(60%) of 10 patients with negative smears (p=0.07).

Data for the 33 people in Group 2 whose cultures were negative for *M. tuberculosis* despite symptoms and suspicion of active tuberculosis are shown in Figure 1, Panel C. For the 25 tuberculosis suspects from whom mycobacteria were not recovered, 56% (14) were positive to either CFP-10 or ESAT-6 in the whole blood IFN-γ assay, a significantly smaller proportion as compare to those with culture-confirmed *M. tuberculosis* infection (89%; Chi square test, p = 0.0001). The whole blood IFN-γ assay with either antigen was positive for all three patients from whom *M. kansasii* was recovered. For one of the 5 patients from whom MAC was recovered, the CFP-10 response was positive (7.5IU/ml IFN-γ).

To examine the effect of age on sensitivity of the whole blood IFN-γ assay and the TST, data from the 110 patients with confirmed tuberculosis, and whose ages were recorded, were stratified as shown in Table 3. Logistic regression analyses were used to estimate the associations between age and QFT response, and between age and TST response. On average, persons were 0.83 times as likely to have a positive QFT and 0.71 times as likely to have a positive TST, compared to persons ten years younger. The 95% confidence interval for the former odds ratio was 0.56 to 1.23, with decline being not statistically significant (P = 0.35), and that for the latter was 0.53 to 0.94, with statistically significant decline (P = 0.015).

DISCUSSION

The current study demonstrates a high degree of accuracy in detecting *M. tuberculosis* infection using the whole blood IFN-γ assay with the *M. tuberculosis*-specific proteins CFP-10 and ESAT-6. The assay was shown to be highly specific (greater than 98%) in BCG vaccinated low risk subjects (Group 1) assumed to be truly free of *M. tuberculosis* infection. Specificity of the whole blood IFN-γ assay was much better than the specificity observed for the TST in the present study (35.4% using a 10 mm induration cut-off), or previously reported for Japan (10%).[11] While we assumed that none of the Group 1 subjects were infected with *M. tuberculosis*, it is probable that some of the 216 subjects had been infected, as the prevalence of *M. tuberculosis* infection in 20 year old people in Japan is estimated at 1%.[12] Thus, the true specificity of the test may be higher than that estimated in the present study.

To estimate sensitivity of the whole blood IFN-γ test, the presence of culture confirmed *M. tuberculosis* infection was used as the standard. This approach has been widely used in sensitivity studies with the TST, often using patients who were receiving, or who had completed, treatment at the time of testing.[3;13-16] However, as it is well documented that both IFN-γ responses can vary in relation to anti-tuberculosis treatment,[3;17-19] we limited this study to patients who had received minimal or no treatment at the time of testing. At the time of enrollment into the study, all 152 Group 2 subjects had radiological and/or clinical signs suggesting tuberculosis and sensitivity was estimated from the 118 who had *M. tuberculosis* recovered subsequently by culture. Both ESAT-6 and CFP-10 demonstrated high positive rates in these patients (65.3% and 81.4% respectively) as compared to that in tuberculin skin testing (65.8%). Combining results from the *M. tuberculosis*-specific antigens improved test sensitivity to 89.0% and had little effect on specificity (98.1%).

The poor skin test specificity of TST (35.4%) seen in this study is likely to be predominantly a result of the extensive use of BCG vaccination, in Japan. However, poor skin test specificity may also be due to exposure or infection with NTM. Exposure to NTM, and not latent *M. tuberculosis* infection, appears to be responsible for the majority of 5-14 mm Mantoux test reactions among U.S.-born health care workers and medical students.[20] The present study was not designed to assess the specificity of the whole blood IFN-γ assay after exposure to NTM. However, given the reported mycobacterial species specificity of ESAT-6 and CFP-10, [5] the assay is likely to be negative for infection with *M. avium* complex (MAC) that is a major source of NTM infection. This was compatible with the study's finding that IFN-γ response to both of ESAT-6 and CFP-10 was negative in all patients who were culture-negative for *M. tuberculosis* and positive for MAC, except one. The latter MAC patient with a positive IFN-γ response could have co-infection with tuberculosis. On the other hand, the positive reactions are expected from people infected with *M. kansasii, M. marinum or M. szulagai* as the genes encoding both ESAT-6 and CFP-10 are present in these NTMs. [7] Therefore, it is not surprising that another three TB suspects positive for *M. kansasii* responeded to ESAT6 and/or CFP10 in the whole blood IFN-γ assay.

It remains to be confirmed whether the enhanced sensitivity of the whole blood IFN-γ assay over the TST, as seen for untreated patients in this study, will also be found for people with latent tuberculosis infection. However, such a possibility can be supported by reports that contacts of tuberculosis patients, who are possibly latently infected with *M. tuberculosis*, have stronger IFN-γ responses to *M. tuberculosis* antigens than do patients with active tuberculosis.[18-19;21-23] Further investigations on the performance of the CFP-10/ESAT-6 based whole blood IFN-γ assay in contact investigations and in other situations where *M.*

tuberculosis exposure can be quantified are required to further estimate the tests performance for detecting latent tuberculosis infection.

Screening for latent tuberculosis infection is most effective if those with positive test are likely to progress to clinical disease. A preliminary study by Doherty et al [24] demonstrated a close relationship between IFN-γ responses and subsequent development of clinical tuberculosis disease in household tuberculosis contacts in Ethiopia, but this needs corroboration in other populations of different immune status and background. In addition, while the current study indicates utility of the IFN-γ assay in screening adults for TB infection, further studies are required, including those in select patient populations such as children, people with x-ray evidence of prior tuberculosis, and those with HIV infection or other immunodeficiencies. Test utility would also be enhanced by studies determining the kinetics of IFN-γ response after infection, and the effect of anti-tuberculosis therapy on IFN-γ test results.

Previous studies have demonstrated the potential of both ESAT-6 and CFP-10 for the specific detection of *M. tuberculosis* infection in humans[4;5-10], although the method generally used to measure IFN-γ responses to these antigens, such as lymphocyte proliferation and IFN-γ ELISpot, are relatively complex and labor intensive to perform.[25] Some of these studies have demonstrated that a combination of results from ESAT-6 and CFP10 provides higher sensitivity than is seen with either antigen alone.[7;8] Additionally, Vordermeier et al demonstrated greater sensitivity with a cocktail of CFP-10 and ESAT-6 over either antigen alone, when used in a IFN-γ ELISpot assay,[26] and Arend et al, showed that use of both antigens increased test sensitivity, as there were variations in responses to CFP-10 and ESAT-6 between individuals with different HLA-DR types.[10] These data suggest that the combined use of both TB-specific

antigens is warranted to increase sensitivity and our results support this conclusion.

In addition to the high diagnostic accuracy resulting from the use of *M. tuberculosis* specific antigens, the whole blood IFN-γ assay offers many methodological and logistical advantages, both over the TST and other laboratory methods of immunological testing. The test requires a single patient visit, does not induce boosting of subsequent test results, and can provide results within one day. Inter-reader variability is low and results are highly reproducible[27] as it is a controlled laboratory assay. Importantly, whole blood testing uses minimal labor and simple equipment, allowing large numbers of samples to be tested concurrently.

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FIGURE LEGEND

Figure 1. Dot plot of individual's responses to CFP-10 and ESAT-6 for 118 culture positive TB patients (a), 213 subjects with a low risk for TB exposure (b), and 33 TB suspects whose TB status could not be determined, as *M. tuberculosis* could not be cultured (c). * For "ESAT/CFP" the data for the antigen (ESAT-6 or CFP-10) giving the highest response is shown. The dotted line is the cut-off of 0.35 IU/ml of IFN-γ.

ANTIGEN	AMINO ACID SEQUENCE
CFP-10 Peptide 1	MAEMKTDAATLAQEAGNFERISGDL
CFP-10 Peptide 2	GNFERISGDLKTQIDQVESTAGSLQ
CFP-10 Peptide 3	DQVESTAGSLQGQWRGAAGTAAQAAV
CFP-10 Peptide 4	AAGTAAQAAVVRFQEAANKQKQELD
CFP-10 Peptide 5	AANKQKQELDEISTNIRQAGVQYSR
CFP-10 Peptide 6	IRQAGVQYSRADEEQQQALSSQMGF
ESAT-6 Peptide 1	MTEQQWNFAGIEAAASAIQG
ESAT-6 Peptide 2	GIEAAASAIQGNVTSI
ESAT-6 Peptide 3	SAIQGNVTSIHSLLDEGKQSLTKLA
ESAT-6 Peptide 4	EGKQSLTKLAAAWGGSGSEAYQGVQ
ESAT-6 Peptide 5	SGSEAYQGVQQKWDATATELNNALQ
ESAT-6 Peptide 6	TATELNNALQNLARTISEAGQAMAS
ESAT-6 Peptide 7	NLARTISEAGQAMASTEGNVTGMFA

TABLE 1. AMINO ACID SEQUENCES OF OVERLAPPING PEPTIDES FOR ESAT-6 AND CFP-10

Cut off (IFN-γ IU/ml)	CFP-10		ESAT-6		CFP-10 and/or ESAT-6	
	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)
0.05	92.5	81.4	94.8	94.9	89.4	97.5
0.10	94.4	77.1	96.2	90.7	92.0	95.8
0.15	95.8	72.9	97.6	88.1	93.9	93.2
0.20	96.7	71.2	99.1	86.4	96.2	91.5
0.25	97.2	67.8	99.1	84.7	96.7	91.5
0.30	97.7	66.9	99.1	83.1	97.2	89.8
0.35	**98.6**	**65.3**	**99.5**	**81.4**	**98.1**	**89.0**
0.40	98.6	61.9	99.5	79.7	98.1	88.1
0.45	98.6	60.2	100.0	78.8	98.6	86.4
0.50	99.1	60.2	100.0	75.4	99.1	83.9

TABLE 2. TEST SENSITIVITY AND SPECIFICITY FOR CFP-10 AND ESAT-6 AT DIFFERENT CUT-OFFS IN THE WHOLE BLOOD IFN-γ ASSAY.

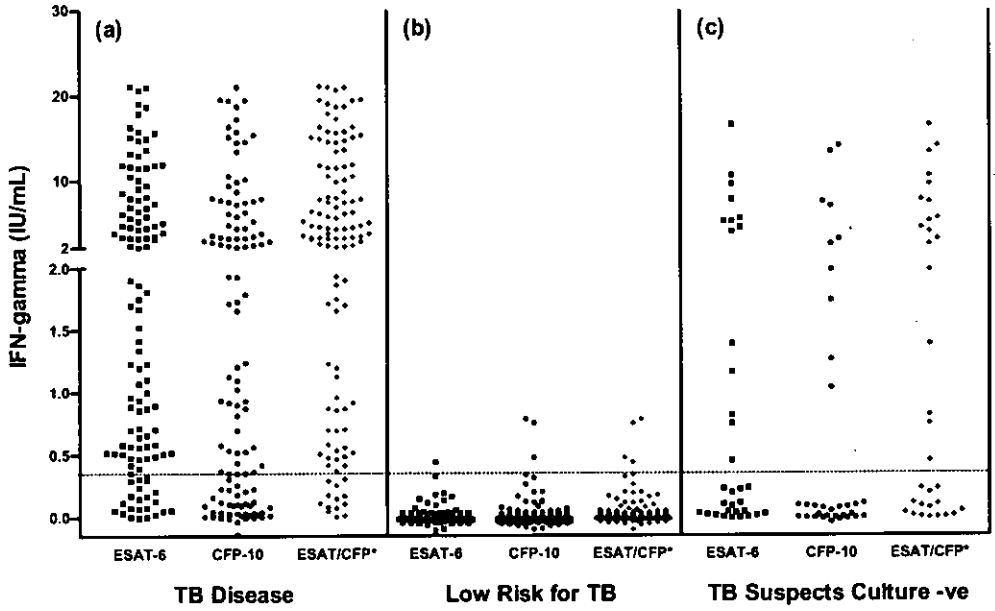
Sensitivity is determined using data from 118 culture positive TB patients, and specificity with data from 213 low risk subjects. The chosen cut-off (0.35) is highlighted.

Age (years)	No. IFN-γ tested	No. IFN-γ positive	% IFN-γ positive	No. Mantoux tested	No. Mantoux positive	% Mantoux positive
13 – 30	19	17	89.5	9	9	100.0
31 – 40	14	14	100.0	12	7	58.3
41 – 50	16	15	93.8	12	9	75.0
51 – 60	19	19	100.0	10	5	50.0
61 – 70	19	17	89.5	12	9	75.0
71 – 80	13	12	92.3	11	6	54.5
> 80	10	8	80.0	6	1	16.7

TABLE 3: CFP-10 AND ESAT-6 IFN-γ ASSAY AND MANTOUX TUBERCULIN SKIN TEST RESULTS, STRATIFIED BY AGE, FOR 110 CULTURE POSITIVE TB PATIENTS.

Results for the Mantoux test are using a 5mm cut-off.

Figure 1.


(a)
(b)
(c)
IFN-gamma (IU/mL)
30
20
10
2
2.0
1.5
1.0
0.5
0.0
ESAT-6
CFP-10
ESAT/CFP*
TB Disease
Low Risk for TB
TB Suspects Culture -ve

厚生労働科学研究補助金(新興・再興感染症研究事業)
分担研究報告書

薬剤耐性結核の迅速診断法の開発に関する研究

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研究要旨
結核の薬剤耐性機構は比較的限定したゲノム上の遺伝子変異であることが知られており、これらの変異を直接シークエンスすることによって同定することが可能である。本研究では、以下の抗結核薬の耐性に関与する遺伝子、*rpoB*（リファンピシン）、*katG* と *inhA*（イソニアジド）、*pncA*（ピラジナミド）、*embB*（エタンブトール）、*rpsL* と *rrs*（アミノグリコシド）、及び *gyrA*（フルオロキノロン）の耐性に関与する領域すべてを一回の操作で PCR 増幅しその塩基配列を決定するダイレクトシークエンス法を開発し、臨床分離株を用いた検討をした。

A. 研究目的

多剤耐性結核菌の出現は、結核医療の中でももっとも深刻な問題の一つである。多剤耐性結核は患者の治療を遅延させるばかりでなく、院内感染の原因ともなり、迅速な診断法の開発と新たな作用機序を持つ抗結核薬の開発が急務となっている。本研究では、多剤耐性結核の診断法の開発を行なう。幸い結核菌は全ゲノム配列が明らかにされており、またその薬剤耐性機構は比較的限定した遺伝子の変異であることが知られている。従って、多剤耐性遺伝子の迅速診断法の開発は大変有望な方法であると期待される。当センター病院とも共同で臨床応用を進め、本診断法の有用性を実証する。具体的には、ダイレクトシークエンス法による薬剤耐性結核の迅速診断法の開発を中心に研究を推進した。

B. 研究方法

喀痰国立国際医療センターなど医療施設から分離された結核菌または患者を J.BEIGE らの方法(J. Clin. Microbiol., 33: 90-95. 1995) に従って前処理し DNA 抽出した。(1)PCR ：8 本の PCR チューブを用いて、抽出した結核菌ゲノム DNA を鋳型とし、PCR による薬剤耐性遺伝子を増幅した。組成は以下の通りである。鋳型 DNA 1.0μl、*Z-Taq* ポリメラーゼ 1.25U (宝バイオ)、dNTP 200μl(宝バイオ)、耐性遺伝子に特異な８組のプライマーペアをそれぞれ 200nM ずつ、さらに 10×Z taq Buffer 5μl (宝バイオ)を加え、滅菌蒸留水にて全量を 50μl とした。これらを geneAmp PCR SYSTEM 9700 thermocycler (Applied Biosystems , Inc.,)を用いて、全て同じコンディションで増幅した。用いたプライマーは、Rifampicin 耐性遺伝子（*rpoB*）、Isoniazide 耐性遺伝子（*katG* 及び *mab-inhA*）、Ethambutol 耐性遺伝子(*embB*)、Pyrazinamide 耐性遺伝子 (*pncA*)、Kanamycin 耐性遺伝子（*rpsL*）、Streptomycin 耐性遺伝子（*rrs*）、Fluoloquinorons 耐性遺伝子（*gyrA*）を増幅するように設計した。PCR 産物はそれぞれ MicroSpin™ Columns (Amersham Pharamacia Biotech)を用いて精製した。(2)塩基配列の決定 ： シークエンス反応は、BigDye terminator cycle sequencing kit (Applied Biosystems)を用いたダイターミネーター法にて行った。組成は以下の通りである。プレミックス[５×Sequencing

Buffer 4μl 、 dNTP mix1μ 、 DyeDeoxy Terminators 0.5μl、AmpliTaq,FS 4μl ; 合計 8 μl (Applied Biosystems)]、それぞれ 1μl の PCR 産物、塩基配列決定用に設計したプライマー3.2 pmol を加え、滅菌蒸留水にて全量を 20μl とした。 増幅反応後、未反応 Dye 及び過剰プライマーの除去には、Centri-sep spin colums (Applied Biosystems)を用いた。次いで、これらを遠心・乾燥させ、さらに loading buffer15μl を加え、95℃ 2 分加熱後、急冷し、96 ウエルプレートに入れ、16 本キャピラリー式ABI 3100 オートシークエンサーを用いて泳動した。(3)塩基配列の解析: 塩基配列の解析及び編集は ABI PRISM 3100 Genetic Analyzer software を用い、これらを既知の薬剤感受性 *M.tuberculosis* H37Rv 株の塩基配列と比較することにより、変異の有無を解析した。

[倫理面の配慮]

研究対象は患者情報と完全に切り離された臨床分離株を使用する。

C. 研究結果

主要抗結核剤 4 剤を含む 7 剤の耐性遺伝子に特異的なプライマーを用いて特定領域を PCR 増幅し、シークエンサーで塩基配列を決定し、変異の有無を解析した（図１）。その結果を表 1 に示す。同時に従来の薬剤感受性試験も実施した。調べた臨床分離株 66 株中 36 株は全ての薬剤に感受性であったが、これらの株全てで耐性遺伝子に変異は認められなかった。INH 耐性株 21 株中 18 株(85. 7%)、RFP 耐性株 13 株中 13 株(100%)、PZA 耐性株 9 株中全て (100%)、EB 耐性株 8 株中 7 株(87. 5%)、SM 耐性株 10 中 8 株(80%)、LVFX 耐性株 2 株中全てに耐性遺伝子に変異が認められた。なお、RFP 耐性株で 1 つの、INH 耐性株で 4 つの、PZA 耐性株で 3 つの、SM 耐性株で 2 つのこれまでに報告のない変異を同定した。現在、この遺伝子をクローニングし、薬剤耐性との詳細な関連を決定した。

D. 考察

RFP 耐性株で 1 株、INH 耐性株で 4 株、PZA 耐性株で 3 株、これまで報告のない新規の変異を同定した。現在、これら新規の変異を有する遺伝子の内、最も臨床上、重宝されている抗結核剤であるイソニアジド耐性に関連する遺伝子 *katG* をクローニングし、薬剤耐性との詳細な関連を調べている。既に、我々は、この遺伝子の大量発現系の構築に成功し、KatG 蛋白を調製した。そこで、この遺伝子内変異に基づくアミノ酸変異を有する KatG 蛋白を用いて、蛋白の生化学的特徴から、この特徴が薬剤耐性と関連するか否かの証明の為、In vitro によるアッセイ系を構築中であり、新規の遺伝子内変異（塩基置換）と薬剤耐性との関連が詳細になりつつある。

E. 結論

本研究では、ダイレクトシークエンス法による薬剤耐性結核の迅速診断法の開発を推進した。今後はこれらの結果を発展させ、製品化をめざす。

F. 健康危惧情報

とくになし。

G. 研究発表

1. 論文発表

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H．知的財産権の出願・登録状況

1. 特許取得

結核菌に含まれる薬剤耐性遺伝子を検出する方法、PCR 用プライマーセット、塩基配列決定用プライマーセット、及び薬剤耐性結核の診断用試薬キット．切替照雄、関口純一朗、大槻隆司．出願番号：PCT/JP03/16941. 出願日：2003 年 12 月 26 日.

2. 実用新案登録

なし

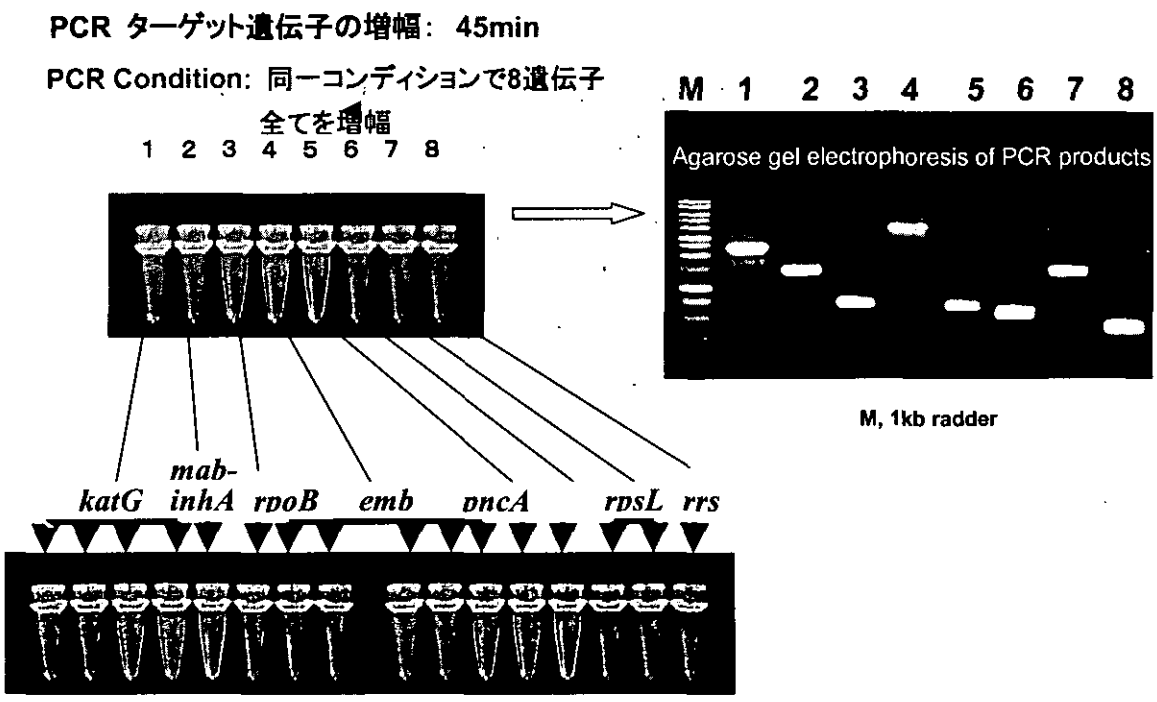

図 1. ダイレクトシークエンス法による結菌薬剤耐性遺伝子変異の検出

結核の薬剤耐性に関与する遺伝子の領域すべてを一回の操作で PCR 増幅しその塩基配列を決定する方法を開発した。具体的には、主要抗結核剤 4 剤を含む 7 剤の耐性遺伝子に特異的なプライマーを用いて特定領域を PCR 増幅し、シークエンサーで塩基配列を決定し、変異の有無を解析した。

表 1. ダイレクトシークエンスによる薬剤耐性遺伝子診断の結果

Direct sequening results	Drugs	Resits of drug susceptibility test(s)		Specificity (%)	Sensitivity(%)
		No. of resistant	No. of susceptible		
rpoB					
Mutation positive	RFP	13	1	100	92.9
Wild type		0	53		
***katG* or *inhA* promoter**					
Mutation positive	INH	19	0	86.4	100
Wild type		3	45		
embB					
Mutation positive	EB	7	0	87.5	100
Wild type		1	59		
pncA					
Mutation positive	PZA	9	0	100	100
Wild type		0	58		
rpsL* or *rrs					
Mutation positive	SM	8	0	80	100
Wild type		2	57		
gyrA					
Mutation positive	LVFX	2	0	100	100
Wild type		0	65		

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分担研究報告書

多剤耐性結核に対する新しい治療方式の開発に関する研究

国立病院・療養所呼吸器ネットワークを利用した患者宿主要因のSNPs解析、T細胞免疫機能解析、マクロファージ機能解析とこれを利用した治療戦略の開発

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研究要旨

(1) 多剤耐性結核（MDR）患者のT細胞免疫機構を解析し結核菌殺傷蛋白（granulysin；この物質に対するモノクローナル抗体をすでに作製し、MDR-TBではこのgranulysin低下が著明に認められた）の低下を明らかにした。

(2) MDR-TB菌のRFLP解析により、多剤耐性結核菌を多くの人に感染させるスーパースプレッダー多剤耐性結核患者の存在を発見した。

(3) Toll-likeレセプター(TLR)の免疫監視機構からスーパースプレッダーMDR-TB菌がエスケープする免疫機構が示唆された。

(4) 一方、MDR-TBの新しい迅速診断法として、結核菌内の16多型配列部位を用いたVNTRを用いた迅速genotypingを開発し、大阪における結核菌解析に必要十分なクラスター抽出能を持つことを明らかにした。さらに我々の今回のVNTRの研究により大阪府内において同一のSM・INH同時耐性菌の複数感染が起こっており、しかもその中の3名が治療の失敗により多剤耐性化していることが判明した。

(5) クラリスロマイシン、ニューキノロンへの感受性について、多数症例を集めて検討した。その結果、多剤耐性結核化学療法においてニューキノロン薬剤は効果的であった。

A. 研究目的

多剤耐性結核の新治療方式の開発：これまで明確な成果の上がっていない免疫療法や姑息的化学療法に一大進歩を印する可能性がある。すなわち、結核や抗酸菌症分野では分担研究者が理研などとの共同でこれまで明確な成果の上がっていない免疫療法に一大進歩を印する可能性がある。また分担研究者の病院は呼吸器疾患の国療ネットワークの全国中核病院として、全国規模で症例にアクセスできる立場にある。したがって、 ①多剤耐性結核患者（国立病院・療養所政策医療呼吸器ネットワークを利用した）リンパ球を用いた新しい技術SNPs解析法による多剤耐性結核の診断法を開発するとともに、新しい治療法を開発する。理化学研究所との共同研究で行う。（すでに非定型抗酸菌症のprojectで実績あり） ②（政策医療呼吸器ネットワークを利用した）糖尿病合併に伴う多剤耐性結核患者の血糖調節ホルモン・サイトカインの測定とT細胞免疫機能解析（結核菌殺傷蛋白等）による新しい多剤耐性結核予防法及び治療法の開発 ③政策医療呼吸器ネットワークを利用した、種々の多剤耐性結核菌によるマクロファージ機能調節機構（SRやTLR等の発現調節）の解明とこの作用機序解明による新しい診断法、治療法の開発 ④政策医療呼吸器ネットワークを利用した多剤耐性結核治療における新しい治療薬（IFN-γ吸入療法や新規化学療法剤）。⑤政