-3-1-3-4-5-5-2-2-2-2-5, respectively. We defined each cluster by complete match of the VNTR profile. To confirm the appropriateness of each cluster, spoligotyping patterns were also considered. The clustering rate was calculated as described elsewhere [17].

Statistical analysis

The chi-squared test was used to compare the proportions between drug-sensitive and drug-resistant groups. The logistic regression models were used to evaluate potential risk factors for drug resistance, and adjusted odds ratios (aORs) and 95% confidence intervals (CIs) were calculated. Therein, each drug-resistance pattern was set as an outcome variable, and factors that could affect the pattern were chosen as independent variables. For RMP resistance and MDR, only variables with biological significance and with significant associations in univariate analysis were included in the multivariate models, because the number of outcome variables was limited. Statistical analysis was performed using Stata version 11 (StataCorp, College Station, TX, USA), and $P < 0.05$ was considered to be statistically significant.

Results

Study samples and patient characteristics

In total, 546 newly diagnosed smear-positive pulmonary TB patients were recruited. From 506 culture-positive cases, microbial isolates were collected from 495 patients (97.8%), of which six were infected with nontuberculous mycobacteria. As a result, 489 MTB isolates were tested for drug susceptibility. Because of insufficient quality of the extracted DNA samples, 467 MTB isolates further underwent spoligotyping and 465 underwent VNTR typing (Figure 1). The median age was 38.6

Table 4. Univariate analysis using the logistic regression model of the associations between potential risk factors and drug resistance (n = 489).

		Any drug resistance		INH resistance		SM resistance		RMP resistance		MDR	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Age (in years)	≥45	1.00		1.00		1.00		1.00		1.00	
	<45	**1.85**	**1.26–2.71**	**1.85**	**1.21–2.83**	**1.77**	**1.16–2.69**	2.53	0.93–6.90	2.25	0.82–6.21
Sex	Male	1.00		1.00		1.00		1.00		1.00	
	Female	0.72	0.45–1.14	1.06	0.65–1.71	0.63	0.38–1.06	0.33	0.08–1.42	0.36	0.08–1.58
Smoking*	No	1.00		1.00		1.00		1.00		1.00	
	Yes	**1.69**	**1.14–2.51**	1.28	0.84–1.96	**2.00**	**1.28–3.14**	1.89	0.69–5.18	1.67	0.60–4.64
HIV status	Negative	1.00		1.00		1.00		1.00		1.00	
	Positive	**1.98**	**1.06–3.70**	**2.07**	**1.10–3.89**	**2.30**	**1.22–4.31**	**4.74**	**1.85–12.16**	**5.40**	**2.07–14.07**
Number of lymphocytes (cells/mm^3)											
	≥1,000	1.00		1.00		1.00		1.00		1.00	
	<1,000	**1.74**	**1.01–3.01**	1.61	0.91–2.85	1.63	0.92–2.89	1.99	0.71–5.54	2.23	0.79–6.30
Smear**		0.91	0.76–1.11	0.90	0.73–1.11	0.88	0.72–1.09	0.89	0.57–1.37	0.92	0.58–1.44
MTB strain	Non-Beijing	1.00		1.00		1.00		1.00		1.00	
	Beijing	**2.00**	**1.35–2.95**	**2.11**	**1.37–3.26**	**1.95**	**1.26–3.00**	1.68	0.68–4.16	1.84	0.70–4.83
Clustered	No	1.00		1.00		1.00		1.00		1.00	
	Yes	**1.66**	**1.13–2.44**	**2.08**	**1.36–3.20**	1.16	0.77–1.75	1.08	0.45–2.62	1.12	0.44–2.83
BMI	18.5–24.9	1.00		1.00		1.00		1.00		1.00	
	<16	0.85	0.49–1.49	0.72	0.38–1.36	0.95	0.52–1.74	0.69	0.19–2.49	0.82	0.22–3.04
	16–18.4	1.02	0.68–1.51	0.99	0.65–1.51	1.03	0.67–1.58	0.64	0.26–1.57	0.76	0.30–1.93
	≥25	1.54	0.21–11.11	2.44	0.34–17.67	0.85	0.09–8.33	-		-	
Residential area											
	Suburban	1.00		1.00		1.00		1.00		1.00	
	New urban	**1.97**	**1.18–3.29**	1.70	0.96–3.03	1.42	0.82–2.45	1.64	0.45–6.01	1.64	0.45–6.01
	Old urban	**1.98**	**1.15–3.40**	**2.15**	**1.18–3.91**	1.38	0.78–2.46	2.14	0.57–7.98	1.69	0.44–6.53

INH: isoniazid; SM: streptomycin; RMP: rifampicin; MDR: multidrug-resistance; HIV: human immunodeficiency virus; BMI: body mass index; MTB: *Mycobacterium tuberculosis*; OR: odd ratios; 95% CI: 95% confidence interval

* Includes ex-smoking.

** OR per unit change of smear positivity (scanty, 1+, 2+, 3+).

Bold type indicates significant associations.

years (range = 16.6–85.4), the proportion of male patients was 78.9%, and HIV coinfection was observed in 9.0% of the patients (Table 1).

Prevalence and patterns of resistance to INH, SM, RMP, EMB, and PZA

Of the 489 MTB isolates, 60.9% were fully sensitive to INH, SM, RMP, and EMB. INH resistance was observed in 138 isolates (28.2%), which included 49 (10.0%) isolates of INH monoresistance; SM resistance was also observed in 138 isolates (28.2%), which included 50 isolates of SM monoresistance (10.2%), and the rest were mostly the combination of INH and SM resistance (Table 2). Primary resistance to RMP was detected in 24 isolates (4.9%), and 22 isolates were MDR-TB, which accounted for 4.5% of all isolates; most of these were also SM resistant. EMB resistance was not frequent (2.9%). The pyrazinamidase assay showed negative results for 12 isolates (2.5%), indicating resistance to PZA. The proportion of PZA resistance among MDR cases was significantly higher than that in non-MDR cases (13.6%, 95% CI 2.9–34.9 vs. 1.9%, 95% CI 0.9–3.6; P = 0.001; Table not provided).

Distribution of MTB lineages and clusters of drug-resistant isolates

Among 467 MTB isolates spoligotyped, the Beijing genotype was most frequently observed [272 isolates (58.2%)]. The East African-Indian (EAI) lineage ranked as the second most frequently observed genotype [93 isolates (19.9%)], of which 84 isolates showed the EAI5 genotype and 9 showed a Vietnamese genotype (EAI4_VNM) (Table not provided). Among 21 of the 22 MDR-MTB strains available for spoligotyping, 15 (71.4%) were of Beijing genotype, 3 (14.3%) were of EAI genotype, and the remaining 3 (14.3%) showed unclassified non-Beijing genotypes but closely resembled EAI4 or 5, according to the spoligotyping database (Table 3).

Of the 465 isolates, in which both spoligotype and VNTR patterns were available, 257 (55.3%) were clustered strains belonging to 55 clusters, indicating that the clustering rate was 43.4% [(257−55)/465]. The proportion of clustered strains was significantly higher in the group with any drug resistance than

Table 5. Results of multivariate analysis using the logistic regression model on the associations between potential risk factors and drug resistance (n = 489).

Factors		Number (%)	Multivariate	
			aOR	95% CI
Any drug resistance*				
Age (in years)	≥45	58/191 (30.4)	1.00	-
	<45	133/298 (44.6)	**1.72**	**1.11–2.66**
Smoking**	No	51/165 (30.9)	1.00	-
	Yes	139/323 (43.0)	**1.87**	**0.99–3.49**
Residential area	Suburban	27/100 (27.0)	1.00	-
	New urban	96/228 (42.1)	**2.06**	**1.17–3.62**
	Old urban	68/161 (42.2)	**2.14**	**1.17–3.91**
MTB strain	Non-Beijing	57/195 (29.2)	1.00	-
	Beijing	123/272 (45.2)	**1.86**	**1.21–2.87**
INH resistance*				
Residential area	Suburban	19/100 (19.0)	1.00	-
	New urban	65/228 (28.5)	1.60	0.85–3.02
	Old urban	54/161 (33.5)	**2.23**	**1.15–4.35**
MTB strain	Non-Beijing	38/195 (19.5)	1.00	-
	Beijing	92/272 (33.8)	**1.91**	**1.18–3.10**
Clustered	No	41/207 (19.8)	1.00	-
	Yes	87/258 (33.7)	**1.69**	**1.06–2.69**
SM resistance*				
Smoking**	No	32/165 (19.4)	1.00	-
	Yes	105/323 (32.5)	**2.47**	**1.18–5.16**
MTB strain	Non-Beijing	39/195 (20.0)	1.00	-
	Beijing	89/272 (32.7)	**2.10**	**1.29–3.40**
RMP resistance***				
HIV	Negative	17/443 (3.8)	1.00	-
	Positive	7/44 (15.9)	**5.42**	**2.07–14.14**
MTB strain	Non-Beijing	7/195 (3.6)	1.00	-
	Beijing	16/272 (5.9)	1.67	0.67–4.20
MDR***				
HIV	Negative	15/443 (3.4)	1.00	-
	Positive	7/44 (15.9)	**6.23**	**2.34–16.58**
MTB strain	Non-Beijing	6/195 (3.1)	1.00	-
	Beijing	15/272 (5.5)	1.84	0.69–4.90

INH: isoniazid; SM: streptomycin; RMP: rifampicin; MDR: multidrug-resistance; TB: tuberculosis; HIV: Human immunodeficiency virus; aOR: adjusted odd ratios; 95% CI: 95% confidence interval

* Only factors showing significant associations were shown.

** Included ex-smoking.

*** The final model included biologically significant variables (MTB lineage) and variables showing significant associations (HIV status) in univariate analysis.

Bold type indicates significant associations.

in the fully-sensitive group [112/178 (62.9%) vs. 145/287 (50.5%), P = 0.009]. Of the 22 MDR isolates, spoligotype and VNTR patterns of MTB were available in 19. Eleven (57.9%) of them belonged to six clusters, I–VI, as determined by a comparison of genotyping patterns observed in the 465 tested isolates, and clusters II and IV were the first (9.5%) and second (3.4%) largest clusters among them (Table not provided). MDR strains in the largest cluster II were observed in all of the old, new, and suburban areas. The VNTR patterns of the clusters I and II were different only in 2 of the 15 loci tested (Table 3).

Factors associated with drug-resistant TB

The logistic regression models were used to identify factors associated with drug resistance. Factors that were analyzed included gender, age, body mass index (BMI), smoking behavior, the patient's residential area, MTB load in the sputum smear before treatment, HIV status, the number of blood lymphocytes, MTB lineage, and clustered strains. Univariate and multivariate analyses (Tables 4 and 5) revealed that age less than 45 years, living in a new or old urban area, and being infected with Beijing strains were significantly associated with any drug resistance (aOR = 1.72, 95% CI 1.11–2.66; 2.06, 1.17–3.62; 2.14, 1.17–3.91; and 1.86, 1.21–2.87, respectively). However, living in an old urban area and being infected with Beijing strains or clustered strains were significantly associated with INH resistance (aOR = 2.23, 95% CI 1.15–4.35; 1.91, 1.18–3.10; and 1.69, 1.06–2.69, respectively), and being a smoker or infection with the Beijing MTB strain showed significant association with SM resistance (aOR = 2.47, 95% CI 1.18–5.16; 2.10, 1.29–3.40, respectively) (Table 5). Younger age was significantly associated with INH and SM resistance in univariate analysis (OR = 1.85, 95% CI 1.21–2.83; 1.77, 1.16–2.69, respectively) (Table 4), but these associations were not significant in multivariate analysis (aOR = 1.59, 95% CI 0.98–2.58; 1.56, 0.97–2.52, respectively) (Table not provided).

Multivariate analyses revealed that only HIV coinfection was significantly associated with RMP resistance (aOR = 5.42, 95% CI 2.07–14.14) and MDR (aOR = 6.23, 95% CI 2.34–16.58) (Tables 4 and 5).

Discussion

We found that the proportion of drug-resistant cases, including MDR, was considerably high among newly diagnosed smear-positive culture-positive pulmonary TB patients residing in Hanoi city. Depending on the type of drug resistance, the drug resistance-associated risk factors showed a pronounced variation and revealed complicated aspects in a large city. The majority of MDR-TB cases revealed that infection with Beijing strains was predominantly spread in this area, while non-Beijing MDR strains were also observed.

INH or SM resistance was not uncommon, and most RMP-resistant strains were also associated with SM and INH resistance, resulting in MDR. These findings were consistent with a previous report in Ho Chi Minh city in Viet Nam [9]. The high prevalence of primary resistance to INH and SM (28.2% and 28.2%, respectively) and moderate prevalence of RMP resistance and MDR (4.9% and 4.5%, respectively) shown in our study might be considered noteworthy, when comparing with those of South East Asian region (10.3%, 8.9%, 3.4%, and 2.8%) [7], and of China (16.0%, 27.7%, 6.7%, and 5.7%) [18]. In this situation, the use of a regimen with RMP for only 2 months of the intensive phase, which is still accepted in Viet Nam, may pose the risk for poor treatment outcome [19] and accumulation of further drug resistance [20].

The association between younger age and anti-TB drug resistance has been reported previously [9,21]. The results of univariate and multivariate analyses performed in our study indicate that primary drug resistance among the younger population may be confounded by the recent transmission of Beijing strains [9,22]. In the current study, living in an old urban area and infection with clustered strains were associated with INH, but not SM, resistance, suggesting that the transmission of INH-resistant strains is concentrated in areas with a high population density, whereas SM-resistant strains are spreading more diffusely throughout the city. Initially, SM was used for treatment of wound infections during the war in Viet Nam in the early 1950s, which may partly explain the widespread development of SM-resistant nonclustered strains, whereas INH was first circulated in 1960s, and RMP was introduced at around 1975 [23,24]. The Beijing genotype was significantly associated with resistance to any drug, INH, and SM, but it was not associated with either RMP resistance or MDR. A direct role of Beijing strains in drug resistance remains controversial [22,25-27].

The spoligotype and VNTR analyses demonstrated that any-drug resistant strains showed a higher tendency for clustering than fully-sensitive strains; and almost half of the MDR strains were clustered and presumably derived from common infection sources or infection with different sources sharing ancestors [16,28]. Three of the MDR strains (13.6%) belonged to the largest Beijing cluster, accounting for approximately 10% of the study population. Although the Beijing genotype was predominant among clustered MDR strains, three non-Beijing genotype strains were closely related to each other based on their VNTR patterns and showed unclassified spoligo patterns resembling EAI5 or EAI4_VNM, a possibly indigenous MTB subtype mainly observed in Viet Nam. Research into the origin and transmission dynamics of these variant MDR strains, as well as their molecular characteristics, may be important, because it is generally believed that the EAI lineage has conferred significantly less drug resistance compared with other genotypes in Asian countries [29,30].

HIV coinfection was significantly associated with only RMP resistance and MDR in multivariate analysis, although it showed significant associations with all types of drug resistance in univariate analysis. This independent association with RMP resistance and MDR has also been reported in other studies [31,32], including one in the northern area [10], but was not observed in a study of the southern area of Viet Nam [9]. The southern study was conducted between 1998 and 2000, when HIV prevalence was low in Viet Nam [33]. This may explain the lower percentage of HIV, compared with ours (2.8% vs. 9.0%), resulting in a low statistical power (20%) [9]. In Hanoi, approximately 25% of injecting drug users tested were HIV positive [31]. Drug use is a risk factor for nonadherent treatment, and it promotes development of drug resistance [34], thus increasing the chance of resistance transmission among the group. HIV coinfection is also associated with pharmacokinetic alteration of RMP, resulting in a 39% reduction of drug concentration [35]. The decreased bioavailability of RMP may contribute to the development of RMP resistance as well. In addition, HIV-coinfected TB patients receiving antiretroviral treatment often suffer from the adverse effects of RMP when an alternative drug is not available, which may cause poor treatment outcomes [36] and facilitate drug resistance. The negative effect of HIV coinfection on RMP resistance, together with the recent spread of Beijing strains associated with INH resistance, may pose a combined risk for the acquisition and transmission of MDR-TB in a large city like Hanoi.

SM resistance was independently associated with smoking, after adjusted for HIV coinfection. The reason for this association is unknown, although smoking is known to be associated with TB [37]. The proportion of PZA resistance tested using the pyrazinamidase assay was low among the total study population [38]. Nevertheless, the proportion of PZA resistance was significantly higher in the MDR group than that in the non-MDR group, indicating a need for evaluation of the susceptibility of MTB strains to this drug.

The clustering rate in Hanoi (43.4%) was high, presumably because our study was conducted in a capital city with high population density and enrolled only patients with smear-positive pulmonary TB. Others have reported relatively lower clustering rates (28.3% in China [39], 37.7% in Zambia [40], and 16.8% in Uganda [41]). However, these studies were conducted in peripheral areas (Zambia) or enrolled patients with smear-negative pulmonary TB (China, Uganda). In addition, it is known that the resolution of 15 MIRU-VNTR for Beijing strains is suboptimal and may overestimate the clustering rate. Addition of more loci to the standard VNTR loci may increase the resolution in a setting where Beijing-genotype strains prevail [42]. Nevertheless, the data can be analyzed using the standard 15 MIRU-VNTR typing method first, since it has been used internationally for a long time [39-41,43,44].

Our study has some limitations. First, we did not have enough information about direct epidemiologic links among clustered patients. In high TB burden countries, however, a TB outbreak is difficult to identify. In addition, we may not have analyzed all representative isolates in Hanoi city. However, the seven districts participating in this study cover old urban, new urban, and suburban areas in this city, and analysis of a relatively large number of isolates definitely provided information that would be useful in the management of drug-resistant TB. Despite the aforementioned limitations, we investigated a variety of host-, bacteria-, and environment-related factors and developed a multidimensional picture of the status of drug-resistance in the studied area.

In conclusion, the transmission status of drug-resistant TB in a large city with a high proportion of Beijing strains, particularly in HIV-prevalent areas, should be carefully monitored to avoid an increase in the incidence of MDR and generation of extensively drug-resistant TB. Drug susceptibility testing should be considered. On the basis of the results, an optimal treatment regimen, together with intensive monitoring of treatment adherence, is suggested to avoid further increases in drug resistance.

Acknowledgements

The authors would like to thank Dr. Bui Thi Nguyet, Ms. Vu Thi Xuan Thu, Dr. Pham Thu Anh (Hanoi Lung Hospital), Dr. Phan

Thi Minh Ngoc, Ms. Nguyen Thi Ha (NCGM-BMH Medical Collaboration Center), and all the healthcare staff of relevant district TB centers for supporting site implementation.

Author Contributions

Conceived and designed the experiments: NTLH LTL SM PHT NVH VCC SS HE NK. Performed the experiments: SM TBT NPH KTTN. Analyzed the data: NTLH AN TM NK. Contributed reagents/materials/analysis tools: NTLH LTL SM PHT NVH TBT NPH VCC KTTN SS HE NK. Wrote the manuscript: NTLH SM NK.

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Received August 28, 2012
Revised December 19, 2012
Accepted December 20, 2012

Short Communication

Multiplex agarose gel electrophoresis system for variable number of tandem repeats genotyping: Analysis example using *Mycobacterium tuberculosis*

As one genotyping method for *Mycobacterium tuberculosis*, variable number of tandem repeats (VNTR) is a promising tool to trace the undefined transmission of tuberculosis, but it often requires large equipment such as a genetic analyzer for DNA fragment analysis or CE system to conduct systematic analyses. For convenient genotyping at low cost in laboratories, we designed a multiplex PCR system that is applicable to agarose gel electrophoresis using fluorescent PCR primers. For tuberculosis genotyping by VNTR, the copy quantities of minisatellite DNA must be determined in more than 12 loci. The system can halve laborious electrophoresis processes by presenting an image of two VNTR amplicons on a single lane. No expensive equipment is necessary for this method. Therefore, it is useful even in developing countries.

Keywords:
Genotyping / Infectious disease / Multiplex PCR / *Mycobacterium tuberculosis* / VNTR
DOI 10.1002/elps.201200471

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Clinical isolates of *Mycobacterium tuberculosis*, an etiological agent of human tuberculosis (TB), cannot be distinguished by phenotypic polymorphisms. Therefore, refined genotyping analysis is indispensable to ascertain their identities. Identification and data accumulation of *M. tuberculosis* strains based on their genotypes are important contributions to public health related to TB, enabling verification of transmission in cases with epidemiological links and supporting the detection of undetected outbreaks.

Variable number of tandem repeats (VNTR) has become a popular genotyping target for *M. tuberculosis* recently [1, 2]. In this method, polymorphic VNTR loci that possess minisatellites (repeat units consisting of 55–110 bp) on a genome are amplified by PCR. Then their number of repeat units is enumerated according to size of their amplicons as a molecular marker. When applied to identification of strains, multiple VNTR loci must be used to achieve sufficient discriminability. Therefore, as many as 15 or 24 loci must be determined as a genotypic profile of each strain even in a promising global standard [2].

Previous reports have described a CE system, including a genetic analyzer for DNA fragment analysis, that is normally used for genotyping to determine amplicon sizes for VNTR typing [3]. Fragment analysis on a genetic analyzer enables to use multiplex PCR with fluorescent primers which can contribute to reduction of reaction tubes and electrophoresis. One merit of VNTR typing is its convenience. Alternatively agarose gel electrophoresis (AE) is applicable to genotyping, in principle [4, 5]. However, in a laboratory where only AE is available, VNTR typing necessitates the electrophoresis of PCR products of many loci one-by-one, which entails an extremely laborious procedure.

This study examined a JATA(12)-VNTR system established to optimize discrimination of TB clinical isolates (especially for Beijing family, a prominent lineage spreading in eastern Asia) [6], as one exemplary case. This system had been constructed with 12 VNTR loci to discriminate clinical strains efficiently. To reduce the effort necessary to perform AE procedures of VNTR typing, both forward and reverse primers were labeled with fluorescein (2-(6-hydroxy-3-oxo-{3H}-xanthen-9-yl) benzoic acid, MW = 389.38) in the 5′ end in advance. Consequently, PCR products were able to

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Abbreviations: AE, agarose gel electrophoresis; **TB**, tuberculosis; **VNTR**, variable number of tandem repeats

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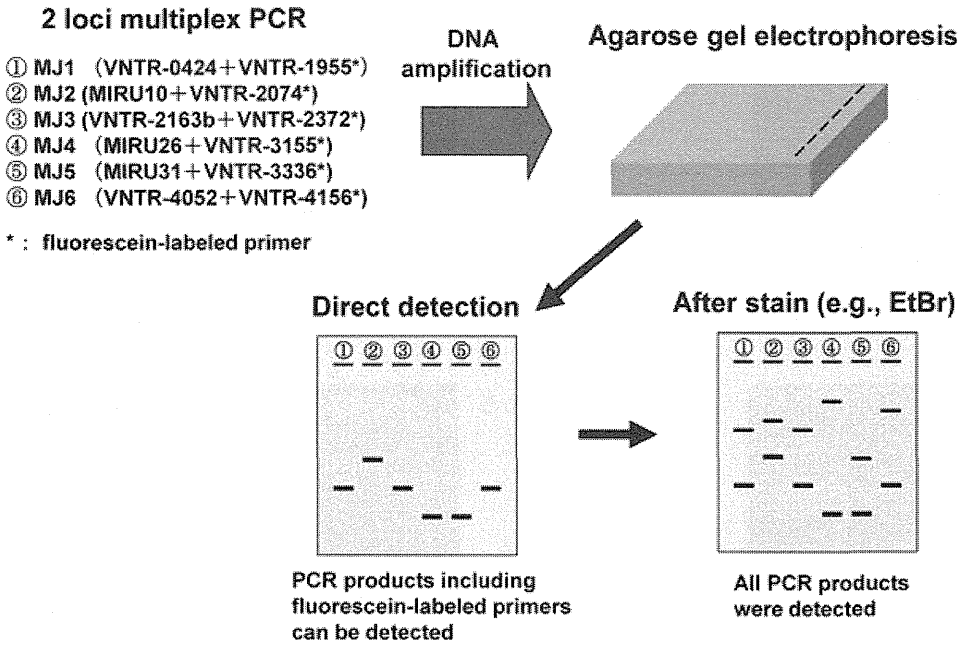

Figure 1. Two loci multiplex PCR were conducted using fluorescein-labeled and non-labeled primer sets. After electrophoresis of each sample, fluorescein-labeled amplicons were detected by putting an agarose gel directly on a UV transilluminator. The agarose gel was stained for detection of all PCR amplicons.

be visualized directly on an agarose gel for six loci of VNTR (Supporting Information Table 1). Nonconjugated pairs of primers were mixed to produce six pairs of multiplex PCR for two VNTR loci per single tube, respectively. Six mixtures were subjected to PCR for an isolate. Then the products were applied, respectively, to AE. The PCR fragments were visualized using a UV transilluminator with excitation (λ = 302 nm) without staining. Then the bands labeled with fluorescein were recorded under a filter (519.5–554.5 nm emission fluorescence). Subsequently, the agarose gel was stained with dyes such as ethidium bromide or other reagents (GelRed, SYBR Gold, etc.) to visualize both multiplex amplicons (Fig. 1).

The PCR was conducted using Takara Ex *Taq* polymerase enzyme with 0.2 mM dNTPs and GC buffer I (Takara Bio, Japan). Conditions of PCR amplification were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1.5 min with final extension at 72°C for 7 min. The PCR products were analyzed using 1 × TBE electrophoresis on a 2% GenePure 3:1 agarose gel (Bioexpress, USA). For this experiment, a 100 bp DNA ladder marker (O'RangeRuler 100 bp + 500 bp; Fermentas) was used.

Each VNTR locus was classified into one of two groups: one for loci in which PCR products were distributed mainly in a high molecular size range (JATA-01, 02, 05, 07, 09, 11) and the other for loci in which PCR products are distributed mainly among low molecular sizes (JATA-03, 04, 06, 08 10, 12). High and low molecular size loci on PCR were mixed in JATA serial order. In mixed loci, primer sets for the loci producing low molecular size were labeled using fluorescein. The primers were designed to distinguish amplicon sizes efficiently in a multiplex product. Typical examples of multiplex PCR analyses are presented in Fig. 2. In most cases, this system functioned with no difficulty. The electrophoresis mobility of amplicons was observed to be unaffected by their conjugation on agarose gels. However, some combinations of multiplex alleles were anticipated to produce undistinguishable fragments as a result of their similar lengths because of low discrimination of AE and overlapping of two multiplex amplicons (< 20 bp differences between two amplicons) (Supporting Information Table 2).

Operations of the multiplex system were simulated using two populations of *M. tuberculosis* clinical isolates: a nationwide collection (n = 325) reported previously [6] and a surveillance study (n = 274) of Tokyo, Japan, between June 2004 and December 2006. Results show that most isolates (>97% of all samples) were estimated in sufficient separation in all six multiplex PCR products by AE. Some isolates (9 of the 325 (2.7%) nationwide isolates; 8 of the 274 (2.9%) Tokyo isolates) were regarded as failing in discrimination of fragments in a multiplex lane by AE (Supporting Information Fig. 1). Results show that these cases are generated in Mix 3 and Mix 5 in both panels. When the system was actually applied to another population of isolates (n = 76, obtained from foreign-registered TB patients at the Hospital of National Center for Global Health and Medicine). When the results were verified using MCE equipment (SV1210; Hitachi High-Technologies Corp., Tokyo, Japan) as done in previous studies [7], the numbers of repetitive units enumerated in this analysis were confirmed to be assigned correctly (Fig. 2). However, four isolates (two isolates in Mix 2, one isolate in Mix 4 and Mix 5, respectively) in 76 isolates (5.3%) showed undistinguishable amplicons in multiplex electrophoresis. In such few cases, it is necessary to operate PCR of nonlabeled primers separately to detect the amplification and to determine accurate VNTR types. It is unnecessary to verify PCR products of fluorescein-labeled

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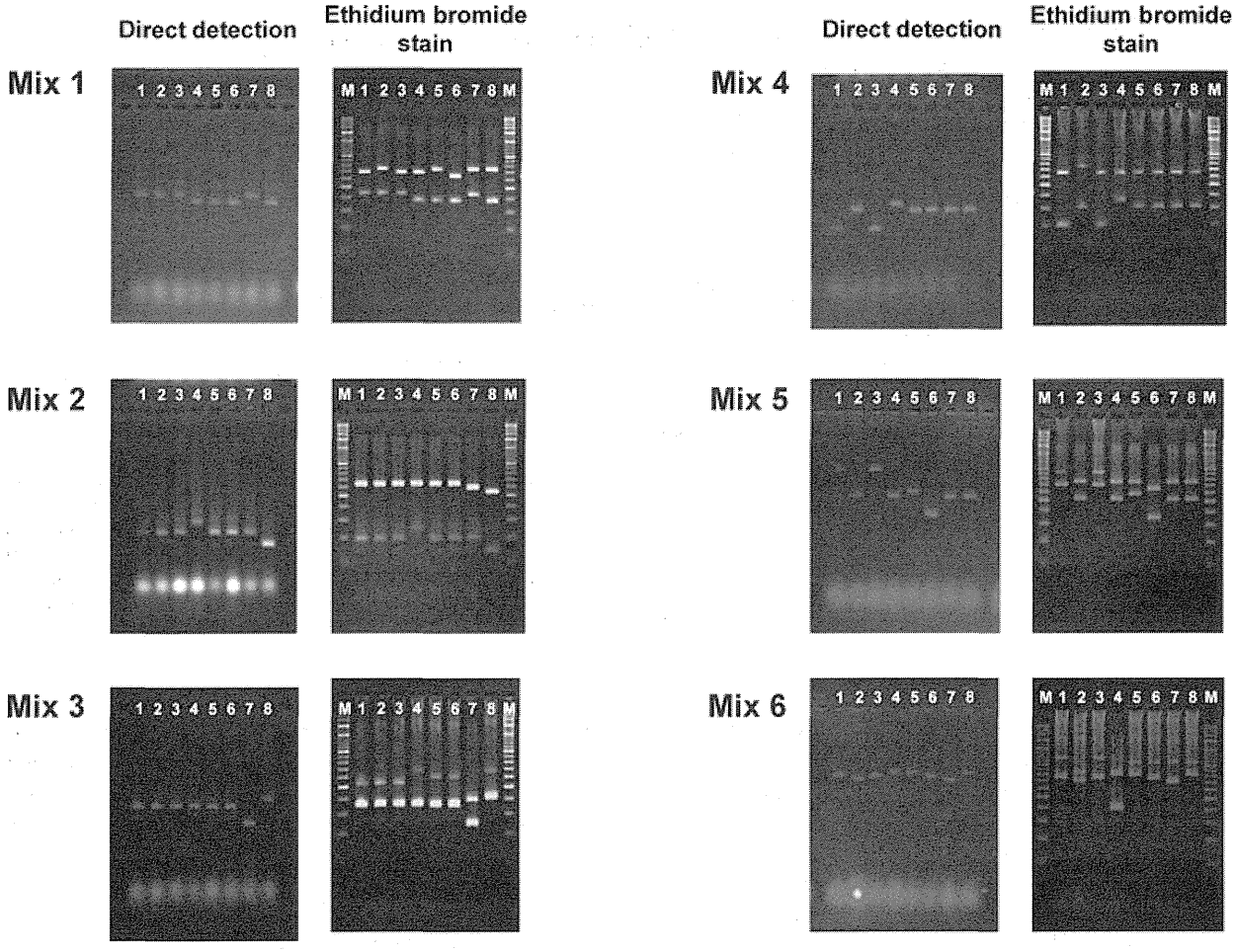

Figure 2. Typical examples in multiplex PCR analyses: Mix 1, VNTRs-0424 and 1955*; Mix 2, MIRU10 and VNTR-2074*; Mix 3, VNTRs-2163b and 2372*; Mix 4, MIRU26 and VNTR-3155*; Mix 5, MIRU31 and VNTR-3336*; Mix 6, VNTRs-4052 and 4156*. *: fluorescein-labeled primer.

primers because they can be detected easily irrespective of overlapping with nonlabeled amplicons.

Length of electrophoresis (an agarose gel), temperature control of gels, and optimal volumes of PCR products to be applied are important factors to achieve high performance and reliable discrimination of AE. However, it consequently demands management to maintain high-quality controls for such conditions. In our system, primers for multiplex VNTR (Supporting Information Table 1) were designed to maintain a low probability of undesirable combination of PCR products, based on a tendency of allelic patterns of *M. tuberculosis*. As a result, the frequency of appearance of undistinguishable bands was held down to 3% or less in the three populations verified in this study (Supporting Information Fig. 1).

The application might be improved using various emission wavelengths of fluorescent primers to conjugate with each primer which can be detected simultaneously. From the perspective of discrimination of electrophoresis, that of microchip-based electrophoresis is sufficient to determine accurate alleles of VNTR of *M. tuberculosis*. Our strategy might also be helpful to reduce running costs for genotyping when its sensor is improved to become capable of determining some wavelengths simultaneously.

Here, we presented one example of the use of a VNTR-analyzing system in Japan. However, this extremely simple method is applicable by anyone for individual VNTR systems in local areas, even in developing countries. For *M. tuberculosis* strains, VNTR genotyping provides digital profiles that enable reliable interlaboratory comparisons. It is important to popularize this method and make it available for use at various laboratories, medical institutes, and public health centers to achieve full benefits from this merit. Reduced dependence on laboratory equipment is desired to further that purpose. Improvement of VNTR typing using AE is a direct approach to hasten the diffusion of this method.

We are grateful to Molecular Epidemiology Project in Research Institute of Tuberculosis in Japan for their support of our analyses. Additionally, we thank Drs. N. Kobayashi and T. Kirikae in NCGHM for permission to use their data. We also thank the members of the Tuberculosis Research Committee

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(RYOKEN) for permission to use their TB isolates. This work was supported by a grant from the Ministry of Health, Labour and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants). This work was also supported in part by a Grant-in-Aid for Young Scientists (A) (24689034) from JSPS.

The authors have declared no conflict of interest.

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PLOS | ONE

Tuberculosis Contact Investigation Using Interferon-Gamma Release Assay with Chest X-Ray and Computed Tomography

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Abstract

Between September 2009 and January 2010, 6 members of the Japanese Eastern Army, who had completed the same training program, were diagnosed with active tuberculosis (TB) on different occasions. The Ministry of Defense conducted a contact investigation of all members who had come into contact with the infected members. The purpose of this study was to verify the efficacy of the TB screening protocol used in this investigation. A total of 884 subjects underwent interferon-gamma release assay (IGRA) and chest X-ray. The 132 subjects who were IGRA positive or with X-ray findings suggestive of TB subsequently underwent chest computer tomography (CT). Chest CT was performed for 132 subjects. Based on CT findings, 24 (2.7%) subjects were classified into the active TB group, 107 (12.1%) into the latent tuberculosis infection (LTBI) group, and 753 (85.2%) into the non-TB group. The first 2 groups underwent anti-TB therapy, and all 3 groups were followed for 2 years after treatment. Although one subject in the active TB group experienced relapse during the follow-up period, no patient in the LTBI or non-TB groups developed TB. IGRA and chest X-ray, followed by chest CT for those IGRA positive or with suspicious X-ray findings, appears to be an effective means of TB contact screening and infection prevention.

Citation: Fujikawa A, Fujii T, Mimura S, Takahashi R, Sakai M, et al. (2014) Tuberculosis Contact Investigation Using Interferon-Gamma Release Assay with Chest X-Ray and Computed Tomography. PLoS ONE 9(1): e85612. doi:10.1371/journal.pone.0085612

Editor: Robert J. Wilkinson, Institute of Infectious Diseases and Molecular Medicine, South Africa

Received August 26, 2013; **Accepted** December 6, 2013; **Published** January 14, 2014

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Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

As a country with an intermediate tuberculosis (TB) burden, Japan occasionally reports an outbreak or case of group infection of TB. Between September 2009 and January 2010, 6 cases of active TB were reported within the jurisdiction of the Eastern Army, Ground Self-Defense Force (SDF). An epidemiological study revealed that all 6 members had completed a training program for new staff members that had been held at the same military post between April and June 2009. To prevent the further spread of TB according to Article 17 of the Japanese Law of Infectious Disease, the Medical Department of the Ground Staff Office, Ministry of Defense, decided to conduct temporary health check-ups as a rapid means of screening surveillance for those who had been exposed to the infected members.

Prior contact TB screening consisted of health check-ups, an interview, chest X-ray, and tuberculin skin test (TST). TST results were obtained by measuring skin redness and the presence or absence of cutaneous induration 48 hours and 8 weeks after intradermal injection of purified protein derivative, using results from a previous TST performed at the time of military enrollment for reference. Increased size of skin redness or presence of cutaneous induration was defined as positive TST. Subjects with a positive TST and suspicious CXR findings were treated as active TB patients. Subjects with positive TST and negative CXR findings were considered to represent latent tuberculosis infection (LTBI) and received preventive TB treatment, depending on their proximity to an index case or clinical symptoms and signs.

In conducting screening for group infection, completing the task as rapidly and accurately as possible is important to preventing the spread of infection and emergence of an epidemic. A major challenge in completing this task has been, and continues to be, the lack of an established screening protocol for contact investigation of TB group infection appropriate for the conditions faced by the Ground SDF members, who are highly mobile and often act in groups over a broad area extending beyond several local municipalities. According to Article 17 of the Japanese Law of Infectious Disease, TB contact screening has been performed in each military post in collaboration with local government; however, the intensification of the management and information-gathering of the disease by the Japan Ground SDF and the SDF Central Hospital has appeared to be insufficient. Furthermore, when the protocol for this contact investigation was created by the Health Management Center of the Japan SDF Central

Hospital, several limitations were expected regarding screening using the traditional TST. First, uncertainty was expected regarding the reliability of the test results because all the subjects had previously received the Bacillus Calmette–Guerin (BCG) vaccination. Second, confusion regarding the results was expected due to inconsistent reading of the TST across military posts, as the participants had been assigned to 35 different military work posts after completing training. Third, much time was expected to be necessary to complete the screening process, and repeated visits by the examinees were anticipated.

In consideration of these limitations, this contact investigation conducted screening for tuberculosis using interferon-gamma release assay (IGRA) rather than the TST. IGRA uses specific antigens of *Mycobacterium tuberculosis,* such as early antigenic target-6 (ESAT-6), culture filtrate protein 10 (CFP-10), and TB 7.7, to stimulate whole blood to induce interferon-gamma production by antigen-specific T cells. Therefore, the IGRA results are not affected by history of BCG vaccination. They are presented in terms of numerical values, allowing for objective interpretation, and are produced within a relatively shorter time frame. Furthermore, studies of contact investigations of TB group infection that used the IGRA and TST have reported that the detection accuracy of the IGRA is equal to or better than that of the TST [1–6]. However, IGRA alone cannot distinguish between active TB and LTBI, which is important for differential treatment of these conditions [7,8]. For this reason, chest computed tomography (CT) was also used to detect possible early pulmonary TB, which may not be detected by chest X-ray [9], in the protocol.

Here, we verified the efficacy of initial screening consisting of IGRA prior to chest X-ray and chest CT for the management of TB group infection in a highly mobile population that often acted in groups.

Methods

The protocol for this contact investigation was approved by the Medical Department of the Ground Staff Office, Ministry of Defense, and the Research Review Committee of the Japan SDF Central Hospital, and was implemented at the initiative of the Army Surgeon, Eastern Army. Written consent was obtained from all subjects. Contact investigation of 884 subjects, including those who had completed training for new staff members and other staff (mean age, 23.4±5.7 years; range, 19–53 years) and excluding 86 people who had resigned their commission before the initiation of the study, began in March 2010. Collection of blood samples for IGRA and performance of medical examinations and interviews at the 35 military posts at which the subjects had been assigned also began in March 2010. Blood samples were sent to Japan SDF Central Hospital for evaluation. Chest X-rays were obtained for all subjects in April 2010. For those found either IGRA positive or IGRA negative but with chest X-ray results suggesting pulmonary TB, chest CT was performed between April and June 2010 (Fig. 1).

(1) Interferon-gamma Release Assay

For IGRA, the QuantiFERON–TB Gold in Tube test (QFT-3G; Cellestis, Carnegie, Australia) was used. Three milliliters of whole blood was drawn from each subject and treated according to the manufacturer's protocol. A QFT-3G test result of less than 0.35 IU/ml was considered negative for TB. Repeated QFT-3G testing of subjects found to have active TB or LTBI was conducted from 1 year after initial diagnosis.

(2) Chest X-ray Examination

A digital radiographic system (RADREX-iDRAD-3000A; Toshiba, Tokyo, Japan) was used to obtain chest X-rays in the posterior–anterior direction. Diagnosis of possible pulmonary TB was made independently using a 3-megapixel monochrome LCD monitor (ME315L plus; Totoku Electric, Tokyo, Japan) by one radiologist with 23 years of experience and 2 respiratory medicine physicians with 34 and 16 years of experience, respectively, who were blind to the QFT-3G results. Each reader read a subset of images and independently classified the result as positive or negative. CXR findings considered suspicious for active TB include infiltration, atelectasis, cavitation, clusters of nodular opacities, diffuse nodular opacities, and pleural effusion. If any findings were suggestive of active pulmonary TB, the subject was categorized as chest X-ray positive.

(3) Chest CT Examination

Chest CT was performed using a 6-row multi-detector row CT machine (SOMATOM Emotion 6; Siemens, Tokyo, Japan) set at the parameters typically used at the facility (130 kVp, effective current 95 mAs, 2-mm collimation × 6, pitch of 1.4). Based on measurements of the dosimetry phantom (diameter 32 cm, length 35 cm) under automatic exposure control by dose-modulation software (Software CARE Dose4D; Siemens, Tokyo, Japan), the radiation exposure of each subject was estimated to be less than 2.8 mSv. Two types of axial images (5-mm thickness/5-mm interval and 2.5-mm thickness/1.5-mm interval, respectively) were obtained and reconstructed before being reviewed on the picture archiving and communication system monitor by 2 radiologists with 23 and 19 years of experience, respectively, who were blind to the chest X-ray results. Positive CT criteria were based on the report by Im et al. [10]. A positive chest CT result was indicated if one or more of the following evaluation criteria was observed: 1) consolidation, 2) cavitation, 3) clusters of non-calcified nodules ≤4 mm in diameter associated with dilated or thickened peripheral airway walls, 4) non-calcified nodules >4 mm in diameter with adjacent small nodules, and/or 5) widespread distribution of small nodules 1–4 mm in diameter. The presence or absence of lymphadenopathy or pleural effusion was also recorded.

(4) Bacteriological Testing

Depending on whether the subject was able to expectorate sputum, a respiratory or gastric fluid sample was collected at least 3 times from all subjects who were chest-CT positive, regardless of QFT-3G status. In addition to acid-fast smear testing, mycobacterial culturing was performed using solid 1% Ogawa egg medium (Nissui, Tokyo, Japan), incubated in tubes at 37°C and observed weekly for up to 8 weeks. In accordance with the manufacturer's protocol, observation of more than one colony was considered a positive result. Subsequent genetic analysis of cultured *M. tuberculosis* was performed using the restriction fragment length polymorphism (RFLP) technique, which consists of agarose gel electrophoresis, Southern blotting, and hybridization after fragmentation of an extracted DNA sample by restriction enzyme to determine the length of the resulting DNA fragments.

(5) Diagnostic Criteria for Active TB and LTBI Groups

After the possibility of community-acquired pneumonia had been excluded, subjects with positive chest-CT results either with or without positive bacteriology were diagnosed with active TB and began a treatment regimen for active tuberculosis consisting of 300 mg isoniazid, 450 mg rifampicin, 750 mg ethambutol, and

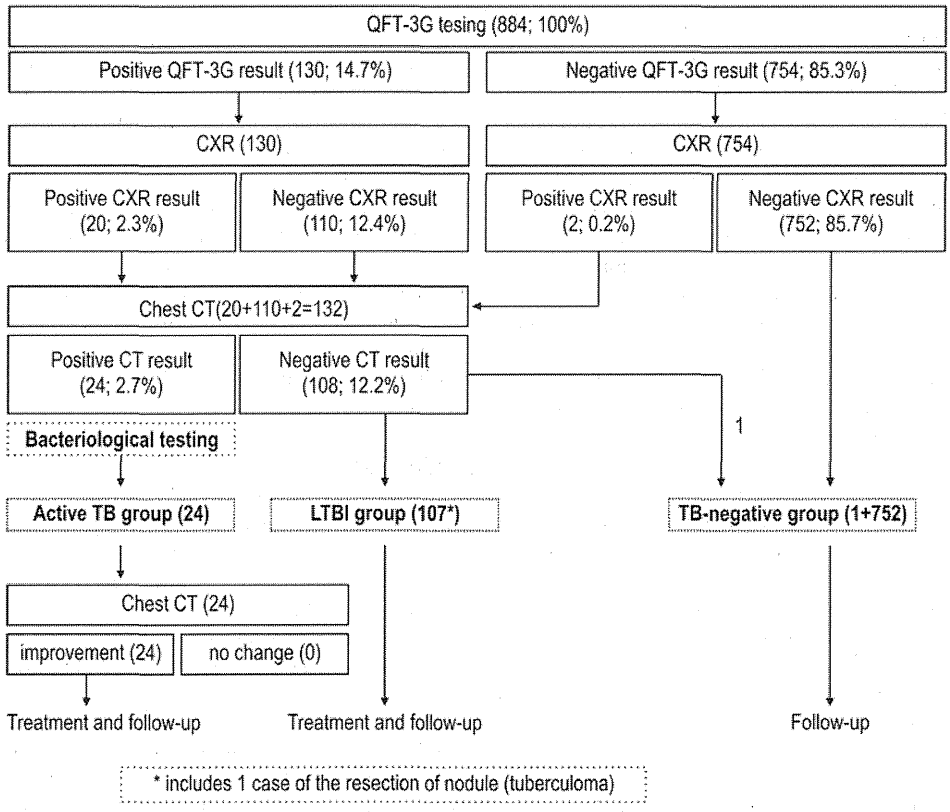

Figure 1. Contact investigation protocol for tuberculosis. The subjects were stratified into 3 groups according to the results of QFT-3G, chest X-ray, and chest CT.
doi:10.1371/journal.pone.0085612.g001

1000 mg pyrazinamide per day for 26 weeks. Chest CT examination was performed 6 months after treatment initiation to determine the extent of improvement.

Subjects with positive QFT-3G results and negative chest CT/bacteriological results were diagnosed with LTBI and, after receiving information regarding the Directly Observed Treatment, Short Course, began a regimen of anti-TB drugs, either 300 mg isoniazid or 450 mg rifampicin per day, for 26 weeks. In the 2 years following completion of treatment, subjects underwent a follow-up examination every 6 months consisting of medical examination, an interview, and chest X-ray, as well as sputum bacteriological examination if any change on chest X-ray was observed.

(6) Comparison of QFT-3G Values

The Mann–Whitney U-test was used to compare the QFT-3G values obtained for the active TB and LTBI groups at screening and the values obtained for the *M. tuberculosis* culture-positive and culture-negative subgroups of the active tuberculosis group at screening. The Wilcoxon signed-rank test was used to compare the values obtained for the active TB and LTBI groups at screening and after treatment if QFT-3G values had been obtained during the follow-up period. Excel 2010 software (Microsoft, Tokyo, Japan) was used for all statistical analyses.

Results

Of the 884 subjects, 130 (14.7%) were QFT-3G positive, and 754 (85.3%) were QFT-3G negative. Of the 22 subjects with chest X-ray images suggestive of TB, 2 were QFT-3G negative and subsequently underwent chest CT. Of the 110 subjects who were QFT-3G positive and chest X-ray negative, 8 subsequently underwent chest CT examination at other facilities because of their remote work locations. Review of the chest CT of the 132 subjects, including 130 QFT-3G-positive subjects and 2 QFT-3G-negative subjects with positive chest X-ray, indicated that 24 were positive and 108 negative for active TB. Review of the chest CT results of the 2 subjects with negative QFT-3G results indicated that one was positive and one was negative for active TB (Fig. 1). All 24 subjects with positive chest CT examination results demonstrated clusters of non-calcified nodules associated with abnormal peripheral bronchioles. While 15 also had non-calcified nodules with adjacent small nodules and 3 also had cavitation, none demonstrated lymphadenopathy or pleural effusion (Fig. 2). No risk factors such as HIV infection or diabetes were noted among the 24 active TB subjects. Twenty of the 24 subjects in the active TB group and 39 of 108 subjects in the LTBI group had resided on the same floor of the barrack. At the time of screening, no significant clinical symptoms or signs were noted, although almost all subjects recalled a passing mild cough similar to a common cold during the training period.

(1) Active TB Group

In this investigation, 12 of 24 active TB subjects were chest X-ray negative, while 9 of 20 subjects who were both QFT-3G and chest X-ray positive, were chest CT negative (Fig. 3). Therefore, 11 of the 23 active TB subjects with positive QFT-3G were both chest X-ray and chest CT positive.

Culture testing of either a sputum or gastric fluid sample from each subject indicated that 10 of the 24 subjects who had been

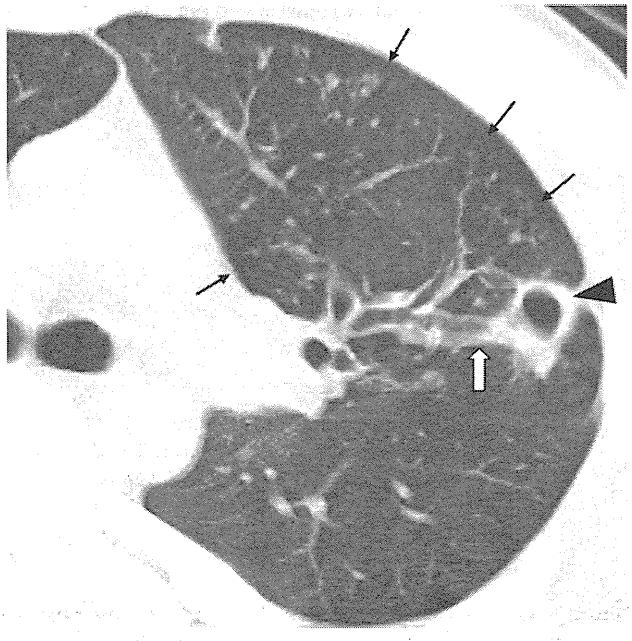

Figure 2. CT findings of active TB. A chest CT image of the left upper lobe of the lung in a 19-year-old subject demonstrated clustered non-calcified nodules associated with dilated peripheral airways (thin arrows) and a cavitation (arrow head) with dilated and thickened subsegmental bronchus (white arrow).
doi:10.1371/journal.pone.0085612.g002

diagnosed with active TB were culture-positive for *M. tuberculosis*. Subsequent RFLP confirmed that the cultured *M. tuberculosis* samples from these 10 subjects were of an identical genotype. The genotype was also identical to that obtained from one of the original 6 active TB cases. Review of the chest X-ray findings of the 10 subjects indicated active TB in 7, of whom one was QFT-3G negative (Fig. 3). The signs of TB in the CT findings of all subjects were found to have either improved or disappeared at the second chest CT examination performed 6 months after treatment initiation. However, one subject who experienced relapse of pulmonary TB 8 months after completion of the initial treatment was required to resume anti-tuberculosis treatment.

(2) LTBI Group

Of the 107 subjects diagnosed with LTBI, 4 who suffered adverse effects from isoniazid were subsequently treated with rifampicin. No TB development was observed in any of the subjects during the 2-year follow-up period after completion of anti-TB treatment. Of the 108 subjects with negative chest CT results, 10 had abnormal findings that did not meet the criteria of active TB: 4 had thick linear attenuation; 2 had solitary small ground-glass attenuation; and 1 each had arteriovenous malformation, pulmonary cyst, solitary calcified nodule, and solitary nodule. When, upon the subject's request for further evaluation, surgical resection of the lesion of one subject with a solitary nodule was performed, a tuberculoma was detected. Subsequent chest CT examination prior to anti-TB drug administration revealed that the ground-glass attenuation that had been observed in 2 of the subjects had disappeared.

(3) Non-TB Group

No TB development was observed in the non-TB group during the 2-year follow-up period.

(4) Comparison of the QFT-3G Values

Repeated QFT-3G testing of 15 subjects in the active TB group and 65 subjects in the LTBI group was performed 1 year after initial diagnosis. Due to the limitations of the sampling date schedule for QFT-3G in our outpatient clinic, subjects' training schedule, and remote work locations, repeated QFT-3G testing could not be performed for all subjects in the active TB and LTBI groups. Comparison of the QFT-3G values of the active TB and LTBI groups at screening indicated that the values of the former were statistically significantly higher than those of the latter (Fig. 4). Comparison of the QFT-3G values for the *M. tuberculosis* positive- and negative-culture subgroups of the active TB group indicated no significant differences between the 2 groups (Fig. 5). Comparison of the QFT-3G values obtained at screening and follow-up indicated that the values for both the active TB and LTBI groups had significantly decreased at follow-up (Fig. 6 and 7).

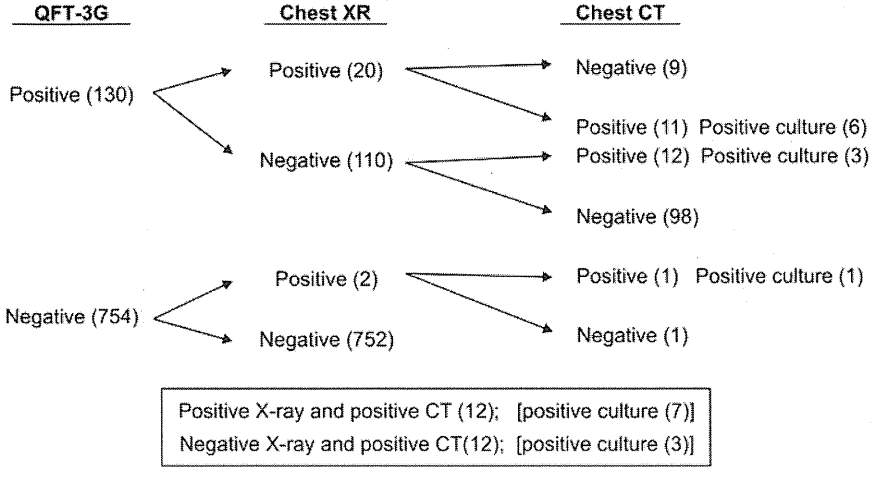

Figure 3. Bacteriologically positive cases of active tuberculosis diagnosed by the investigation protocol. Ten of 24 subjects diagnosed with active TB in the protocol showed positive culture for *M. tuberculosis*. Of these 10 subjects, 3 were missed by chest X-ray.
doi:10.1371/journal.pone.0085612.g003

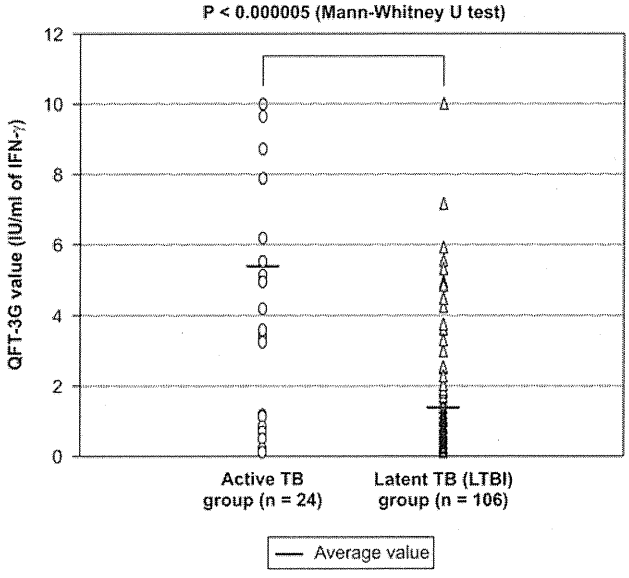

Figure 4. Comparison of QFT-3G values at screening between the active TB group and latent (LTBI) group. Comparison showed a statistically significantly higher value in the active TB group than in the LTBI group.
doi:10.1371/journal.pone.0085612.g004

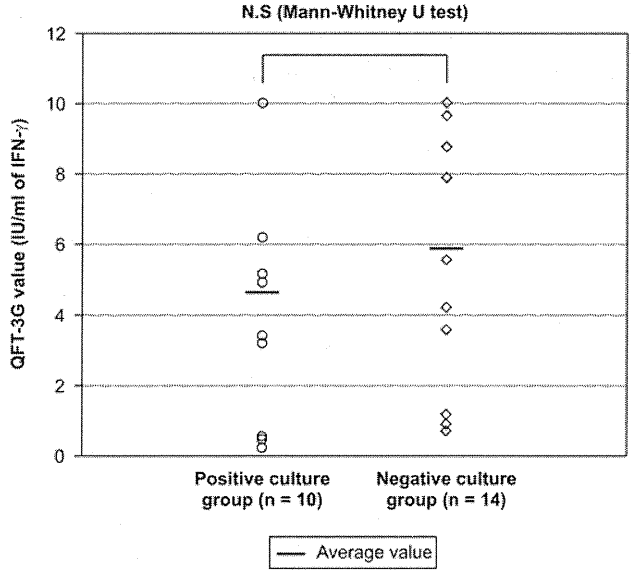

Figure 5. Comparison of QFT-3G values between the positive and negative culture groups at diagnosis of active TB. Comparison showed no significant difference between the 2 groups.
doi:10.1371/journal.pone.0085612.g005

(5) Determination of the Sensitivity and Specificity of the QFT-3G Test

Because it was uncertain whether the case with tuberculoma had any correlation with this group infection, the case was excluded from this analysis. The sensitivity of the QFT-3G screening test in detecting active tuberculosis was found to be 95.8% (23/24), and the specificity was 87.7% (753/859).

Discussion

As no signs of new infection were detected in either the LTBI or TB-negative groups during the 2-year follow-up period, the contact surveillance conducted in this study, which was composed of the complementary screening methods of QFT-3G testing, chest X-ray, and chest CT, appears to have achieved effective prevention of infection spread.

The QFT-3G-positive rate of the subjects in this study was 14.7%, which, being much higher than the estimated rate of approximately 1% in the young Japanese adult population, indicates that the subjects had likely experienced very close group contact. We acknowledge the possibility of some of the active TB subjects diagnosed in this investigation, might have had a prior contact [11].

IGRA alone cannot be used to distinguish between active TB and LTBI. Previous contact investigations have reported the rate of IGRA-positive subjects who develop active TB to range from 10% to 30% [12,13]. In the current study, subjects found to be QFT-3G positive but chest-CT negative were diagnosed with and treated for LTBI, although the only gold standard for diagnosis of LTBI is development of active TB [8]. As the population studied here is very mobile and maintains close contact within groups, treating LTBI members is important for group infection management, leading to an inevitable tendency toward overdiagnosis. Interestingly, our findings that QFT-3G values were higher in the active TB group than in the LTBI group and that these values decreased after treatment are consistent with previous studies investigating the quantitative discrimination between active TB and LTBI [14–16] (Fig. 4, 6, 7). However, because all subjects diagnosed with LTBI were treated, we were unable to establish a cut-off value by which to determine the necessity of preventive therapy for subjects with positive QFT-3G test results but negative chest CT results.

The definitive gold standard for diagnosing active TB is positive *M. tuberculosis* culture of a sputum or gastric fluid sample. A major challenge in TB testing has been underdiagnosis due to factors such as test technique, test accuracy, or patient disease state [17,18]. Of the 24 subjects diagnosed with active TB, 10 tested positive for *M. tuberculosis*, yielding a culture-positive rate (41.7%)

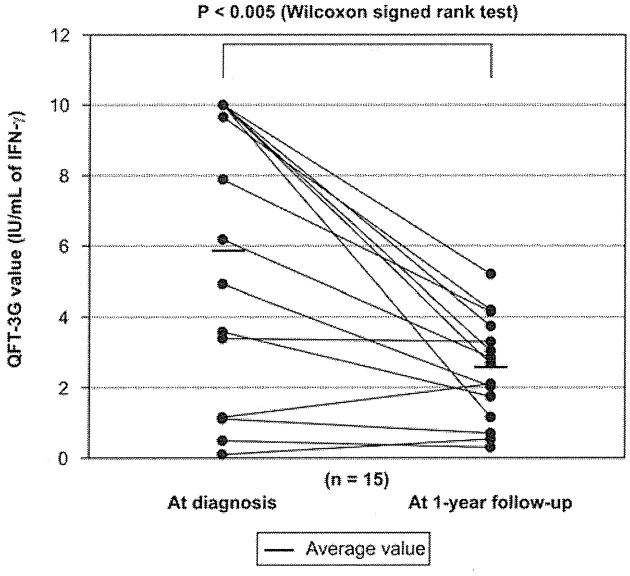

Figure 6. Comparison of QFT-3G values at diagnosis and after treatment of active TB. Comparison showed a statistically significant reduction of QFT-3G values after treatment.
doi:10.1371/journal.pone.0085612.g006

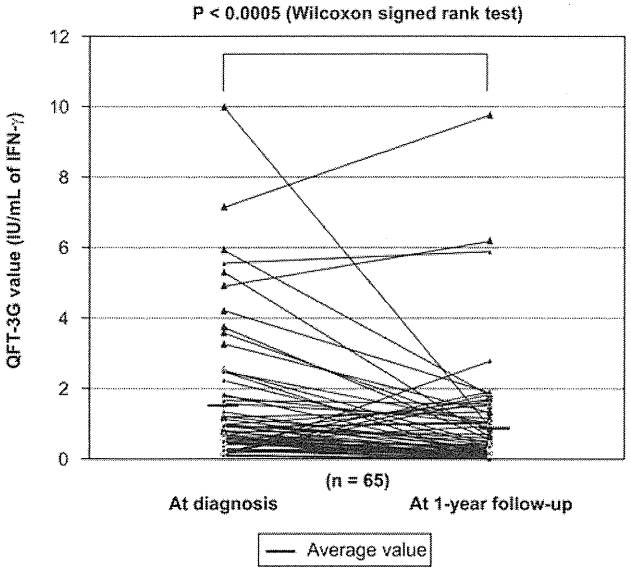

Figure 7. Comparison of QFT-3G values at diagnosis and after treatment of latent TB. Comparison showed a statistically significant reduction of the QFT-3G values after treatment.
doi:10.1371/journal.pone.0085612.g007

similar to that reported by Lee et al. [19]. The early active TB cases detected using chest CT may have been culture negative because early active TB is believed to be relatively more common in contact investigations. The lack of overt symptoms of TB also can be suggestive of early infection. Furthermore, no significant differences between the QFT-3G values obtained for the culture-positive and culture-negative groups were observed among subjects who had been definitively diagnosed with active TB based on CT findings (Fig. 5). Nonetheless, we acknowledge the possibility that not all subjects in the active TB group would necessarily progress to severe disease, which should be aggressively treated. However, we emphasized control of the disease in our target population.

One advantage of chest CT may be the prevention of development of isoniazid-resistant *M. tuberculosis*, a risk posed by the use of isoniazid therapy alone for active TB subjects who were misdiagnosed with LTBI by positive QFT-3G and negative X-ray results. Another advantage of chest CT is the identification of false-positive chest X-ray results.

Although it is a very effective tool in group infection management, CT screening poses the risk of excessive radiation exposure. Use of dose-modulation software has been estimated to achieve an approximate 25% reduction in the effective dose of 2.8 mSv per chest CT examination [20]. Considering that the average natural background radiation level in the US is 3.1 mSv, the positive benefits of chest CT appear to outweigh its risks. Moreover, the cost-effectiveness of the methods used in this contact investigation have been verified by a recent study reporting that a combined QFT-3G-testing and CT-examination strategy yielded the combined greatest benefit at the lowest cost of all possible combinations of TB screening tests [21].

Several aspects of this study may limit the generalization of the findings. One limitation is the study sample, which was composed of healthy adults approximately 20 years of age and among whom the percentage found to be QFT-3G negative and chest X-ray negative was relatively low. Older populations are expected to have higher positive rates of QFT-3G or chest X-ray findings in countries with an intermediate or high TB burden. Thus, it is possible that the need for chest CT examination to detect active TB increases with age. A second limitation is the inability to follow subjects who had resigned their commission before the initiation of the investigation. A third limitation is that results obtained using IGRA and TST were not compared. However, considering the characteristics of the target population, such as history of BCG vaccination in all subjects and time and personnel constraints, IGRA can be considered useful.

In conclusion, the screening methodology of TB contact investigation used in this unique setting for a highly mobile population, after very close group contact, was appropriate and effective. Specifically, use of a combination screening protocol of QFT-3G testing and chest X-ray examination appeared to be effective in ruling out infection in subjects with no signs of TB, while a protocol of QFT-3G testing and chest CT examination also appeared to be effective in detecting active TB and, thus, differentiating between cases of active and latent TB.

Acknowledgments

We would like to express our sincere appreciation to Kyuma Ota, MD, Department of Internal Medicine, Niigata Prefectural Central Hospital, for providing support in the conduct of this study and for performing chest CT of 8 subjects.

Author Contributions

Conceived and designed the experiments: AF TF. Performed the experiments: AF TF SAM RT MS SS YK SHM. Analyzed the data: AF TF SAM RT SHM. Contributed reagents/materials/analysis tools: TF YU SHM TM. Wrote the paper: AF TF TM. Collection and assembly of data: SAM MS SS YK SHM YU.

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講　座

電子顕微鏡観察で可視化される結核菌の素顔

Visualization and Characterization of Tubercle Bacilli through Electron Microscopy

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要　旨　結核菌（*Mycobacterium tuberculosis*）は，かつて世界で最も高い死亡率を記録した感染症である結核の原因菌である．1882年，Robert Kochにより発見された．幅約0.5 μm，長さ約3 μmのやや湾曲した普通の桿菌であるが，結核菌を含む抗酸菌属の細菌はcarbol fuchsin（石炭酸フクシン）やauramine Oを用いる染色で，塩酸アルコールによる脱色に耐える「抗酸性（acid-fastness）」を示す．また，結核菌のコロニーはコード形成と呼ばれる特異的な形態を呈し，感染宿主細胞内ではphagosome-lysosome fusion（P-L fusion）を阻害して殺菌プロセスから逃れるなど極めて特徴的な性質を示すが，その発現機序は未だ不明な部分が多い．本講座では結核菌が示すこれらの形態学的な特徴を電子顕微鏡観察を用いて検討した結果を紹介する．

キーワード：病原性細菌，結核菌，抗酸性，コード形成，phagosome-lysosome fusion

1. はじめに

エジプトのミイラの骨に骨カリエスと類似した病変が見つかり，さらにその病変部からは抗酸性を示す桿菌が検出されている．また，同じくエジプトから出土した別のミイラの骨病変からはDNAの抽出が行われ，PCRで結核菌特異的なDNAであることが証明されている[1]．一方，米国ワイオミング州にあるNatural trap caveで見つかった絶滅したバイソンの17,000年前の骨化石からは結核菌特異的なDNA配列に加え，毒力因子である脂質の存在がHPLC分析により明らかにされている[2,3]．これらの報告は，結核菌が既に少なくとも17,000年前から哺乳類を宿主として感染症を惹起し，2000年～5000年前には既に人類に脅威を及ぼす感染症であったことを示している．

結核菌を発見したRobert Kochはその業績により1905年にノーベル賞を受賞したが，受賞式での講演で，当時ドイツ国内には排菌のため入院を要する結核患者が約200,000人存在すると推定している[4]．一方，日本ではその約40年後，1943年に年間17万人を超える結核死が記録され，その年の結核による死亡順位は1位であった[5]．現在に目を向けてみると，WHOの統計によれば2011年に全世界で年間約1200万人の結核患者が存在し，約140万人が死亡している[6]．一方，日本国内では2011年の統計で年末登録者が約55,000例で，約2,200人が死亡しており，死亡順位は25位になっている[7]．これら統計的データを見ると，世界的に結核が高蔓延であった長い時代の後，ストレプトマイシンやイソニアジドなどの抗生物質による化学療法が患者の減少に大きく寄与してきたことがうかがえる．しかし，他の感染症と同様に抗生物質が効かない耐性菌が出現し，現在は一次薬であるイソニアジドとリファンピシンの両剤に耐性を示す多剤耐性（MDR）菌に加え（全患者の0.7～0.8%），二次薬のフルオロキノロンと注射薬にも耐性を示す超多剤耐性菌（MDRの約10%）も出現し，今なお死に至る感染症としての脅威を維持している[8]．

本講座では，バイオセーフティーレベル3領域での取扱を義務付けられた病原性結核菌の特徴を光学顕微鏡と電子顕微鏡を用いた観察で紹介させて頂きたい．

2. 結核菌の形態学的基礎データ

結核菌は，原核生物の真正細菌ドメイン，放線菌門，放線菌綱，放線菌目，コリネバクテリウム亜目のマイコバクテリア科，マイコバクテリア属に属するグラム陽性桿菌である．抗酸菌属には100種以上の抗酸菌が含まれ，ヒトに対して病原性を有する種は結核菌を含めて50種以上ある[9]．ハンセン病の原因菌である*M. leprae*は現時点でも培養不可能であるが，これまでに同定された他の抗酸菌は液体培地，寒天培地の他に，全卵を用いる小川培地，Löwenstein-Jensen培地などを用いて培養できる（図1a）．

光学顕微鏡観察では抗酸菌は一般的な細菌の鑑別染色であるグラム染色陽性で，更に抗酸菌を鑑別するための抗酸菌染色で陽性を示す．グラム染色では他のグラム陽性菌の様に明瞭な陽性所見を示さない（塗抹標本中の抗酸菌が均一に明瞭

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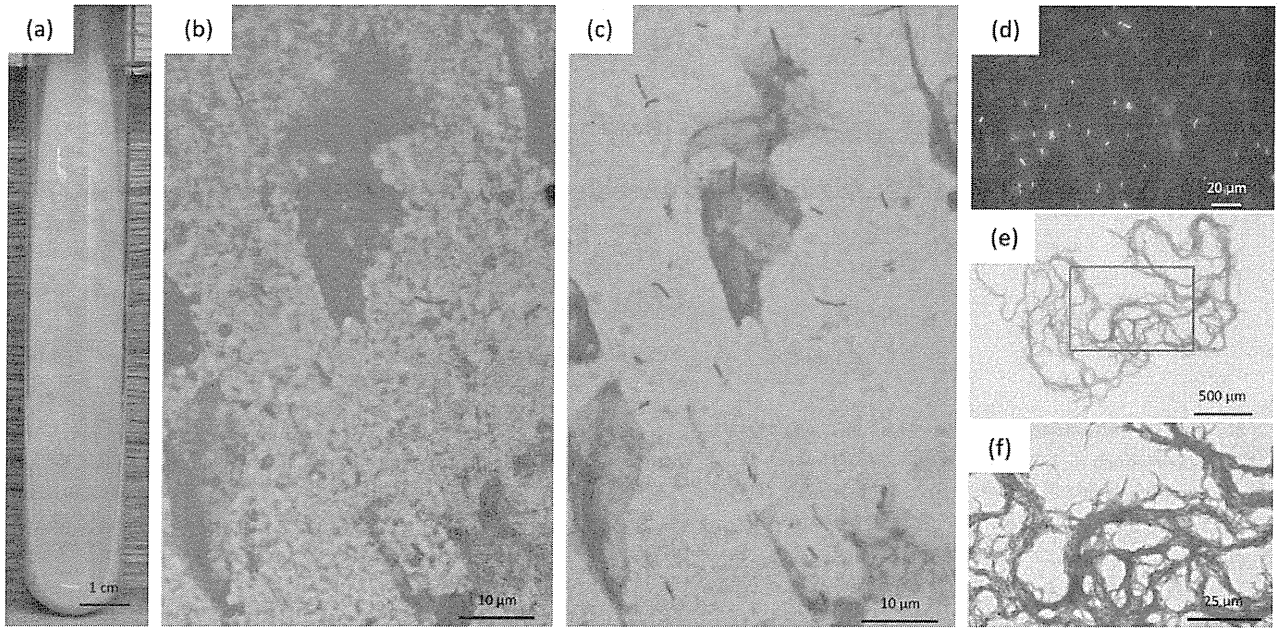

図1　結核菌の肉眼及び光学顕微鏡像．(a) 1%小川培地に接種後4週間目の結核菌コロニー．(b) 結核菌の人工痰の塗抹標本のGram染色像．(c) (b) と同じ視野を脱色後抗酸菌 (Ziehl-Neelsen) 染色した光顕像．(b) で青く染色され (Gram陽性)，(c) で赤く染色されている桿菌が結核菌 (抗酸菌染色陽性)．(d) auramine 0を用いた蛍光抗酸菌染色．(e) detergentを含まない液体培地で長期間培養した結核菌に見られたコード形成像．(f) (e) の青い長方形部分を拡大した像．結核菌が集合してコード状の塊を形成している．

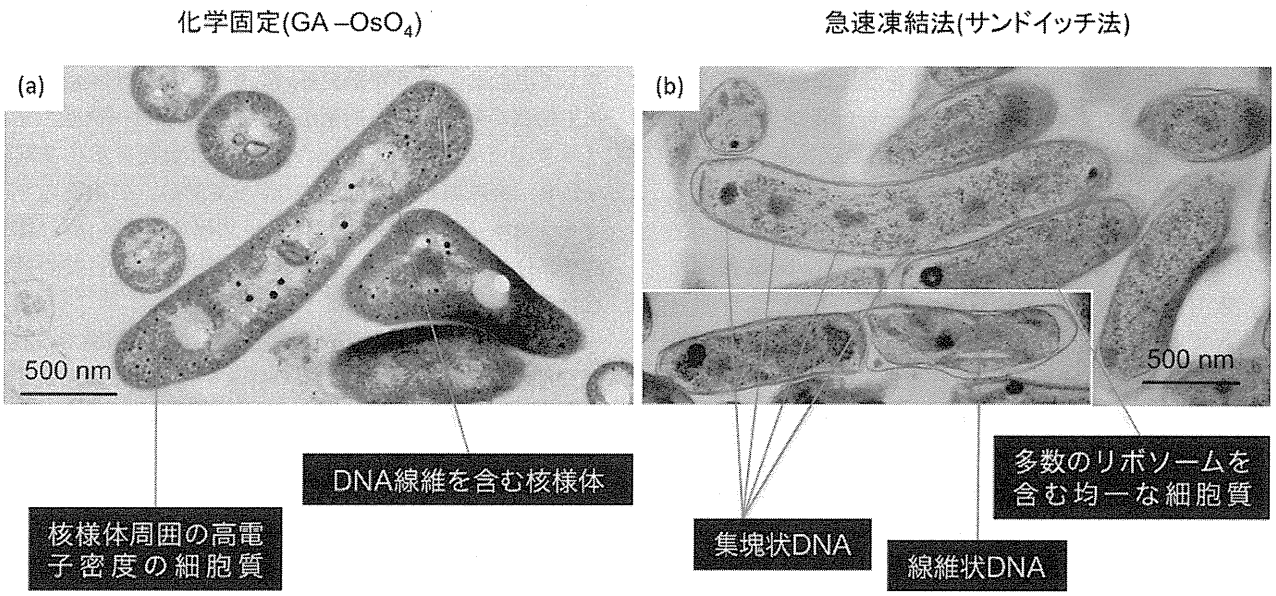

図2　化学固定 (a) と急速凍結置換固定法 (b) で調製した結核菌超薄切片の透過電子顕微鏡像．化学固定では菌体周辺領域と中心部で電子密度が異なり，いわゆる核様体にDNAと思われる線維状構造が見られる．一方，急速凍結置換固定法で調節した標本では，菌体内は均一で多数のリボソームが一様に分布し，DNAと思われる線維状構造がその中に埋め込まれたように見える．

な陽性を示さない) ため「不定性」と記されることもあるが，標本中に陽性菌が少なからず存在するため，グラム陽性菌として扱われる．「抗酸菌 (acid-fast bacilli)」という名称は，抗酸菌染色の染色過程でcarbol fuchsinやauramine Oによる主染色の後，3%塩酸アルコールによる脱色に耐えて，主染色色素が菌体内に残るため，明視野観察ではfuchsinの赤，蛍光観察ではauramine Oの蛍光により検出できることが所以である．図 (1b～d) は著者らが開発したポリアクリルアミドを基材とした人工痰の塗抹標本である[10]．

また，結核菌の光学顕微鏡所見におけるもう一つの重要な特徴としてコロニーのコード形成が挙げられる (図1e, f)．コード形成は結核菌を発見したKochの論文にもその形跡が

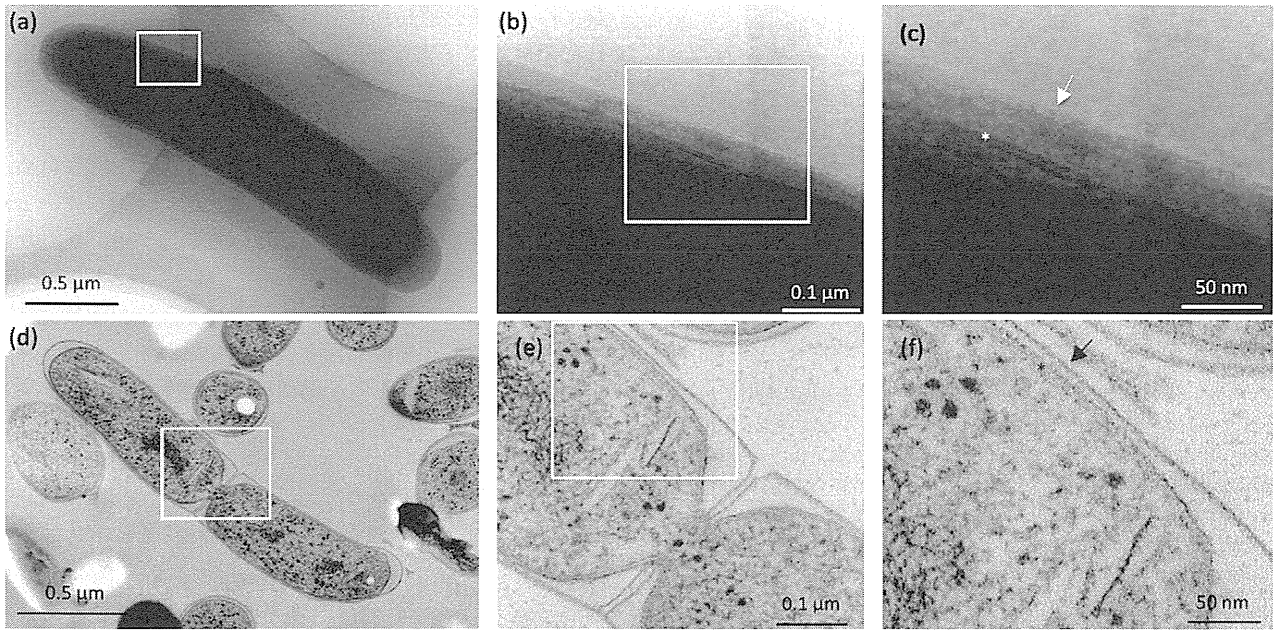

図3　氷包埋（a–c）と急速凍結置換固定法（d–f）で調製した結核菌標本での細胞壁外膜の観察．（c）と（f）で細胞膜（*）の外側に明瞭な細胞壁外膜（矢印）が観察出来る．特に（c）では，細胞壁外膜が2重層として観察できる．

記されており，初期の研究では細胞壁成分のtrehalose dimycolate（TDM）が結核菌の病原性と特有のコード形成を誘導する細胞壁成分と考えられたが，後に，結核菌や病原性抗酸菌以外の非病原性，非結核性抗酸菌の細胞壁にもTDMが存在することが明らかになり，コード形成とTDMの直接の関連については否定されている．なぜこのような構造が形成されるのか未だ不明な点が多いが，結核菌体表面にミコール酸を主成分とする多量の脂質が存在するため，隣接する菌体が密着したまま増殖することが原因と考えられ，現時点では細胞壁構造を決定する複数の遺伝子により支配されていると考えるのが妥当であろう[11]．

以上が光学顕微鏡観察により得られる結核菌の形態学的特徴であるが，以下に電子顕微鏡観察で得られる基礎的データと上記の「抗酸性」と「コード形成」及び実験動物を用いた感染実験で得られたサンプルで電子顕微鏡を用いて観察したデータを紹介する．

3.　結核菌の透過電子顕微鏡による基礎的所見

結核菌の透過電子顕微鏡観察は，一般的なグルタルアルデヒドと四酸化オスミウムによる化学固定とエポキシ樹脂包埋により調製されたサンプルの超薄切片で行われてきた（図2a）．近年，急速凍結法や氷包埋およびCEMOVIS（cryo-electron microscopy of vitreous section）を用いた観察も行われるようになった[12～18]．これらの報告では従来の化学固定サンプルでは観察されなかったいくつかの特徴が明らかにされた．研究者により細かな見解の違いはあるが，共通した観察結果の一つは，従来，膜構造による境界が存在しないため不明瞭ではあるが遺伝情報DNAが存在する「核様体」として一つの区画された細胞質内の領域が想定されていたが（図2a），急速凍結法や氷包埋，CEMOVISによる観察では細胞質内は極めて均一で，多数のリボソームとゲノムDNAと思われる線維が観察されるだけであった（図2b）．また，これらの手法で調製された標本の観察で得られたもう一つの重要な知見は，結核菌の細胞壁に外膜が存在することを証明したことである（図3a，b）．グラム陰性菌には細胞壁外膜が存在することは既に明らかにされていたが，急速凍結法，氷包埋やCEMOVISで観察するとグラム陰性菌と結核菌の両者には極めて類似した細胞壁外膜が存在することが明らかにされた[15,16,19]．

4.　抗酸性発現の機序に関する電子顕微鏡による検討

既に述べたように結核菌を含む抗酸菌の極めて特異的な特徴の一つが「抗酸性」である．抗酸性の発現の基になる詳細な機序は未だ不明であるが，抗酸菌の主要かつ特異的な細胞壁構成成分であるミコール酸（mycolic acid）の存在が重要な役割を果たしていることは確かである．抗酸菌の脂肪酸・ミコール酸の合成経路の一つであるFAS-II（type II fatty acid synthases）酵素群には2つのβ-ketoacyl-AcpM synthase, KasA，KasBが存在する．両酵素は連携してミコール酸β鎖の伸長を行うが，KasAは伸長の開始とその後のβ鎖の伸長，KasBはβ鎖伸長の後半過程に関与するとされている．KasAをコードする遺伝子*kasA*を欠損した株は，溶菌を起こし増殖できないため実験材料としては使用できないが，KasBをコードする遺伝子*kasB*をトランスポゾン導入によりknock-outした変異株は動物感染実験における毒力の低下と抗酸性を失うことが明らかになった（図4）[20～22]．著者は，この