Table 2 Discrepant and unidentified results in identification of *Mycobacterium* species, including 9 isolates of *M. lentiflavum*.

Isolate No.	Cobas Amplicor system	AccuProbe	DDH	INNO-LiPA	16S rRNA gene		ITS	
					Identity (%)			Identity (%)
9 isolates	Negative	Negative	Unidentified**	MYC genus	*M. lentiflavum* DSM44418T	429/429 (100)		
2	Negative	Negative	Unidentified**	*M. kansasii* 3	*M. kansasii* Borste 8875/99, sqv. VI-3	441/441 (100)	*M. kansasii*, MkaF	277/277 (100)
19	Negative	Negative	Unidentified**	*M. kansasii* 3	*M. kansasii* Borste 539/99, sqv. III	440/440 (100)	*M. kansasii*, MkaC	279/279 (100)
14	Negative	NT	Unidentified**	*M. gordonae*	*M. gordonae* Borste 11340/99, sqv. III	440/440 (100)	*M. gordonae*, MgoC	270/270 (100)
22	Negative	NT	Unidentified**	MYC genus	*M. interjectum* ATCC51457T	430/430 (100)		
7	Negative	NT	Unidentified***	*M. abscessus*	*M. abscessus* or *M. chelonae* (*M. abscessus* by ITS)	428/428 (100)	*M. abscessus* DSM44196	294/294 (100)
6	Negative	NT	*M. fortuitum*	*M. chelonae*	*M. abscessus* or *M. chelonae* (*M. chelonae* by ITS)	428/428 (100)	*M. chelonae* Mche B	293/294 (99.7)
5	Negative	NT	*M. fortuitum*	MYC genus	*M. fortuitum* DSM46621T	428/428 (100)		
23	*M. intracellulare*	MAC*	NT	MAIS	*M. intracellulare* ATCC35770 sqv. III	442/442 (100)		
13	Negative	NT	Unidentified***	*M. fortuitum*	*M. mucogenicum* ATCC49650T	423/428 (98.8)		
18	Negative	NT	*M. scrofulaceum*	*M. intracellulare* 2	*M. paraffinicum* DSM44181T	439/439 (100)		

M. avium complex **slow growers ***rapid growers NT: not tested T, Type strain sqv., sequevar

められたが菌種の特定には至らず，DDH法とシークエンス解析では *M. fortuitum* と同定された。菌株23はコバス アンプリコア マイコバクテリア法で *M. intracellulare*，アキュプローブ MAC法で MAC，INNO-LiPA法で MAIS と判定され，シークエンス解析で *M. intracellulare* ATCC 35770 sqv. Ⅲ（Mac-D）と100%相同と判定された。

シークエンス解析結果といずれかの方法の結果が異なった株（菌株13，18）は各々 *M. mucogenicum* 近縁種と *M. paraffinicum* と同定された。

小川培地上で遅発育菌と観察され，コバス アンプリコア マイコバクテリウム法，アキュプローブ法，DDH法でも同定不能となり，INNO-LiPA法で MYC genus にしかバンドの発色が見られなかったがシークエンス解析で *M. lentiflavum* と同定された株が9株認められた（Table 2）。

複数菌種が混在していた3混合培養は INNO-LiPA法でも複数のバンドパターンが認められた（*M. tuberculosis* ＋*M. gordonae*，*M. avium*＋*M. fortuitum*，*M. kansasii*＋*M. gordonae*）。

考　察

分子遺伝学的に近縁な菌種であり16S rRNA遺伝子に違いが見られない場合，ITS領域のほうが進化速度は速いため，より多様性のある配列結果が得られる。ITS領域をターゲットとした INNO-LiPA法は ITS領域で高い多型性が知られている MACに対して4種類の亜型プローブを使って亜型判定を可能としている。菌株23はアキュプローブ MAC法で MAC，コバス アンプリコア マイコバクテリウム法で *M. intracellulare*，シークエンス解析で *M. intracellulare* ATCC 35770 sqv. Ⅲ（Mac-D）と判定された。INNO-LiPA法では同タイプに対応する亜型プローブは設計されていないために MAISプローブのみの反応となった。Lebrun らも ATCC 35770の検討で同じく MAISプローブにのみ発色が認められたと報告している[13]。したがって，ITS領域において菌種内多型性を示す菌種に対しては，シークエンス解析で相同性を確認することが重要となってくる。

シークエンス解析といずれの方法とも結果が食い違った2株のうち菌株18はアキュプローブ MAC法陰性，DDH法で *M. scrofulaceum* となり，INNO-LiPA法で MAIS と MIN-2に発色が見られ *M. intracellulare* sqv. Mac-A と判定された。遅発育菌である同菌株は16S rRNA解析では100%の相同性で *M. paraffinicum* DSM 44181と判定され，同じく *M. scrofulaceum* DSM 43992とは99%の相同性が見られた。Tortoli らも *M. paraffinicum* は MAIS と MIN-2に発色が見られたがアキュプローブ MAC法は陰性であったと報告している[10]。一方 Lebrun らはアキュプロー

ブMAC法陰性，INNO-LiPA法ではMAISのみにバンドに発色があり，シークエンス解析で*M. paraffinicum*と判定されたが同時に*M. scrofulaceum* DSM 43992と98.9%の相同性があったと報告している[13]。今回アキュプローブMAC法でMAC，コバス アンプリコア マイコバクテリウム法により*M. avium*と同定された菌株はすべてINNO-LiPA法で明確にMAVに発色が見られ，アキュプローブMAC法でMAC，コバス アンプリコア マイコバクテリウム法により*M. intracellulare*と判定された菌株も上記の菌株23以外はMIN-1に発色が見られた。したがって唯一MIN-2にバンドを示した菌株18は*M. intracellulare* sqv. Mac-Aとかなり相同性が高い近縁菌種と考えられた。

菌株13は16S rRNAシークエンス解析で*M. mucogenicum* ATCC 49650Tと5 bpの違い（98.8%の相同性）が見られ*M. mucogenicum*の近縁種と推定された。小川培地上で迅速に発育しDDH法で同定不能，INNO-LiPA法で*M. fortuitum*と判定されており，結果に乖離が見られた。*M. mucogenicum*は古くは*M. chelonae*-likeとして知られていたが，16S rRNA遺伝子では*M. chelonae*よりも*M. fortuitum*に近い系統に位置しており[15]，現在では*M. chelonae-abscessus*グループと*M. fortuitum*グループに近縁の迅速発育菌として独立したグループと定義されている。Ballardらは同じくATCC 49650Tと5 bp違いでなおかつATCC 49649と1 bp違いの*M. mucogenicum* N248を解析しており，新しいsubspeciesの可能性があると報告している[16]。迅速発育菌は多様性に富んでおり，菌株13も*M. mucogenicum*のvariant typeの可能性が考えられた。

同じく迅速発育菌であった菌株5はINNO-LiPA法ではMYC genusのみ発色が見られ，DDH法で*M. fortuitum*，シークエンス解析で*M. fortuitum* DSM46621とDSM44220に100%の相同性が認められた。PadillaらはINNO-LiPA法で同じタイプのDSM46621株は*M. fortuitum*と同定されたと報告している[12]。われわれの検討では同菌種のDSM44220株（*M. fortuitum subspecies acetamidolyticum*）はDDH法とINNO-LiPA法で*M. fortuitum*と同定できた（データ未掲載）。*M. fortuitum*はITSシークエンス解析でsqv. Ⅰ～Ⅳが認められており高い多型性を示すため[17]，迅速発育菌の詳細な亜型解析にはシークエンス解析が重要であると思われた。

遺伝子を用いた同定キットによる判定と併行して従来法やコロニー性状から菌種を鑑別することは非常に重要である。菌株22はコバス アンプリコア マイコバクテリウム法，アキュプローブ法，DDH法で同定不能となった遅発育菌である。INNO-LiPA法ではMYC genusの反応が見られたが，シークエンス解析では*M. interjectum*と判定された。*M. interjectum*は非光発色性の遅発育菌でかつ16S rDNA配列が特異的であり，遺伝子を用いた同定キットによる菌種同定は困難である[18]。INNO-LiPA法においても該当プローブが固相化されていないため同菌種の同定はできず，培養でのコロニー性状の観察や生化学的性状試験が鑑別上重要になってくる。同様に菌株2と19は，3種類のプローブで*M. kansasii*の亜型を判別可能であるINNO-LiPA法でMKA-3に発色した。16S rRNA遺伝子のシークエンス解析から*M. kansasii* sqv. ⅢとⅥとに判定されたが研究用試薬アキュプローブ カンサシで陰性となるため*M. kansasii*と判定されなかった。日常検査では光発色試験に及んでいなかったが，改めて実施した結果*M. kansasii*と同定できた。

コロニーの光発色試験での光発色菌，暗発色菌，非光発色菌の鑑別は純培養を用いるため可変的，主観的であり，熟練を要する。*M. szulgai*は37℃で暗発色性，25℃培養で光発色性になる。*M. simiae*の光発色性の出現は通常1時間の光照射のところ6～24時間の光照射が必要であり注意を要する。培養時のコロニー性状の観察において，S型，R型，その移行型（SR型，RS型）の性状が継代を重ねることで変化してくることがある。また発育速度の観察は，遅発育菌でも大量の菌を接種すれば7日ぐらいで発育は見られる場合はあるし，迅速発育菌での分離培養の時にはコロニーの発生までに時間がかかる場合もある。したがって培養条件により変化する菌の性状を十分考慮して，なるべく初代分離菌について詳細に観察することが望ましい。

INNO-LiPA法の製造元であるINNOGENETICS社の本社がベルギーに位置するため，欧米のAIDS患者から分離された*M. genavense*[19]や，イギリス，スコットランド，ウェールズ，スウェーデン，フランスで分離が増えている*M. malmoense*[20]といった菌種に対する同定が可能となっている。わが国では現時点でのこれらの菌種による感染症の報告は非常にまれであるため，今後これら稀少菌種の同定の際には大きな威力を発揮すると思われる。一方，最近わが国で分離の報告が増加している遅発育菌の*M. lentiflavum*[14]が今回シークエンス解析により9株確認されたが，対応プローブが配置されていないINNO-LiPA法ではMYC genusにしかバンドの発色が見られず同定に至らなかった。臨床での有用性をより高めるために，わが国の抗酸菌分離状況にあわせたINNO-LiPA法の仕様改良を切望したい。

今回有用性が認められたINNO-LiPA法は手技面でもPCR増幅後約3時間で判定可能であり，迅速性が証明された。ハイブリダイゼーションから洗浄，発色までを行う自動化ハイブリダイゼーション装置Auto-LiPAを利用すれば労力の軽減が可能であると思われる。またINNO-LiPA法はストリップ上に得られるバンドの有無で判定するため，DDH法のような読み取り時の測定誤差は少

なくなると考えられる。複数菌混合培養における複数菌種同定も可能であることから，単一分離培養に要する時間や手間が省かれ，迅速に同定結果が得られることが明らかとなった。

抗酸菌における遺伝子検査の進歩は特に目覚ましく，今回用いた検査法も含めて多様な検査キットが市販されている。各種キットの特徴を熟知したうえでそれぞれの施設に適した検査法を選択し，各キット間に生じる結果の乖離や同定不能な株が存在する場合を考慮して菌種同定を行うことが望まれる。またこれらキットは定性用検査であり，検体内の菌量を反映できないため，塗抹・培養検査の結果と同定結果とを鑑みて治療方針を決定することが重要である。特にNTMを分離した場合には非結核性抗酸菌症の診断基準[21)22)]と合わせて総合的に判断するべきである。

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Original Article

EVALUATION OF THE INNO-LiPA MYCOBACTERIA v2 FOR MYCOBACTERIAL IDENTIFICATION

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Abstract [Purpose] Evaluation of the INNO-LiPA MYCOBACTERIA v2 (the INNO-LiPA assay) for mycobacterial identification.

[Materials and Methods] The laboratory identifications consisting of Cobas Amplicor systems, AccuProbe, and DDH, are commonly used to identify mycobacterial isolates in Japan. We compared the results between the INNO-LiPA assay and the common methods. A total of 122 clinical isolates from NHO Kinki-chuo Chest Medical Center from 1 February to 30 June 2006 were tested.

[Results] There was agreement between the INNO-LiPA assay and the common methods for 112 mycobacterium isolates. The six discordant isolates have showed same results between sequencings and the INNO-LiPA assay. The one *M. fortuitum* isolates was indicated correctness by DDH and the one *M. intracellulare* isolates was recognized by Cobas Amplicor systems and as MAC by AccuProbe MAC. Moreover, discrepant results between sequencings and mycobacterial identifications including the INNO-LiPA assay were 2 isolates (*M. paraffinicum*, *M. mucogenicum* variant type).

[Conclusion] The INNO-LiPA assay could provide rapid and correct identification results with clear-cut and easy interpretation.

Key words: Mycobacteria, INNO-LiPA MYCOBACTERIA v2, Identification, 16S rRNA gene, ITS sequencing

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2010, p. 1793–1799 Vol. 54, No. 5
0066-4804/10/$12.00 doi:10.1128/AAC.01691-09

Identification of *katG* Mutations Associated with High-Level Isoniazid Resistance in *Mycobacterium tuberculosis*▽†

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Received 2 December 2009/Returned for modification 28 December 2009/Accepted 25 February 2010

Isoniazid (INH) is an effective first-line antituberculosis drug. KatG, a catalase-peroxidase, converts INH to an active form in *Mycobacterium tuberculosis*, and *katG* mutations are major causes of INH resistance. In the present study, we sequenced *katG* of 108 INH-resistant *M. tuberculosis* clinical isolates. Consequently, 9 novel KatG mutants with a single-amino-acid substitution were found. All of these mutants had significantly lower INH oxidase activities than the wild type, and each mutant showed various levels of activity. Isolates having mutations with relatively low activities showed high-level INH resistance. On the basis of our results and known mutations associated with INH resistance, we developed a new hybridization-based line probe assay for rapid detection of INH-resistant *M. tuberculosis* isolates.

Isoniazid (INH) is an effective drug used in the treatment of tuberculosis and has been in common use to treat tuberculosis since its introduction in 1952 (4). However, the emergence of INH-resistant (Inh^r) *Mycobacterium tuberculosis* is jeopardizing the continued utility of INH (10).

Drug resistance in *M. tuberculosis* is caused by mutations in restricted regions of the genome (36). Mutations in *katG*, the upstream region of the *fabG1-inhA* operon ($P_{fabG1\text{-}inhA}$), and *inhA* are responsible for INH resistance (36). The *katG* gene encodes the bifunctional catalase-peroxidase enzyme that converts INH to an active form (35).

Previously, we developed a DNA sequencing-based method to detect mutations in regions associated with INH resistance in *M. tuberculosis*, including *katG* and $P_{fabG1\text{-}inhA}$ (28). Consequently, five novel mutations in *katG* associated with INH resistance were found (28). In the present study, we cloned 21 *katG* mutants, including 15 novel mutants, and compared their INH oxidase activities. Certain *katG* mutations were shown to cause high-level INH resistance, which suggests the possibility of determining the degree of INH resistance, such as high- or low-level resistance, by detecting these *katG* mutations. Furthermore, to detect these mutations in ordinary-scale clinical laboratories without sequencing, we developed a new hybridization-based line probe assay (LiPA) for INH resistance in *M. tuberculosis* isolates, which can be applied easily in clinical use.

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 8 March 2010.

MATERIALS AND METHODS

Bacterial strains and plasmids. One hundred eight Inh^r *M. tuberculosis* isolates were obtained from single patients at the International Medical Center of Japan and National Hospital Organization Tokyo National Hospital from 2003 to 2008. INH-susceptible (Inh^s) *M. tuberculosis* strains H37Rv and IMCJ 2751 were used. The IMCJ 2751 isolate has a *katG*(*G1388T*) [KatG(R463L)] neutral mutation. The *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *E. coli* TOP10F′ (Invitrogen, Carlsbad, CA) was used as the host for cloning. *E. coli* UM262 (17) was used as the host for expression of *katG* derived from clinical isolates and H37Rv.

Drug susceptibility testing. All clinical isolates, H37Rv, and IMCJ 2751 were tested for drug susceptibility. Strains were analyzed by an agar proportion method with egg-based Ogawa medium (Vit Spectrum-SR [Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan] or Wellpack [Japan BCG Laboratory, Tokyo, Japan]), which is based on a slightly modified WHO protocol (3) and is recommended by the Japanese Society of Tuberculosis (3, 12). The medium contained INH (0.2 μg/ml and 1.0 μg/ml), rifampin (RIF) (40 μg/ml), ethambutol (EB) (2.5 μg/ml), kanamycin (KM) (20 μg/ml), *p*-aminosalicylic acid (PAS) (0.5 μg/ml), streptomycin (SM) (10 μg/ml), ethionamide (TH) (20 μg/ml), enviomycin (EVM) (20 μg/ml), cycloserine (CS) (30 μg/ml), and levofloxacin (LVFX) (1.0 μg/ml). The results of drug susceptibility testing are shown in Table S1 in the supplemental material.

Isolation of genomic DNA. Genomic DNA from *M. tuberculosis* was extracted as described previously (22).

DNA sequencing of INH resistance-related genes. The *furA-katG* operon and its upstream region were amplified by PCR with primers −129*furA* (5′-GCTCA TCGGAACATACGAAG-3′) and *katG*+50 (5′-GTGCTGCGGCGGGTTGTG GTTGATCGGCGG-3′). The *fabG1-inhA* operon and $P_{fabG1\text{-}inhA}$ were also amplified, using primers −200*fabG1* (5′-TTCGTAGGGCGTCAATACAC-3′) and *inhA*+40 (5′-CCGAACGACAGCAGCAGGAC-3′). PCR products were used as templates for direct DNA sequencing. DNA sequences were compared with the H37Rv sequence using Genetyx-Mac, version 14.0.2 (Genetyx Corporation, Tokyo, Japan).

Construction of plasmids. The coding regions of *katG* from H37Rv, IMCJ 2751, and Inh^r clinical isolates with *katG* mutations were amplified by PCR with the primers *katG*-F-ccc (5′-CCCGAGCAACACCCACCCATTACAGAAAC-3′) and *katG*-R (5′-TCAGCGCACGTCGAACC-3′) and cloned into pTrcHis2-TOPO (Invitrogen) using the TA cloning method. The pTrcHis2-TOPO vector encodes a C-terminal peptide containing a c-*myc* epitope and a 6×His tag. However, the expressed recombinant KatG protein did not have any additional amino acid residues, such as the c-*myc* epitope and the 6×His tag, because the

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
E. coli strains		
TOP10F′	F′ [*lacI*^{q} Tn*10* (Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74* *recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
UM262	*katG*::Tn*10 recA pro leu rpsL hsdM hsdR endl lacY*	17
Plasmids		
pTrcHis2-TOPO	TA cloning and expression vector; Ap^r Km^r	Invitrogen
p*katG*-wt	pTrcHis2-TOPO carrying *katG*	This study
p*katG*-1	p*katG*-wt carrying G1388T (neutral mutation)	This study
p*katG*-2	p*katG*-1 carrying C379G	This study
p*katG*-3	p*katG*-1 carrying C694T	This study
p*katG*-4	p*katG*-wt carrying A398C	This study
p*katG*-5	p*katG*-1 carrying T1147C	This study
p*katG*-6	p*katG*-1 carrying 1297::C, Δ1305C	This study
p*katG*-7	p*katG*-1 carrying a290g	This study
p*katG*-8	p*katG*-1 carrying C1465A	This study
p*katG*-9	p*katG*-wt carrying G944C	This study
p*katG*-10	p*katG*-1 carrying T1259C	This study
p*katG*-11	p*katG*-wt carrying G944C, G1159C	This study
p*katG*-12	p*katG*-1 carrying G368A, G895A	This study
p*katG*-13	p*katG*-1 carrying G1255C	This study
p*katG*-14	p*katG*-1 carrying C195T (silent mutation), T527C	This study
p*katG*-15	p*katG*-wt carrying Δ(478–479)	This study
p*katG*-16	p*katG*-1 carrying G944C	This study
p*katG*-17	p*katG*-wt carrying Δ371G	This study
p*katG*-18	p*katG*-1 carrying C1894T	This study
p*katG*-19	p*katG*-wt carrying C945A	This study
p*katG*-20	p*katG*-1 carrying Δ(571–576)	This study
p*katG*-21	p*katG*-1 carrying G1624C	This study

katG-R reverse primer included the native stop codon. The DNA sequences of all clones were confirmed by sequencing.

RFLP. IS*6110*-probed restriction fragment length polymorphism (RFLP) was performed as described previously (22). Patterns with more than 70% similarity were postulated to form a cluster.

Immunoblotting. Proteins separated by SDS-PAGE were transferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The proteins on the membranes were detected using primary antibodies specific for KatG (28). KatG was visualized with horseradish peroxidase-conjugated secondary antibodies.

Enzyme assays. KatG mediates free-radical formation from INH oxidation in the presence of H_2O_2. The activities of KatG were detected spectrophotometrically by following the reduction of nitroblue tetrazolium (NBT) at A_{560} (28, 32). Peroxidase activity was monitored spectrophotometrically by following the oxidation of 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at A_{405} (21). Catalase activity was measured spectrophotometrically by following the degradation of H_2O_2 at A_{240} (21). The catalase activity is shown as values subtracted from that of the vector control. All assays were carried out at 25°C. The absorbance was read 200 s after the initiation of the reaction.

LiPA. The line probe assay (LiPA) was performed as described previously (1, 29). In brief, 41 oligonucleotide probes were designed to cover mutations in the *furA-katG* (35 probes for *katG* and 2 for *furA*), $P_{fabG1\text{-}inhA}$ (2 probes), and *fabG1* (2 probes) regions (Table 2). These probes were immobilized on two strips. Six regions, located within $P_{fabG1\text{-}inhA}$ (477 bp), *fabG1* (209 bp), *furA* (256 bp), and *katG* (612 bp, 698 bp, and 907 bp), were amplified by nested PCR. Immobilized probes on the two strips were hybridized with the biotinylated PCR products and then incubated with streptavidin labeled with alkaline phosphatase. The color development was performed by incubation with 5-bromo-4-chloro-3′-indolylphosphatase *p*-toluidine and NBT.

RESULTS

Drug susceptibility profiles. As shown in Table S1 in the supplemental material, among 108 Inh^r isolates, 65 (60%) were resistant to INH at 0.2 μg/ml but susceptible to INH at 1.0 μg/ml. The remaining 43 (40%) were resistant to INH at 1.0 μg/ml. Among the 108 isolates, 44 (41%) were resistant to INH but susceptible to other antituberculosis drugs. Thirteen (12%) were multidrug-resistant (MDR) isolates and five (5%) were extensively drug resistant (XDR).

IS*6110*-probed RFLP. The results of IS*6110*-probed fingerprinting of the 108 Inh^r isolates are shown in Fig. S1 in the supplemental material. Five clusters were detected, consisting of a total of 63 isolates (58%), including 12 (11%) in cluster I, 22 (20%) in cluster II, 12 (11%) in cluster III, 12 (11%) in cluster IV, and 5 (5%) in cluster V. These observations suggested that the majority of Inh^r isolates in Japan expanded in a clonal manner.

Correlation between drug susceptibility and IS*6110*-probed RFLP. With regard to the degree of INH resistance, the proportions of high-level Inh^r isolates, i.e., isolates resistant to INH (1.0 μg/ml), were 1 (8%) in cluster I, 8 (36%) in cluster II, 4 (33%) in cluster III, 4 (33%) in cluster IV, and 5 (100%) in cluster V. These results indicated that the majority of isolates belonging to cluster I were resistant to INH (0.2 μg/ml) and susceptible to INH (1.0 μg/ml) and that those belonging to cluster V were highly resistant to INH. Six of 13 MDR isolates (46%) and 1 of 5 XDR isolates (20%) belonged to the clusters, but other MDR and XDR isolates did not belong to any clusters, indicating that they emerged sporadically in Japan.

Mutations in *furA-katG*, *fabG1-inhA*, and their upstream regions. We sequenced the *furA-katG* operon, the *fabG1-inhA* operon, and their upstream regions in all Inh^r isolates tested. Of the 108 isolates, 105 had at least one mutation (see Table S1

TABLE 2. Locations of 41 oligonucleotide probes designed to cover a mutation(s) associated with INH resistance

Probe	Amino acid (nucleotide) region covered by probe
inhA-1	(−17 to −3)[a]
inhA-2	95–100
fabG1-1	202–206
fabG1-2	230–235
furA-1	12–17
furA-2	6–12
katG-1	45–51
katG-2	63–68
katG-3	92–97
katG-4	94–99
katG-5	105–111
katG-6	123–127
katG-7	132–137
katG-8	135–140
katG-9	140–145
katG-10	157–163
katG-11	170–174
katG-12	174–179
katG-13	178–183
katG-14	190–194
katG-15	228–236
katG-16	247–252
katG-17	256–261
katG-18	271–277
katG-19	294–299
katG-20	313–318
katG-21	323–327
katG-22	326–330
katG-23	383–387
katG-24	389–391
katG-25	417–422
katG-26	457–462
katG-27	479–482
katG-28	486–490
katG-29	522–528
katG-30	539–543
katG-31	553–558
katG-32	565–569
katG-33	591–596
katG-34	631–635
katG-35	707–712

[a] Nucleotide position relative to the initiation codon of *fabG1*.

in the supplemental material), while the remaining 3 had no mutations in the regions sequenced. Of the 105 isolates with mutations, 64 had mutations in the *furA-katG* operon, 62 had mutations in *fabG1-inhA* operon, and 21 had mutations in both regions. Of the 64 with mutations in the *furA-katG* operon, six had a large-scale deletion adjacent to the *furA-katG* operon (Fig. 1; see also Table S1 in the supplemental material). As shown by genetic maps (Fig. 1), these isolates had large-scale deletions, ranging in size from 2.3 to 34.4 kb. The remaining 58 isolates did not have large-scale deletions.

Twenty-eight different mutations were found among the 58 isolates with mutations in the *furA-katG* operon (see Table S1 in the supplemental material). Twenty-three were in *katG*, two were in *furA*, and three were in the intergenic region. Seven different mutations were found among the 62 isolates with mutations in the *fabG1-inhA* operon (see Table S1 in the supplemental material). Three were in the upstream region, two were in *fabG1*, and two were in *inhA*. Of the 28 different mutations found in the *furA-katG* operon, 22 were novel (2 in *furA*, 3 in the intergenic region of the *furA-katG* operon, and 17 in *katG*). Of the seven different mutations found in the *fabG1-inhA* operon, four were novel: one in the upstream region of the *fabG1-inhA* operon, two in *fabG1*, and one in *inhA* (see Table S1 in the supplemental material).

Correlation between INH resistance and mutations. We recently reported 5 novel mutations in *katG* (28). Including these mutations, 280 different mutations in *katG* were found in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) when articles were searched by the keywords "*katG*," "mutation," and "tuberculosis." In addition, six mutations in the upstream region of the *fabG1-inhA* operon, including C−15T, and seven in *inhA* cause INH resistance (27, 28, 36). In this study, we found an additional 17 novel mutations in *katG*. One was a silent mutation (C195T [A65A]), while the other 16 caused amino acid substitutions. These mutations and amino acid substitutions are shown in Table 3. Furthermore, several novel mutations were detected in the present study: one in *fabG1* (G609A [L203L]), one in *furA* (C41T [A14V]), and three in the intergenic region of the *furA-katG* operon (G−7A, A−10C, and G−12A).

We will report elsewhere that these mutations in *furA* and the intergenic region are associated with INH resistance induced by downregulation of *katG* expression (H. Ando and T. Kirikae, unpublished results), and those in *fabG1* are also associated with INH resistance induced by upregulation of *inhA* expression (Ando et al., unpublished). In the present study, we examined whether novel mutations in *katG* are associated with INH resistance.

Correlation between mutations and IS*6110*-probed RFLP. As shown in Fig. S1 and Table S1 in the supplemental material, all isolates belonging to cluster I detected in the IS*6110*-probed RFLP analysis, 11 (50%) in cluster II, and 8 (67%) in cluster III had a C−15T mutation in the *inhA* promoter region. All isolates in cluster IV had a C41T mutation in *furA*. All isolates in cluster V had a G944C/G945A (S315T/R) mutation. Isolates harboring *katG* mutations, except those with the G944C/G945A (S315T/R) mutation, did not cluster in the IS*6110*-probed RFLP.

Enzymatic activity of the novel KatG mutants. We cloned a wild-type (WT) *katG* gene (p*katG*-wt) from H37Rv, a *katG* gene carrying a G1388T neutral mutation (p*katG*-1) from IMCJ 2751, and 20 *katG* genes harboring mutations causing amino acid substitutions (p*katG*-2 to -21) from Inh^r isolates (Tables 1 and 3). Among the mutants, 15 were novel and 6 had been reported previously (the *katG*-1, -7, -9, -13, -16, and -19 mutants) (Table 3). These *katG* genes were expressed in *katG*-deficient *E. coli* UM262. As shown in Fig. 2, *E. coli* isolates with *katG*-wt expressed KatG (lanes 1 and 15), whereas *E. coli* isolates with an empty vector did not (lanes 2 and 16). *E. coli* isolates carrying *katG* mutants other than the *katG*-15 (lane 8) and *katG*-17 (lane 24) mutants expressed KatG proteins at levels similar to those observed for *E. coli* isolates carrying p*katG*-wt. *E. coli* isolates with *katG*-15 (lane 8) and *katG*-17 (lane 24), which had a frame shift mutation (Table 3), did not express *katG*.

INH oxidase, peroxidase, and catalase activities were assessed using these clones (Table 4). Of the cloned mutants, one with KatG(R463L) from IMCJ 2751 showed levels of these activities similar to those observed for the wild type, and the KatG(R463L) mutation was not associated with INH resis-

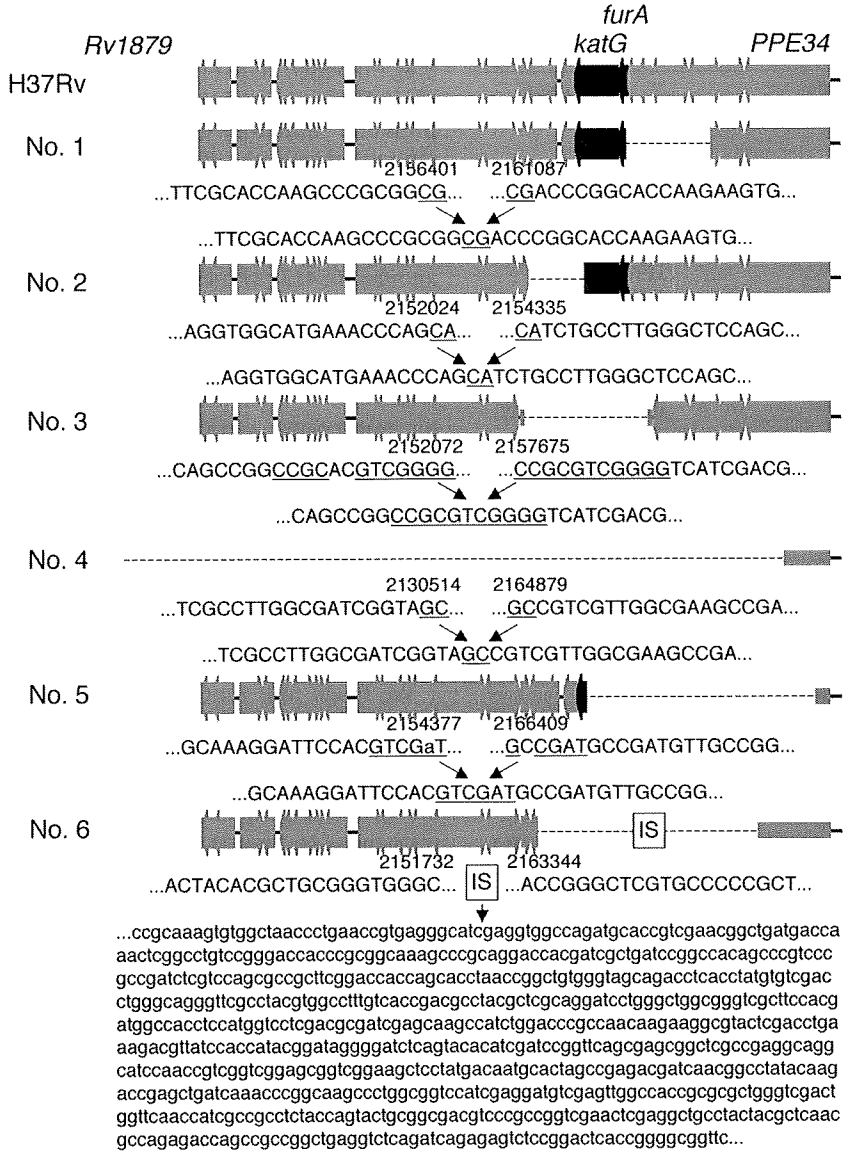

FIG. 1. Maps of large-scale deleted regions adjacent to *katG* in six Inh^r *M. tuberculosis* isolates. Bold arrows indicate the open reading frames annotated in the H37Rv genome sequence (http://genolist.pasteur.fr/TubercuList/). The dotted lines correspond to the deleted regions, with the end sequences and H37Rv genome coordinates given below. Underlined sequences are possible substrates for recombination. The box labeled "IS" represents the 750-bp fragment of IS*6110*. Numbers 1 to 6 represent the names of the isolates and correspond to the numbers shown in Table S1 in the supplemental material. A nucleotide shown in lowercase in region 5 indicates a mutation.

tance (Table 4). With regard to INH oxidase activity, *E. coli* isolates with *katG*-2 to -8 showed 1/3 to 1/17 less activity than those with *katG*-wt. *E. coli* isolates carrying *katG*-9 to -13 showed reduced activity compared to those carrying *katG*-2 to -8. *E. coli* isolates carrying *katG*-14 to -21 showed no activity (i.e., levels similar to those observed for vector controls). These results indicated that the degree of INH oxidase activity is correlated with that of INH resistance. *E. coli* isolates with *katG*-wt and *katG*-1 showed the highest levels of INH oxidase activity, and *M. tuberculosis* isolates with these genes were sensitive to INH. *E. coli* isolates carrying *katG*-2 to -8 showed slightly weaker activities, and *M. tuberculosis* isolates with these genes were resistant to INH at 0.2 μg/ml but susceptible to INH at 1.0 μg/ml. *E. coli* isolates with *katG*-9 to -21 showed weak or no activity, and *M. tuberculosis* isolates with these genes were resistant to INH at 1.0 μg/ml.

The peroxidase and catalase activities of *E. coli* isolates with mutations were correlated well with each other and also with INH oxidase activity (Table 4). However, in *E. coli* isolates carrying some clones, peroxidase/catalase activities were different from INH oxidase activity, i.e., *E. coli* isolates with *katG*-16 and -9 showed weak activity.

Development of a LiPA for detection of INH resistance. To detect novel mutations associated with INH resistance, we developed a new LiPA based on the reverse hybridization principle (25). Forty-one oligonucleotide probes were designed for the LiPA to detect mutations containing the *furA-katG* operon, the *fabG1-inhA* operon, $P_{fabG1\text{-}inhA}$, and *fabG1* (Table

TABLE 3. *katG* mutations found in Inh^r isolates

Clone	Mutation(s)	
	Nucleotide	Amino acid
katG-1[a]	G1388T	R463L
katG-2[c]	C379G[b]	Q127E[b]
katG-3[c]	C694T[b]	P232S[b]
katG-4	A398C[b]	N133T[b]
katG-5[c]	T1147C[b]	S383P[b]
katG-6[c]	1297::C[b], Δ1305C[b]	KQT433-435QAD[b]
katG-7[c]	A290G	H97R
katG-8[c]	C1465A[b]	R489S[b]
katG-9	G944C	S315T
katG-10[c]	T1259C[b]	M420T[b]
katG-11	G944C, G1159C[b]	S315T, D387H[b]
katG-12[c]	G368A[b], G895A	G123E[b], G299S
katG-13[c]	G1255C	D419H
katG-14[c]	C195T[b], T527C[b]	A65A[b], M176T[b]
katG-15	Δ(478–479)[b]	Frame shift[b]
katG-16[c]	G944C	S315T
katG-17	Δ371G[b]	Frame shift[b]
katG-18[c]	C1894t[b]	R632C[b]
katG-19	C945A	S315R
katG-20[c]	Δ(571–576)[b]	Δ(191W-192E)[b]
katG-21[c]	G1624C[b]	D542H[b]

[a] *katG*-1 carrying a G1388T (R463L) neutral mutation was cloned from the Inh^s strain IMCJ 2751.

[b] These mutations have not previously been reported. Other mutations were previously reported in references 36 (G1388T), 7 (A290G), 36 (G944C), 7 (G895A), 6 (G1255C), and 36 (C945A).

[c] This clone also had a G1388T neutral mutation.

2). As shown in Fig. S2 in the supplemental material, the LiPA could detect all mutations found in this study.

DISCUSSION

The results of RFLP and sequence analysis in the present study indicated that there are several predominant strains of Inh^r *M. tuberculosis* with different genetic backgrounds in Japan (see Fig. S1 and Table S1 in the supplemental material). These strains had *katG*(*G944C*) (S315T), an *inhA* promoter mutation, *fabG1*(*G609A*) (L203L), and *furA*(*C41T*) (A14V) (see Table S1 in the supplemental material). Inh^r isolates were reported to expand clonally in several regions, including northwestern Russia (20), the Netherlands (30), San Francisco, CA (13), Venezuela (2), and Sierra Leone (15). These clonal Inh^r strains had a KatG(S315T) or *inhA* promoter mutation. Gagneux et al. (13) reported that the strains carrying the KatG(S315T) or *inhA* promoter mutation were more likely to spread than those carrying other mutations; our results were consistent with these previous findings. In addition, strains with *fabG1*(*G609A*) (L203L) and *furA*(*C41T*) (A14V) mutations were also more likely to spread in Japan.

Of Inh^r isolates, a smaller number (22%) had S315T/R mutations in Japan (Table S1). The prevalences of the KatG(S315T) mutation in *M. tuberculosis* strains from around the world differ, especially with regard to the prevalence of tuberculosis. In regions where the prevalence of tuberculosis is low or intermediate, the mutation has been reported relatively infrequently: it occurred in 26% to 30% of 95 isolates from Singapore (16) and Madrid (23) and rarely in isolates from Scotland (11) and Finland (19). In contrast, the S315T mutation accounted for INH resistance in 52% to 64% of strains in Africa (8, 14, 31), 79% in Peru (9), 91% in Russia (18), and 58% in New York, NY. (23).

We found four KatG mutations (D419H, M420T, D542H, and R632C) that are associated with high-level INH resistance, and we also found three KatG mutations (H97R, N133T, and P232S) that are associated with low-level INH resistance (Table 4). The S315 mutation is known to confer high-level INH resistance (24, 26, 33). KatG is a functional homodimer, and each monomer is composed of two domains that are mainly α-helical. The N-terminal domain contains a heme binding site, whereas the C-terminal domain lacks this feature (34). The high-level INH resistance-associated mutations D419H and M420T are located in the region connecting the N-terminal and C-terminal domains (5). The interdomain interactions between the N-terminal and C-terminal domains of the two monomers are essential for forming the functional homodimer (5). The changes in the interdomain interactions due to the D419H and M420T mutations may result in loss of enzymatic activities of KatG. D542H and R632C are located in the 16th

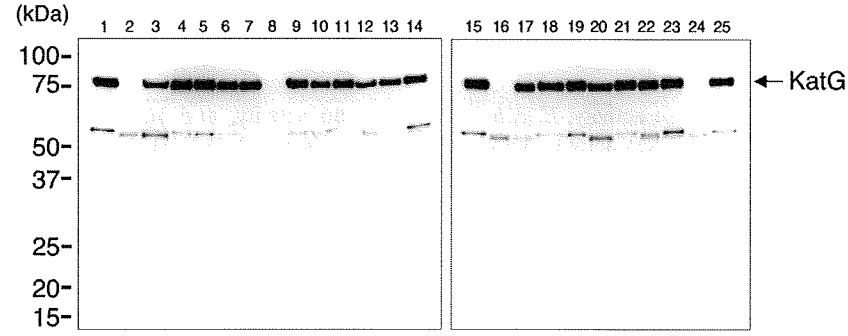

FIG. 2. Western blot of whole-cell extracts from *katG*-deficient *E. coli* strain UM262 transformed with the empty vector, pTrcHis2-TOPO, or recombinant plasmids expressing various KatG mutations as follows: lanes 1 and 15, WT; lanes 2 and 16, empty vector; lane 3, R463L and D542H; lane 4, S315T and R463L; lane 5, Q127E and R463L; lane 6, P232S and R463L; lane 7, G123E, G299S, and R463L; lane 8, frame shift mutation from position 160; lane 9, S315T and D387H; lane 10, R463L and R489S; lane 11, S315R; lane 12, M420T and R463L; lane 13, A65A, M176T, and R463L; lane 14, H97R and R463L; lane 17, Δ(191W-192E) and R463L; lane 18, N133T; lane 19, R463L; lane 20, R463L and R632C; lane 21, S315T; lane 22, D419H and R463L; lane 23, S383P and R463L; lane 24, frame shift mutation from position 124; lane 25, in-frame insertion and deletion and R463L. The positions of molecular mass markers are shown on the left.

TABLE 4. Enzymatic activities of KatG mutants detected in this study

Plasmid	Amino acid mutation(s)		Mean activity ± SD[a]			Additional mutation associated with INH resistance	INH resistance level[b]
	Not previously reported	Previously reported	INH oxidase (10^3 A_{560} units)	Peroxidase (10^2 A_{405} units)	Catalase (10^2 A_{240} units)		
pTrcHis2-TOPO[c]			4.84 ± 0.17	6.89 ± 0.70	0.00 ± 0.24		
p*katG*-wt			177.16 ± 18.50	286.08 ± 0.43	142.26 ± 0.16		S
p*katG*-1		R463L	162.00 ± 11.31	289.62 ± 1.40	141.85 ± 0.13		S
p*katG*-2	Q127E	R463L	60.18 ± 0.95	256.07 ± 7.80	143.21 ± 0.35	$P_{fabG1\text{-}inhA}$C−15T	0.2
p*katG*-3	P232S	R463L	54.47 ± 0.36	62.25 ± 0.05	76.63 ± 0.52		0.2
p*katG*-4	N133T		40.67 ± 6.31	36.00 ± 0.26	100.61 ± 5.55		0.2
p*katG*-5	S383P	R463L	38.49 ± 0.04	42.24 ± 3.64	107.65 ± 4.13	$P_{fabG1\text{-}inhA}$C−15T	0.2
p*katG*-6	KQT433-435QAD[d]	R463L	20.02 ± 0.48	106.47 ± 1.17	142.81 ± 0.11	$P_{fabG1\text{-}inhA}$C−15T	0.2
p*katG*-7		H97R, R463L	17.42 ± 0.35	26.80 ± 0.44	27.86 ± 2.01		0.2
p*katG*-8	R489S	R463L	10.40 ± 0.16	27.34 ± 0.27	14.83 ± 0.93	$P_{fabG1\text{-}inhA}$C−15T	0.2
p*katG*-9		S315T	8.83 ± 0.04	102.00 ± 2.54	71.26 ± 1.71		1.0
p*katG*-10	M420T	R463L	8.42 ± 0.14	21.02 ± 0.37	46.45 ± 0.20		1.0
p*katG*-11	D387H	S315T	7.93 ± 0.08	34.75 ± 0.61	35.71 ± 0.41		1.0
p*katG*-12	G123E	G299S, R463L	6.87 ± 0.66	6.02 ± 0.17	−0.70 ± 1.42	$P_{fabG1\text{-}inhA}$T−8C	1.0
p*katG*-13		D419H, R463L	6.30 ± 0.52	7.67 ± 0.01	4.49 ± 0.39		1.0
p*katG*-14	M176T[e]	R463L	5.14 ± 0.01	4.67 ± 0.07	1.06 ± 0.30	$P_{fabG1\text{-}inhA}$C−15T	1.0
p*katG*-15	Frame shift[f]		5.02 ± 0.24	4.01 ± 0.57	−1.75 ± 1.16		1.0
p*katG*-16		S315T, R463L	3.83 ± 0.18	84.41 ± 0.17	117.07 ± 7.56		1.0
p*katG*-17	Frame shift[g]		3.30 ± 0.69	4.59 ± 0.09	2.07 ± 1.51		1.0
p*katG*-18	R632C	R463L	3.26 ± 0.13	1.56 ± 0.08	−7.41 ± 0.76		1.0
p*katG*-19		S315R	3.19 ± 0.76	3.24 ± 0.02	−2.36 ± 0.71		1.0
p*katG*-20	Δ(191W-192E)[h]	R463L	2.78 ± 0.09	2.09 ± 0.04	2.61 ± 1.86		1.0
p*katG*-21	D542H	R463L	1.63 ± 0.49	0.32 ± 0.17	−7.00 ± 0.69		1.0

[a] Mean (n = 3) ± SD.
[b] The INH susceptibility levels for clinical isolates with *katG* mutations are shown, as follows: S, INH sensitive; 0.2, resistant to INH (0.2 μg/ml) and susceptible to INH (1.0 μg/ml); and 1.0, resistant to INH (1.0 μg/ml).
[c] A vector control.
[d] 1297::C and Δ1305C.
[e] This isolate had an additional A65A silent mutation.
[f] Δ(478–479).
[g] Δ371G.
[h] Δ(571–576).

and 19th α-helices in the C-terminal domain, respectively, and showed no enzymatic activities, although the functional role of the C-terminal domain in KatG remains unclear (5, 34). The mutations associated with low-level INH resistance, H97R, N133T, and P232S, are located adjacent to the INH binding pocket (5). They may weakly affect the binding affinity of INH. The S315T mutation located at the INH binding pocket could block binding of INH without interfering with catalysis (5).

The new LiPA was able to distinguish high-level INH resistance (resistant to 1.0 μg/ml) from low-level INH resistance (resistant to 0.2 μg/ml and sensitive to 1.0 μg/ml) in clinical isolates without sequencing. Thus, we were able to determine the degree of INH resistance using this LiPA. This assay would be useful in clinical application in combination with culture-based drug susceptibility tests. We have recently developed a LiPA to detect a *pncA* mutation(s) for rapid detection of pyrazinamide-resistant *M. tuberculosis* (29), which was shown to be readily usable in clinical applications (1). The whole procedure takes only 9 h, and the estimated cost per sample is $35. The clinical trials for *in vitro* diagnosis are in progress (from April 2009 to March 2010) in Japan. The trials will reveal the specificity of the LiPA. It will be beneficial especially in developing countries where the laboratories are scarcely equipped because of the high cost of setting them up.

Assessment of INH oxidase activities of *M. tuberculosis* isolates may provide useful information about INH resistance. The INH oxidase activities of KatG mutants showed good correlations with the degree of INH resistance (Table 4). Other enzymatic activities of KatG mutants, i.e., peroxidase and catalase activities, were also correlated with the degree of INH resistance (Table 4). However, the activities of the S315T mutant were not, i.e., this mutant showed catalase-peroxidase activities but no INH oxidase activity (Table 4). Other S315 mutants, such as the S315R (Table 4) and S315N (32) mutants, have lost all three kinds of enzymatic activity. Thus, the Inh^r isolates with KatG(S315T), retaining catalase-peroxidase activities, may have a survival advantage, and this may explain the global spread of strains with the KatG(S315T) mutation.

ACKNOWLEDGMENTS

We thank Akiko Seshimo for excellent technical assistance.

This work was supported by Health Sciences Research grants (H21-SHINKO-IPPAN-016) and a Grant for International Health Research (21A-105) from the Ministry of Health, Labor and Welfare of Japan.

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10.1111/j.1469-0691.2009.03078.x

Pyrazinamide resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates in Japan

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Abstract

Thirty-six multidrug-resistant (MDR) *Mycobacterium tuberculosis* isolates collected in Japan were examined for pyrazinamide susceptibility and pyrazinamidase activity, and analysed by *pncA* sequencing and a hybridization-based line probe assay (LiPA), which was used to detect *pncA* mutations for the rapid identification of pyrazinamide-resistant isolates. Pyrazinamide resistance was found in 19 (53%) of them. All pyrazinamide-resistant isolates had no pyrazinamidase activity and at least one mutation in *pncA*. Among the *pncA* mutations, 11 had not been previously reported. The results of the LiPA were fully consistent with the DNA sequencing results. A majority of MDR *M. tuberculosis* isolates in Japan were resistant to pyrazinamide.

Keywords: Line probe assay, multidrug resistance, *Mycobacterium tuberculosis*, *pncA*, pyrazinamide

Original Submission: 30 April 2009; **Revised Submission:** 7 July 2009; **Accepted:** 13 July 2009
Editor: G. Greub

Clin Microbiol Infect

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The emergence and spread of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis*, which are defined as strains resistant to both rifampin and isoniazid, constitute a serious threat to the control of tuberculosis (TB) [1].

Pyrazinamide is a first-line anti-TB drug that exhibits strong activity against semidormant bacilli sequestered within macrophages, and is used in short-course treatment in combination with rifampin, isoniazid, and ethambutol [2,3]. Pyrazinamide is also one of the most important drugs used in the treatment of MDR TB. It is a prodrug that requires conversion into its active form, pyrazinoic acid, by the bacterial enzyme pyrazinamidase (PZase), which is encoded by *pncA* [4–6]. Mutations in *pncA* lead to the loss of PZase activity and constitute the mechanism of pyrazinamide resistance in *M. tuberculosis* [5,7,8].

It is essential that rapid drug susceptibility testing (DST) be developed to prevent the spread of MDR *M. tuberculosis*. DST of *M. tuberculosis* produces reliable results for most anti-TB drugs [9]. However, conventional DST for pyrazinamide is hampered by poor bacterial growth, because pyrazinamide requires acidic conditions to be active; therefore, DST must be performed under such conditions [5]. Liquid culture-based methods have recently been developed to resolve this problem [10].

Previously, we described a DNA sequencing-based method for detecting mutations in the genome of drug-resistant strains, including pyrazinamide-resistant *M. tuberculosis* [11]. However, the use of this method in ordinary-scale clinical laboratories can present a problem because of its high cost. Therefore, we developed a hybridization-based line probe assay (LiPA) for the rapid detection of *pncA* mutations in pyrazinamide-resistant *M. tuberculosis* that can easily be used for clinical applications [12].

Thirty-six MDR *M. tuberculosis* isolates were collected during the national surveillance study, from June to November 2002, by the Tuberculosis Research Committee (Ryoken), Japan [13]. These isolates were obtained from patients with TB in nine hospitals in Japan located in various regions: one in Hokkaido, one in Tohoku, four in Kanto, two in Kinki and one in Kyushu. *M. tuberculosis* strain H37Rv (ATCC 27294), which is susceptible to pyrazinamide and positive for PZase, was used as a positive control for the assay. *Mycobacterium bovis* strain BCG (Japanese strain 172), which is resistant to pyrazinamide and negative for PZase, was used as a negative control.

Pyrazinamide susceptibility testing was performed with a broth method (BD BACTEC MGIT 960; BD Biosciences, Sparks, MD, USA) [9]. PZase activity was determined using Wayne's method [14], with some modifications [12]. Rifampin and isoniazid susceptibility testing were performed with an egg-based Ogawa medium method. These assays were performed in one institution (Japan Anti-Tuberculosis Association).

Nested PCR was performed to amplify a 670-bp fragment that includes the entire *pncA* gene, as described previously [12]. For DNA sequencing, only the second PCR was

performed. DNA sequences of *pncA* and its promoter region (nucleotides −80–572 relative to the initiation codon) were determined as described previously [11] and compared with those of H37Rv using GENETYX-MAC (Genetyx Corporation, Tokyo, Japan).

Forty-seven oligonucleotide probes designed to cover the entire *pncA* gene of H37Rv were immobilized on two strips and used for the LiPA (Table S1). The LiPA was conducted as described previously [15]. Biotinylated PCR products from test samples were hybridized to the immobilized probes, and the strips were washed. The presence or absence of bands on all strips was judged visually.

Of 36 clinical isolates of MDR *M. tuberculosis* tested with the LiPA, 17 were wild type, and the other 19 showed at least one mutation (Table 1 and Fig. 1). As shown by the data in Fig. 1, the 17 wild-type isolates (lanes 1–17) and H37Rv (lane 37) hybridized to all probes. The other 19 isolates did not hybridize to at least one probe (lanes 18–36). Regarding the pyrazinamide resistance profile, the LiPA yielded results that were 100% in agreement with those obtained by culture-based susceptibility testing (Table 1). All PZase-positive bacilli tested were sensitive to pyrazinamide, and all PZase-negative bacilli were resistant to pyrazinamide (Table 1). These data are consistent with those of previously published reports [11,12]. All of the 19 pyrazinamide-resistant isolates were correctly identified as being pyrazinamide-resistant by the LiPA, and all of the 17 pyrazinamide-susceptible isolates were identified as being pyrazinamide-susceptible.

The *pncA* genes of all isolates tested were sequenced (Table 1). One or more *pncA* mutations were identified in 24 isolates, and 12 isolates had no mutation. Among the 24 isolates with *pncA* mutations, we found 20 different mutations, of which 11 have not been previously reported. Of these 11 novel mutations, four were frameshift mutations (Δ59, Δ(129–130), 261::AC, Δ(374–389)), five were mutations causing an amino acid substitution (G232A, A340G, G400T, G419A, G493A), one was a non-

TABLE 1. **Identification of *pncA* mutations by line probe assay (LiPA) among 36 multidrug-resistant *Mycobacterium tuberculosis* isolates**

Strain	LiPA profile[a]	Pyrazinamide susceptibility	PZase activity	Mutation	
				Nucleotide change	Amino acid change
2A-3-16	Wild type	S	+	–	–
2A-3-83	Wild type	S	+	–	–
2A-3-84	Wild type	S	+	–	–
2A-4-30	Wild type	S	+	–	–
2B-7-38	Wild type	S	+	–	–
2C-1-46	Wild type	S	+	–	–
2G-2-5	Wild type	S	+	–	–
2I-11-4	Wild type	S	+	–	–
2P-5-113	Wild type	S	+	–	–
2P-5-280	Wild type	S	+	–	–
2P-5-420	Wild type	S	+	–	–
2R-1-48	Wild type	S	+	–	–
2C-3-89	Wild type	S	+	G419A[b]	R140H
2O-2-16	Wild type	S	+	G419A[b]	R140H
2A-3-142	Wild type	S	+	G419A[b], G493A[b]	R140H, A165T
2P-5-269	Wild type	S	+	G419A[b], G493A[b]	R140H, A165T
2C-4-48	Wild type	S	+	G419A[b], C450A[b], G493A[b]	R140H, G150G, A165T
2A-3-11	Δ16	R	–	T175C	S59P
2A-3-14	Δ7, Δ20, Δ21	R	–	Δ59[b], G232A[b]	Frameshift, G78S
2A-3-137	Δ13, Δ14	R	–	C153A, G493A[b]	H51Q, A165T
2B-7-33	Δ15	R	–	C161T	P54L
2C-3-105	Δ33	R	–	A410C	H137P
2E-1-3	Δ1, Δ28	R	–	T-7C, A340G[b]	T114A
2E-1-93	Δ1, Δ28	R	–	T-7C, A340G[b]	T114A
2O-4-41	Δ2	R	–	C8A	A3E
2P-1-57	Δ12	R	–	Δ(129–130)[b], G493A[b]	Frameshift, A165T
2P-1-114	Δ23	R	–	261::AC[b]	Frameshift
2P-1-118	Δ10	R	–	T100G	Y34D
2P-1-120	Δ4	R	–	T26G	V9G
2P-5-58	Δ2	R	–	C8A, G419A[b]	A3E, R140H
2P-5-108	Δ23	R	–	261::AC	Frameshift
2P-5-167	Δ10	R	–	T100G	Y34D
2P-5-230	Δ10	R	–	C102A[b]	Y34[c]
2P-5-233	Δ2	R	–	C8A	A3E
2P-5-254	Δ10	R	–	T100G, G400T[b]	Y34D, A134S
2V-5-5	Δ31, Δ32	R	–	Δ(374–389)[b]	Frameshift

[a]Δ indicates a negative signal at a probe.
[b]Mutation not previously reported.
[c]Ochre mutation.

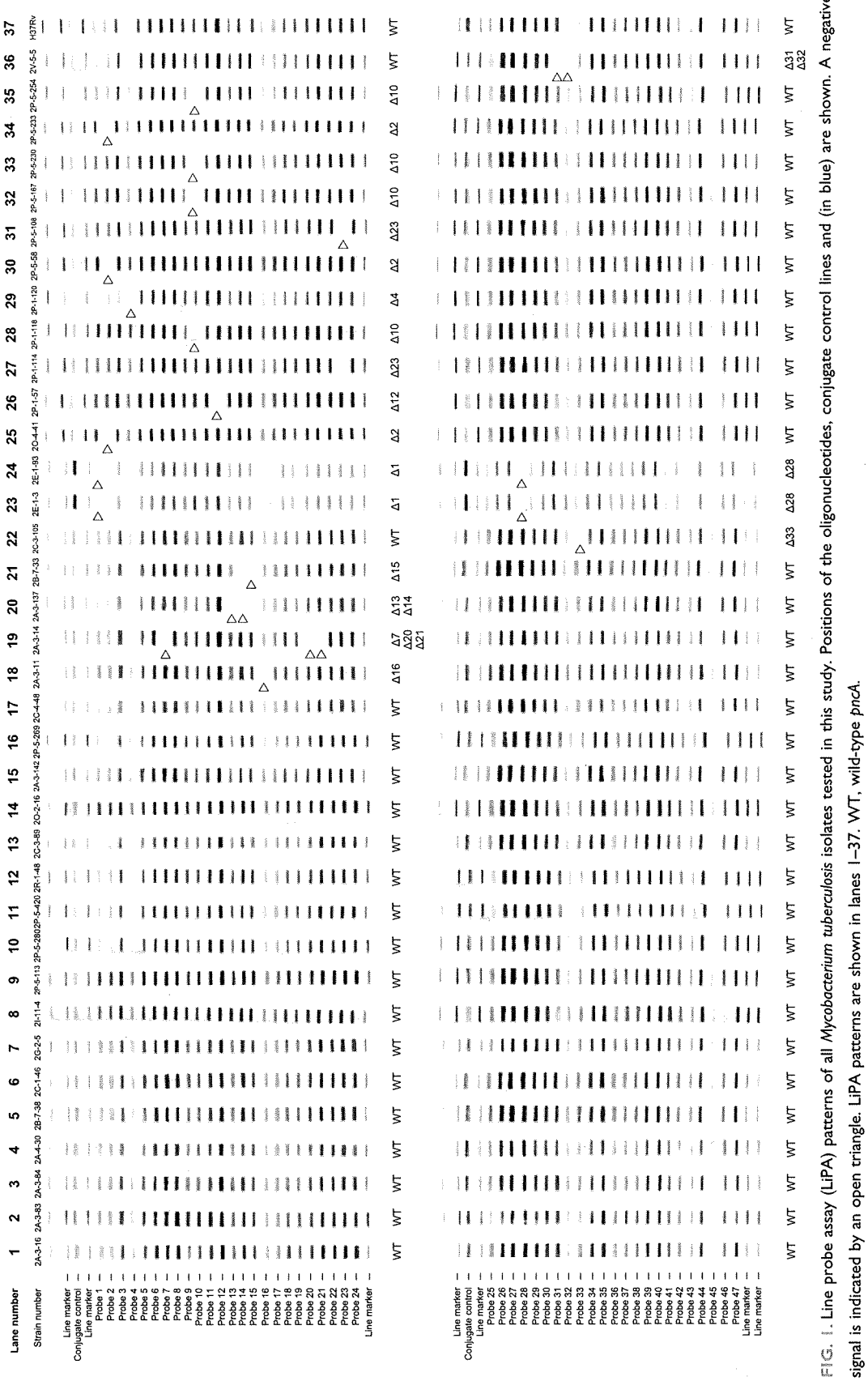

FIG. 1. Line probe assay (LiPA) patterns of all *Mycobacterium tuberculosis* isolates tested in this study. Positions of the oligonucleotides, conjugate control lines and (in blue) are shown. A negative signal is indicated by an open triangle. LiPA patterns are shown in lanes 1–37. WT, wild-type *pncA*.

sense mutation (C102A), and one was a silent mutation (C450A) (Table 1). The G419A and G493A mutations are not associated with pyrazinamide resistance, because the isolates with these mutations were pyrazinamide-sensitive. It is unknown whether G232A, A340G and G400T are associated with pyrazinamide resistance, because isolates with these mutations had an additional mutation in *pncA* that conferred pyrazinamide resistance. As shown in Table 1, the isolate with the C102A mutation (strain 2P-5-230) was resistant to pyrazinamide and exhibited no PZase activity. The C102A mutation changed the 34th amino acid of PZase into a stop codon, suggesting that the C102A mutation is associated with pyrazinamide resistance. The results of the LiPA were fully consistent with the DNA sequencing results (Table 1). The LiPA correctly identified pyrazinamide susceptibility and resistance in all strains in which a mutation occurred.

We found that 53% of MDR *M. tuberculosis* isolates (19 of 36) obtained in Japan were resistant to pyrazinamide. Although the number of MDR isolates detected in this study was small, the results suggest that the majority of MDR *M. tuberculosis* isolates in Japan are resistant to pyrazinamide. Mphahlele *et al.* [16] reported that 52% of South African MDR *M. tuberculosis* isolates are resistant to PZA. Thus, a majority of MDR *M. tuberculosis* isolates in other countries may also be resistant to pyrazinamide. Clinical trials of the LiPA for *in vitro* diagnosis in Japan started in April 2009.

Acknowledgements

The authors thank the Tuberculosis Research Committee (Ryoken), Japan, for supporting the collection of clinical MDR *M. tuberculosis* isolates.

Transparency Declaration

This study was supported by Health Sciences Research grants from the Ministry of Health, Labour, and Welfare of Japan (H21-SHINKO-IPPAN-016) and a Grant for International Health Research (21A-105). H. Ando is supported by the Japan Health Sciences Foundation.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Location of 47 oligonucleotide probes designed to cover *Mycobacterium tuberculosis pncA*.

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複数の薬効きにくい結核

迅速判別キット

厚労省研究班

結核のなかで複数の薬が効きにくい「多剤耐性結核」かどうかを約9時間で見分ける診断キットを、厚生労働省研究班（主任研究者＝加藤誠也・結核予防会結核研究所副所長）が開発した。現在は患者が医療機関を受診してから1～2カ月間かかっていた。開発したキットを使えば、効きにくい薬を治療の選択肢から外し、素早く適切な治療ができるようになる。

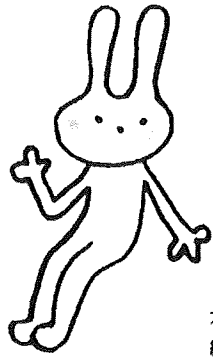
研究班に参加した国立国際医療センターやニプロが共同開発した。すでに臨床試験（治験）を終えており、ニプロを通じて2012年初頭までの製品化を目指す。

多剤耐性結核は少なくとも「イソニアジド」「リファンピシン」の2つの結核治療薬に耐性を示す。国内では年間2万人以上が結核を新たに発症しているが、2%程度が多剤耐性結核と考えられている。世界では毎年約50万人が多剤耐性結核を発症しているとされる。

キットは薄いフィルムの上に、結核菌の薬剤耐性にかかわる遺伝子と反応する塩基を配列した。患者から採取した喀痰（かくたん）の中の結核菌の遺伝子を増幅し、フィルムと反応させると、フィルムに線が現れ、4種類の薬に対する耐性の有無が分かる。

現在、多剤耐性かどうかを見極めるには結核菌と薬を実際に反応させて長期間培養しなければならない。検査には感染を防ぐための空調設備などを持つ施設が必要で、途上国で実施するのは困難だった。国内以外に途上国での薬剤耐性検査用としても需要が見込めるとみている。

日本経済新聞　2010年（平成22年）2月22日（月曜日）



シリーズ

これ知っとこう！ ⑥

北京株

大阪市立環境科学研究所　微生物保健担当　和田崇之
結核予防会結核研究所抗酸菌レファレンス部結核菌情報科　前田伸司

「北京株」―非常になじみの薄い言葉で，一体結核と何の関係があるのかと思われる方も多くいらっしゃると思います。北京株とは，遺伝型別解析の発展に伴って見いだされてきた，ある特殊な遺伝的特徴を示す結核菌株に付けられた名称です。正確には，その遺伝的特徴を「北京型（Beijing family type）」，北京型を示す結核菌株を「北京株（Beijing strain）」と呼びます。日本の結核と密接な関係があり，特に分子疫学や臨床分離株に関する基本情報として，いまや欠かすことのできない知識となってきています。

結核菌はすべてが同一ではない

結核菌株の差異を遺伝子レベルで判定する遺伝子型別法が確立する1990年代まで，結核菌の臨床分離株は基本的にほとんど違いが無く，同じ個性を持った菌株が全世界に拡散していると認識されていました。しかし，制限酵素断片長多型（RFLP）分析や反復配列多型（VNTR）分析を応用することによって，結核菌も他の生物と同様に多くの遺伝的多型をもった集団であることがわかってきました。

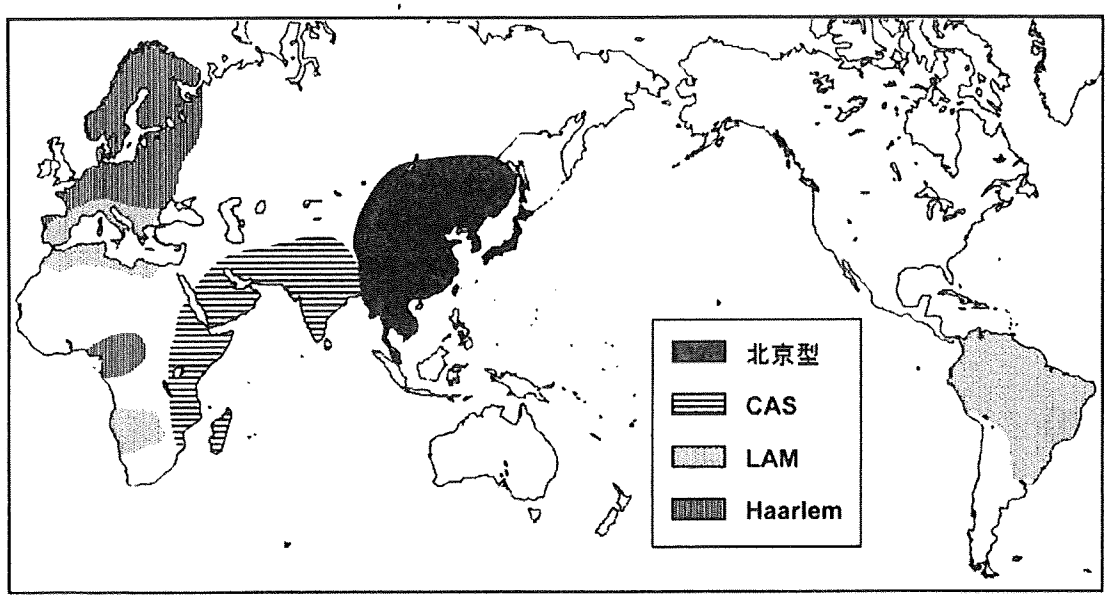
例えばヒトの世界において遺伝子型を用いると，人種などのグループごとに分類することができます。同様に結核菌を遺伝子型に従って分類すると，世界各地で分離された結核菌株はおおむね7つの系統群[1]（clade）に大別され，各系統群にはさまざまな違いがあることがわかってきました。これらの系統群は数万年前に大きく分岐した後，ヒトへの感染・伝播を経て現代まで残ってきたと推定されています。

興味深いことに，いくつかの系統群は特定の地域で優先的にまん延していることが知られています（図1）。最近の研究では，地域性のある分布を示す系統群は，高頻度で分離される地域の人々に強く適応しているため，他の系統群よりも発症しやすいのではないかと考えられています[2]。北京株は，中国・北京の結核患者から極めて高頻度に分離されることが報告され[3]，このような名前が付けられています。しかし実際には，中国のみならず東アジア全域で分離される系統群であり，日本でも臨床分離株の約8割を占めることが明らかになっています[4]。

北京株の特徴

北京株はその発見経緯からもわかるように，

図1　結核菌系統群の分布地図



特定の地域で優先的にまん延している結核菌系統群（4群）の代表例を地図上に示した。北京型は東アジア一帯での定着が認められる系統群である

遺伝型に大きな特徴があります。従来の遺伝型別法でも判定できますし，現在普及が進んでいるVNTR法でも特徴のあるパターンを示すことがわかっています（図2）。したがって，遺伝型別解析を利用して北京株を見いだすことは容易です。

一方で，病原体としての北京株の特徴についてはまだ研究が始まったばかりで，詳しいことはほとんどわかっていません。マウスなどを用いた動物実験では他の結核菌株よりも病原性が高いという報告[5]もありますが，その要因は現時点でははっきりしていません。BCGによる獲得免疫に対しても抵抗性が高い（つまり，BCGワクチンが効きにくい）[5,6]と考えられており，現在の結核対策が今後継続的に有効であるか否かを判断する上で，微生物学・分子疫学的だけでなく，公衆衛生学的にも重要視されることが予想されます。

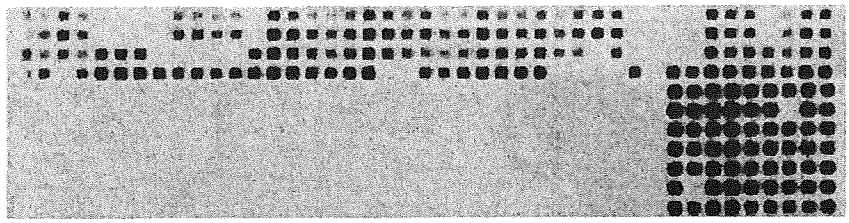
日本特有の北京株の分布

結核菌株が遺伝型別によって分類されることは上述しましたが，北京株そのものも同様で，遺伝型の違いによってさらに細分類できます。祖先型（ancient型）と新興型（modern型）という分類が最も代表的で，日本では祖先型北京株が優先的に分離されます（北京株の7～8割程度）[7]。逆に，中国をはじめとした諸外国では新興型の分離率が高く，日本の特異性が際立っています（表1）。なぜ日本でのみ祖先型北京株が多いのか，その理由はまったくわかっていません。

一次結核が疑われる若年層のケースでは，日本でも新興型北京株が分離されることが多く，その拡散・流行が懸念されるところです。臨床分離株を継続的に遺伝子型別解析し，そのような兆候を見逃さないような監視体制を確立することが，結核対策における重要課題の一つになりつつあると言えるでしょう。また，北京株を

図2　北京株にみられる遺伝子型の特徴

(a)



(b)

M04	M10	M16	M26	M31	M40	ETR A	ETR C	t04	t21	t30	t39	Q11b	Q26	Q4156	
1	5	1	1	3	2	2	4	2	2	4	3	2	4	4	
2	2	3	5	2	1	3	4	2	1	2	5	4	4	3	非北京株
2	2	2	5	2	2	3	4	2	2	2	3	3	7	3	
2	2	3	5	3	3	3	4	2	2	2	3	3	7	3	
2	3	4	7	5	3	4	4	3	3	4	3	7	2	3	
2	3	3	10	5	3	4	4	4	3	4	3	6	8	5	
2	1	3	7	5	4	4	4	4	3	4	3	7	7	4	北京株
2	3	3	6	5	3	4	4	4	4	4	3	5	8	3	
2	3	3	7	5	3	4	4	4	4	4	3	5	7	3	
2	3	3	7	5	3	4	4	4	4	4	3	8	8	3	

北京株にはさまざまな遺伝子の特性があり，遺伝型別解析によって判別が可能である。a）スポリゴタイピング法による結核菌株の型別例。北京株ではまとまった領域でシグナルの欠損が生じており，これを指標にして容易に判別可能である。b）VNTR法による結核菌株の型別例。ここでは，国際標準法である15領域を示している。北京株にみられる特徴は，M26＞6（主に7），M31＝5，ETR A＝4など。VNTR型別では例外も多いため，実際には全体的な傾向から判断する

ターゲットとした世界規模の調査研究[8]と連携することにより，国際的な結核感染症の理解への貢献が期待されます。

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表1　各地域において報告されている北京株の分離率（臨床分離株全体を100%とした時）および祖先型・新興型北京株の比率（概算値）

国 / 地域	北京株の分離率	祖先型	新興型
日本	80%	80%	20%
中国 (北京)	93%	5%	95%
香港	70%	14%	86%
台湾	52%	4%	96%
ベトナム	54%	25%	75%
ロシア (北西部)	55%	5%	95%
南アフリカ	20%	1.5%	98.5%

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