The compound in the first study is *N*-(3-oxocyclohex-1-enyl)octanamide (named J8-C8) (**Figure 3**), which was isolated from Smith's library (Smith et al., 2003a) and was characterized as an inhibitor for TofI, a C_8-HSL generator of the LuxI-family protein in *Burkholderia glumae* (Chung et al., 2011). J8-C8 significantly inhibited C_8-HSL production by TofI in a dose dependent manner, furthermore the effect was enhanced in the presence of MTA. Generally, the LuxI-family protein synthesizes a specific AHL from an acylated ACP and SAM, eventually AHL is released with holo-ACP and 5′-methylthioadenosine (MTA) as by-products from the protein. In a TofI/J8-C8/MTA ternary crystal structure, J8-C8 binds to TofI occupying the binding site for the acyl chain of the cognate substrate C_8-ACP. Simultaneously, a second substrate (MTA) binds to the binding site for SAM, which accounts for the synergistic effect of MTA.

The latter study describes AHL synthesis inhibitors that are transition state analogs of MTAN. The MTAN enzyme catalyses the hydrolytic deadenylation of MTA and *S*-adenosyl homocysteine (SAH) and produces 5′-methylthioribose (MTR) and *S*-ribosylhomocysteine (SRH) which are steps in SAM biosynthesis. MTAN enables AHL-producing bacteria to recycle SAM from the MTA released as a by-product after AHL syntheses. In addition, SRH also becomes a precursor for AI-2 generation (Xavier and Bassler, 2003). Thus, the MTAN inhibition provides a method of blocking not only AHL production, but also AI-2. Three analogs of the transition state during the reaction from the MTA substrate into MTR and adenine, have been designed and are named 5′-methylthio- (MT-), 5′-ethylthio- (EtT-), and 5′-butylthio- (BuT-) DADMe-ImmucillinAs (**Figure 3**; Gutierrez et al., 2009). According to a 3D-structural analysis of MTAN in *Vibrio cholerae* with BuT-DADMe-ImmucillinA, the inhibitor binds to the catalytic active site of the protein producing hydrophobic stacking interactions. These analogs, including BuT-DADMe-ImmucillinA, have been shown to inhibit MTAN activity with IC50 values at the nM level and reduce AI-2 production and biofilm formation in *Vibrio cholerae* and *Escherichia coli* O157. Although this study principally described the AI-2 effect, the researchers might investigate AHL quorum sensing in the near future.

DEGRADATION ENZYMES

In addition to small molecules which interfere with signal sensing or generation, signal breakdown by catalytic enzymes is an alternative strategy. Two classes of enzymes, lactonase and acylase, are known to perform this function. The former is a catalytic enzyme that cleaves the homoserine lactone ring and the latter catalyzes the hydrolysis of an amide bond between the homoserine lactone moiety and a fatty-acyl group. The degraded AHL products are no longer active in quorum sensing, therefore the phenomenon is often called "quorum quenching." A lactonase was originally identified and purified from a Gram-positive *Bacillus* strain and the enzyme was designated "AiiA," meaning autoinducer inactivation. The protein sequence has no significant similarity to any known sequences, but contains a HXHXDH zinc-binding motif that is conserved in glyoxalase II, metallo β-lactamase and arylsulfatase (Dong et al., 2000). The purified AiiA protein cleaves the homoserine lactone ring in C_4 to C_{12}-HSLs, with or without substitution at carbon three position including 3-oxo-C_6-HSL produced by a plant pathogen, *Erwinia carotovora*. Heterologous expression of the *aiiA* gene in *Erwinia carotovora* resulted in a remarkable decrease in quorum sensing-activating gene expression and less virulence to plants. Also, *aiiA*-expressing transgenic tobacco and potato were tolerant to the bacterial infection (Dong et al., 2001). There are similar bioengineering studies utilizing AiiA lactonase. For example, AiiA overexpression in *Pseudomonas aeruginosa* and *B. thailandensis* impaired their quorum sensing activities through the degradation of signals (Reimmann et al., 2002; Ulrich, 2004). Following these AiiA studies, *aiiA* homologs genes have been identified from other species such as *Agrobacterium tumefaciens* (Zhang et al., 2002) and *Arthrobacter* sp., (Park et al., 2003) and their enzymatic activities have been demonstrated. In addition, a subclass of AHL lactonases has been recently discovered in a species of soil bacterium. Unlike AiiA, they have no conserved HXHXDH zinc-binding motif. For example, QsdA in *Rhodococcus erythropolis* is a phosphotriesterase (PTE)-like protein that has other zinc-binding domains instead of the HXHXDH motif and can degrade AHLs which have an acyl chain of C_6–C_{14} in length (Uroz et al., 2008). However, unlike typical PTE enzymes, the protein is unable to cleave the phosphotriester bond. AiiM in *Microbacterium testaceum* isolated from a potato leaf (Wang et al., 2010) and three BpiB isomer proteins from soil metagenomic clones (Schipper et al., 2009) do not have any putative zinc-binding domains. AiiM has been deduced to belong to alpha/beta hydrolase fold family. The protein prefers C_6 to C_{12}-HSLs with 3-oxo substitution to those without substitution as degradation substrates. The expression of AiiM in the plant pathogen *Pectobacterium carotovorum* subsp. *carotovorum*, which is a 3-oxo-C_6-HSL producer, reduced virulence against the potato tissue (Wang et al., 2010). The series of BpiB proteins were originally isolated from soil-derived metagenomic libraries with a *traI-lacZ Agrobacterium tumefaciens* reporter strain that can respond to 3-oxo-C_8-HSL, then protein expressing clones which attenuated *traI-lacZ* activity were isolated. Two out of the three BpiB proteins, designated BpiB01 and BpiB04, show no similarity to any known proteins while the other protein, BpiB07 shares some sequence similarity with the esterase-lipase superfamily proteins. In addition to the degradation of 3-oxo-C_8-HSL, all the clones inhibited *Pseudomonas aeruginosa* swarming motility and biofilm formation controlled by 3-oxo-C_{12}-HSL and C_4-HSL (Schipper et al., 2009). AHL lactonase was originally characterized as a zinc-binding protein and it has been shown that mutations in the HXHXDH zinc-binding domain in some AiiA family lactonases result in them losing their function. However, based on their amino acid sequences, it is still unclear whether AiiM and BpiBs bind zinc. Recently, a new type of AHL lactonase has been isolated from the marine bacterium *Pseudoalteromonas byunsanensis*. The identified ORF appears to encode a hybrid membrane protein that has a GDSL (consensus Gly-Asp-Ser-Leu motif) hydrolase domain at the N-terminal of a RND (resistance-nodulation-cell-division)-type multidrug efflux transporter (Huang et al., 2012). The truncated form, including the GDSL hydrolase function, is designated as QsdH and has a catalytic activity for the C_4 to C_{12}-HSLs (with or without 3-oxo substitution) lactonase reaction, and co-inoculation of the plant pathogen *Erwinia carotovora* with a recombinant QsdH-overexpressing *Escherichia coli* has resulted in

milder lesions on potato tissues compared to a co-inoculation without QsdH.

In addition to environmental microorganisms, mammalian enzymes also have AHL lactonase activities (Chun et al., 2004). Human has three paraoxygenases (PON1, PON2, and PON3) with a distinct substrate specificity and expression pattern. There are reports that they cleave lactone rings in a series of AHLs (Draganov et al., 2005; Ozer et al., 2005)

The other family of AHL degradation enzymes is AHL acylase. This was first described in *Variovorax paradoxus*, although the gene which is responsible for the reaction has not been yet identified (Leadbetter and Greenberg, 2000; Leadbetter, 2001). The organism was isolated from soil based upon its ability to utilize 3-oxo-C_6-HSL as both an energy and nitrogen source. Hypothetically, AHLs is initially cleaved into a fatty acid and homoserine lactone moiety by an uncharacterized acylase in first reaction step, and subsequently the fatty acid is subjected to beta-oxidation as an energy material, while the homoserine lactone is degraded into ammonium chloride and carbon dioxide. The first AHL acylase to be characterized is AiiD from *Ralstonia eutropha* (Lin et al., 2003). The polypeptide is most similar to the aculeacin A acylase (AAC) from *Actinoplanes utahensis* and it also shares significant similarities with the cephalosporin and penicillin acylases, which are members of the N-terminal (Ntn) hydrolase superfamily. AiiD has been purified as a glutathione *S*-transferase (GST) fusion protein and its AHLs cleavage spectrum has been investigated. The GST-AiiD protein effectively hydrolyzes an amide bond on AHLs with longer fatty acyl side chains, such as 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL and 3-oxo-C_{12}-HSL whereas it is less active against shorter side chain substrates as 3-oxo-C_6-HSL. Heterologous AiiD expression in *Pseudomonas aeruginosa* has also been shown to abolish the accumulation of both 3-oxo-C_{12}-HSL and C_4-HSL and the killing of *Caenorhabditis elegans*. Based on the AiiD sequence, homologues have been identified in other organisms. Three acylases in *Pseudomonas aeruginosa* and closely related species, designated PvdQ, QuiP, and PA0305 (alternatively named HacB), respectively, are well-characterized (Huang et al., 2003, 2006; Wahjudi et al., 2011). They participate in the degradation of 3-oxo-C_{12}-HSL, but not C_4-HSL. However, their expression is considered to be highly regulated and turned off in standard experimental conditions (usually aerobically growth at 37 degree in rich medium) because wild-type *Pseudomonas aeruginosa* accumulates a large amount of 3-oxo-C_{12}-HSL during early stationary phase. When these acylases are constitutively produced from exogenous plasmids, a significant reduction of 3-oxo-C_{12}-HSL accumulation in the medium is observed. Some other homologous AHL acylases have also been identified in *Streptomyces* sp. from soil samples, the fish-associated bacterium *Shewanella* sp. and the nitrogen-fixing cyanobacterium *Anabaena* sp. (Park et al., 2005; Morohoshi et al., 2008; Romero et al., 2008). These studies provide us with not only extensive ideas for quorum sensing inhibitor applications, but also stimulate our general biological interest as to why AHLs-degrading organisms are widespread in nature. Thus far, the physiological benefits of degradation enzymes are presumed to be specific to AHL utilization as a nutrient resource, the detoxification of lactone ring compounds, the jamming of quorum sensing in pathogens as an innate bio-defense mechanism, and the modulation of the quorum sensing activity.

SIGNAL TRAPPING

An alternative technique for the attenuation of quorum sensing based on the trapping of AHLs has been created. This method arose from the observation that quorum sensing does not occur when the AHL concentration is maintained below a threshold level, thus an AHL interceptor would act as a quorum sensing inhibitor. Cyclodextrins are well known to form stable aqueous complexes with many organic compounds. In an initial study, C_4-HSL was reported to be an entry substrate for a cyclodextrin donor, and a bacterial culture containing the cyclodextrin suppressed RhlR-activated *rhlA* gene expression in *Pseudomonas aeruginosa* (Ikeda et al., 2002). The C_6-HSL, C_7-HSL, C_8-HSL, and 3-oxo-C_6-HSL from *Serratia marcescens* and C_4-HSL from *Pseudomonas aeruginosa* can be trapped and this results in the decrease in the production of the quorum sensing-induced red-pigment (prodigiosin; Kato et al., 2006). Currently, the use of cyclodextrin as a method of quorum sensing interference is still immature, although it is well studied as a cholesterol remover and as a carrier for medical applications such as in Niemann–Pick disease (See review Vance and Peake, 2011). For applications to antibacterial and bio-fouling materials, further technical studies are required, for example, a chemical engineering approach to increase the solubility and the stability of the cyclodextrin-AHL inclusion complex may be successful in the future.

UNKNOWN MECHANISM: MACROLIDE ANTIBIOTICS AS A CASE OF STUDY

Macrolide antibiotics have been shown to inhibit *Pseudomonas aeruginosa* quorum sensing. Since macrolides are relatively hydrophobic and are large sized-molecules, these antibiotics are generally believed to be ineffective against Gram-negative bacteria due to their low permeability and exclusion from the bacterial cytoplasm (Nikaido and Vaara, 1985; Nikaido, 1996). However, surprisingly, there have been reports from clinical trials showing that long term treatment with macrolide antibiotics at sub MIC eases the chronic lung infectious diseases caused by *Pseudomonas aeruginosa* in patients with CF and diffuse panbronchiolitis (DPB; Keicho and Kudoh, 2002; Southern et al., 2011). A number of mechanisms for the macrolide action on the bacterium and host have been proposed (See reviews, Tateda et al., 2007; Kanoh and Rubin, 2010). One proposal is that the drug influences quorum sensing. Azithromycin, the 15-membered ring macrolide has been shown to repress the activity of *Pseudomonas aeruginosa* quorum sensing based on both the levels of 3-oxo-C_{12}-HSL and C_4-HSL syntheses and the expression of *las* and *rhl*-activated gene/protein such as elastase, rhamnolipid, and pyocyanin (Tateda et al., 2001; Wagner et al., 2005; Nalca et al., 2006; Skindersoe et al., 2008). The azithromycin efficacy at sub-MIC is presumably attributed to the reduction of 3-oxo-C_{12}-HSL and C_4-HSL levels because a subset of genes involving SAM biosynthesis is partly repressed by azithromycin (Kai et al., 2009). Elucidation of its exact molecular action and target is the next question to be answered.

SUPPRESSORS OF QUORUM SENSING

It is known that some quorum sensing bacteria have regulatory elements which impede their quorum sensing. It is speculated that the physiological implications of an intrinsic modulation mechanism in quorum sensing is a tightly controlled repression of quorum sensing-controlled genes under a threshold population, a delay in quorum sensing initiation, and a slowing of its regulatory circuit or fine-tuning of its activity at a specific level. Apart from their actual roles, it might be possible to apply these suppressors to a quorum sensing inhibitory method, because if we are able to artificially manipulate the function and cellular level of these elements, quorum sensing will be controlled.

Anti-LuxR activators inhibiting quorum sensing activation have been reported in *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*. TrlR from *Agrobacterium tumefaciens* is a homologue of TraR, an AHL receptor protein, but lacks a DNA binding domain,(Chai et al., 2001). The protein forms an inactive heterodimer with TraR. The other anti-TraR proteins, TraM and its homologue TraM2, also interact with TraR to prevent its DNA binding (Fuqua et al., 1995; Hwang et al., 1995; Swiderska et al., 2001; Wang et al., 2006). These mutants confer constitutive AHL signal accumulation even in the absence of octopine, which is a quorum sensing initiator, and also confer hyper-plasmid conjugative transfer efficiency with excessive activation of the quorum sensing. Like anti-TraR in *Agrobacterium tumefaciens*, QslA is an anti-LasR protein in *Pseudomonas aeruginosa* (Seet and Zhang, 2011). The *qslA* null mutant is able to respond to much lower levels of the quorum sensing signal than the parent, resulting in higher quorum sensing activity, such as elevated exo-protease and elastase production. QscR, an orphan LuxR-family protein in *Pseudomonas aeruginosa,* inhibits a number of LasR- RhlR-activated genes by protein–protein interaction with LasR and RhlR, respectively, and suppresses virulence in a *Drosophila* infection model (Chugani et al., 2001; Ledgham et al., 2003). The unique small protein QteE controls the stability of LasR protein in *Pseudomonas aeruginosa,* but affects neither its transcription nor translation (Siehnel et al., 2011). In the absence of the *qteE* gene, LasR is more stable at a low cell density culture and overproduction of QteE reduces the LasR stability. In addition to these anti-activators, RsaL is a repressor and simultaneously binds to the *lasI* promoter with LasR in *Pseudomonas aeruginosa* (Rampioni et al., 2006, 2007). The *rsaL* mutant results in unlimited 3-oxo-C_{12}-HSL production, however, overproduction of the protein produces a lower level of virulence proteins, thereby RsaL manages homeostasis of the quorum sensing.

Non-coding regulatory RNAs are also involved in quorum sensing suppression. YenS from *Y. enterocolitica* is a non-translated *trans*-RNA (Tsai and Winans, 2011). At low cell densities, the signal-free receptor protein apo-YenR activates the *yenS* transcription binding to a particular sequence on the *yenS* promoter. The YenS base-pairs with 5′ region of the signal generator YenI mRNA and then inhibits YenI translation. At high densities, a signal-bound YenR (holo-YenR) cannot do so, resulting in the induction of the quorum sensing. Thus, YenS is a suppressor for the quorum sensing in *Y. enterocolitica*. The photosynthetic soil bacterium *Rhodopseudomonas palustris* produces a non-coding *cis*-RNA that affects the quorum sensing signal receptor expression (Hirakawa et al., 2012). The *cis*-RNA (named asrpaR) is an anti-sense transcript of *rpaR*, a *luxR*-family signal receptor gene. The transcript is induced by the quorum sensing signal *p*-coumaroyl-HSL and the RpaR protein. asrpaR inhibits RpaR translation, presumably by base-pairing with sense transcripts, thus suppressing the quorum sensing activity. Off-targeting technology to disrupt specific target functions utilizing RNA interference with siRNA (small interfering RNA) is undergoing extensive development in the mammalian area, but the major challenge of developing therapeutic applications is currently ongoing. Studies on RNA interference will also be carried out on bacteria.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Blocking bacterial cell-to-cell communication activity is a novel strategy in antibacterial therapy. In the last 20 years, several approaches to disrupting quorum sensing have been attempted, and these include antagonizing signal sensing, inhibition of signal generation, inactivation of signals, and a variety of agents have been discovered from natural and synthetic libraries. In addition, there are inhibitors like the macrolides where the mode of action has not yet been addressed. The aim of inhibiting quorum sensing is to suppress bacterial virulence and reduce drug resistance/tolerance accompanied with quorum sensing-activated biofilm formation and other innate bio-defense mechanisms by means other than killing bacteria. This strategy is the opposite of bacteriocidal therapies using antibiotics. The benefits might be the suppression of the development of antibiotic resistance, and the ability to expand chemotherapeutic strategies to combat multidrug resistant (MDR) pathogens. Some of the inhibitors have been evaluated in animal and plant infection models. However, there are many hurdles to overcome for this approach to be used in clinical applications. For example, do these agents only target quorum sensing without any critical and unexpected side effects in addition to their pharmacokinetics (ADME: absorption, distribution, metabolism and excretion)? If they do, can they be administrated with authentic antibiotics to promote healing against multidrug-resistant infections? Recently, a pilot study in clinical trial has been made (Smyth et al., 2010). They used garlic as a quorum sensing inhibitor for 13 CF patients, however, no significant effects were observed compared to placebo group. As they suggested in the preliminary study, reorganization of the study with some modifications (for example, to test in a larger trial) should be necessary. We also need to investigate the potential for selective pressure by quorum sensing inhibition (As we mentioned above, there is a report describing a *mexR* mutation that increases resistance to a furanone inhibitor). Quorum sensing cheater with mutations will be also a critical issue to keep in mind. We will need to answer these questions in the near future to enable us to use these agents as novel antibacterial agents.

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Simultaneous and Longitudinal Comparison of Interferon Gamma Release Assay Data from Health Care Workers in Japan

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Abstract

Background: Tuberculosis is one of the serious occupational diseases among health care workers, especially those who work with patients suffering from respiratory disorders. It is important to assess latent tuberculosis infection status in such workers using interferon- release assays, including QuantiFERON-TB Gold and its successor, the QuantiFERON-TB Gold in-Tube test. Although the relative efficacies of these two methods have been evaluated in patients with tuberculosis, data from health care workers in Japan have not been extensively examined.

Purpose: In the present study, we examined the utilities of the QuantiFERON-TB Gold and QuantiFERON-TB Gold in-Tube tests to detect latent tuberculosis infections in staff working in a respiratory disease hospital in Japan. We also longitudinally compared data from these subjects obtained using the QuantiFERON-TB Gold assay.

Methods: We collected blood samples from 120 staff members and performed both the QuantiFERON-TB Gold and QuantiFERON-TB Gold in-Tube assays. A total of 58 subjects had previously been tested 5 years prior using the QuantiFERON-TB Gold assay, and we compared these data with our more recent information.

Results: The QuantiFERON-TB Gold in-Tube test tended to yield higher test values than did the QuantiFERON-TB Gold test, suggesting that the former test may be more sensitive when used to detect latent tuberculosis infection. In both tests, the results differed in 32 instances (26.7%), associated with significant difference ($p<0.001$, $=0.55$). In 94 subjects with negative QuantiFERON-TB Gold test results, 16 (17.0%) were intermediate and 11 (11.7%) positive by QuantiFERON-TB Gold in-Tube test. The longitudinal comparison confirmed this suggestion. The number of subjects rated "intermediate" in terms of tuberculosis status differed, with statistical significance, when the two datasets were compared.

Conclusion: Health care workers should be screened for possible tuberculosis infections using the QuantiFERON-TB Gold in-Tube test, which is more sensitive than the QuantiFERON-TB Gold test.

Keywords: Interferon-gamma release assay; Latent tuberculosis infection; Health care workers; Phlebotomy

Abbreviations: BCG: Bacillus Calmette-Guérin; CFP-10: Culture Filtrate Protein; ESAT-6: Early Secreted Antigenic Target; HCW: Health Care Workers; IFN-γ: Interferon-Gamma; IGRA: Interferon-Gamma Release Assay; LTBI: Latent Tuberculosis Infection; Mtb: *Mycobacterium Tuberculosis*; NTM: Non-Tuberculosis Mycobacteria; PBMC: Peripheral Blood Mononuclear Cells; QFT-G: Quantiferon-TB Gold; QFT-GIT: Quantiferon-TB Gold In-Tube; TB: Tuberculosis; TST: Tuberculin Skin Test

Introduction

The incidence of tuberculosis (TB) in Japan is less than 20 per 100,000 population and is continuing to decline. However, TB remains a major occupational disease of health care workers (HCW) [1,2]. Several reports have shown that HCW were at several-fold higher risk for TB than the general population [3-6]. One practical way to control TB is routine screening of HCW for latent tuberculosis infections (LTBIs) and administration of chemoprophylaxis to HCW suspected to have LTBIs. Therefore, evaluation of *Mycobacterium tuberculosis* (Mtb) infective status is crucial in HCW working in hospitals dedicated to TB patients [2,7,8]. In such contexts, screening of HCW should be routine and data should be evaluated longitudinally [2,9].

Recently, methods detecting *Mycobacterium tuberculosis* (Mtb)-specific antigens have been developed. The target antigens include culture filtrate protein 10 kD (CFP-10), early secreted antigenic target 6 kD (ESAT-6), and TB7.7 [10-12]. The QuantiFERON-TB Gold (QFT-G) test uses CFP-10 and ESAT-6, whereas its successor, the QuantiFERON-TB Gold in-Tube test (QFT-GIT), additionally uses TB7.7 [13-15]. The results of the two assays cannot be directly compared, because not only do the stimulating antigens used vary, but some differences are evident in the methods used to stimulate lymphocytes. Thus, in the QFT-G tests, lymphocytes are separately stimulated by CFP-10 and ESAT-6 [16,17], whereas stimulation by a mixture of CFP-10, ESAT-6, and TB7.7 is commenced just after blood is drawn for the QFT-GIT assay [16,18-21]. Several investigators have reported that the sensitivity of QFT-GIT was higher than that of QFT-G, but that the specificity values were similar [10,11,20,21]. One issue that must be considered is the intermediate results introduced in some countries including Japan, but not most developed countries [20]. In Japan, an intermediate result

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(0.10 IU/ml IFN- level <0.35 IU/ml) is recognized but the definition of intermediate varies among countries [17,19]. One study concluded that intermediate results were more frequent by QFT-GIT than by QFT-G [20].

In the present study, we compared simultaneously derived QFT-G and QFT-GIT assay data from HCW in Japan. We also compared our results with those of QFT-G tests performed five years prior in some subjects.

Subjects and Methods

Study design

This cross-sectional study involved 120 HCW of the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Osaka, Japan. No subject had any underlying illnesses such as acute infection, autoimmune disorder, or any other chronic disease. No subject had an abnormal chest X-ray. The study protocol was approved by the Review Board of the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, and written informed consent was obtained from all participants.

QFT-G and QFT-GIT assays

The QFT-G and QFT-GIT assays were performed following the instructions of the manufacturer (Cellestis Limited, Carnegie, Australia). Blood samples were collected by normal phlebotomy into both evacuated 4 ml sterile sodium heparin tubes for QFT-G assay and into three 1 ml-volume QFT-GIT blood collection tubes [17-19]. When QFT-G tests were run, incubation with Mtb-specific antigens was initiated within 12 h of blood collection. After incubation for 24 h at 37°C, samples were centrifuged at approximately 500 g for 10 min to facilitate plasma collection. Plasma samples were stored at -70°C prior to conduct of ELISA detecting IFN-γ.

QFT ELISA assay

The concentrations of IFN- in plasma samples were determined via ELISA according to the manufacturer's protocol. All ELISAs were performed by the same trained staff. QFT-G and QFT-GIT test responses were automatically calculated using QFT-G ELISA Analysis software (Cellestis Limited) after input of ELISA plate optical density values. QFT-G and QFT-GIT test data were interpreted as suggested by the manufacturer. An IFN- response to Mtb-specific antigens that was at least 0.35 IU/ml and greater than the nil control value was considered positive. Samples with 0.10 ≤ IFN-level <0.35 IU/ml were regarded as intermediate, according to guidelines of Committee for Prevention of the Japanese Society of Tuberculosis [17,19]. Mitogen stimulation was used to positively control the quality of both blood samples and laboratory technique. If a sample IFN- value was <0.35 IU/ml when the positive control value (upon mitogen stimulation) was ≥ 0.5 IU/ml, the test result was considered to be negative.

Data analyses

The extent of agreement between data yielded by the two tests was evaluated using the McNemar approach, and agreement was expressed in terms of both a kappa coefficient and the level of overall agreement (the proportions of samples yielding positive or negative results in both tests). Non-parametric statistics (Wilcoxon's ranked sign test and Spearman's ranked correlation coefficient) were employed when the means of IFN- measurements obtained using either method were compared. A difference associated with a p value <0.05 was considered to be statistically significant.

Results

Comparison of QFT-G and QFT-GIT assay data from 120 Japanese HCW

When comparing QFT-G and QFT-GIT assay data from 120 blood samples obtained at the same time, QFT-GIT yielded higher IFN-levels than did QFT-G in most samples (Figure 1). However, the results (positive, intermediate, or negative) differed in 32 instances (26.7%), associated with a significant difference in the results of the two assays ($p<0.001$ by Wilcoxon's ranked sign test; =0.5503 by weighted value).

Thus, in 94 subjects with negative QFT-G test results, 16 (17.0%) were intermediate and 11 (11.7%) positive by QFT-GIT (Table 1); only 67 subjects (71.2%) were negative on both tests. Similarly, of eight subjects diagnosed as intermediate by the QFT-G test, two (25%) were positive by QFT-GIT and only four (50%) yielded the same intermediate results. Moreover, two subjects (25%) scored as intermediate by the QFT-G test were negative by QFT-GIT, probably reflecting the indeterminacy of 'intermediate' results. Seventeen of 18 (94%) subjects positive by QFT-G were also positive by QFT-GIT, although the IFN-levels yielded by the latter test were higher (Figure 1). However, one of these 18 (6%) subjects was diagnosed as intermediate by QFT-GIT, although the IFN- level measured by QFT-G was just above the cut-off

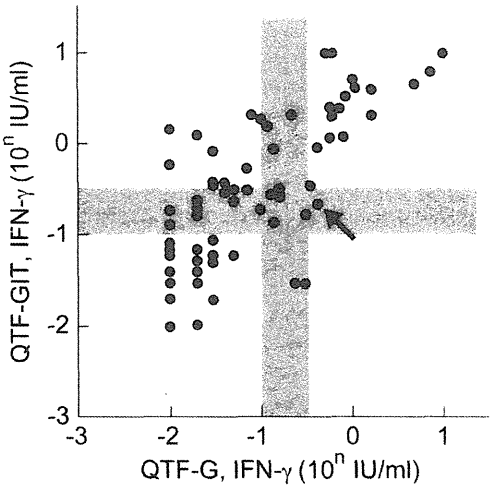

Figure 1: Comparison of two simultaneous IGRAs. Blood samples from 120 HCW were simultaneously examined using two different IGRAs. IFN- concentrations are shown. In both assays, a level of IFN- less than 0.1 IU/ml constituted a negative result. IFN- levels falling in the shaded area (0.1 IU/ml ≤ IFN- level <0.35 IU/ml) are regarded as intermediate in Japan. An arrow indicates one case that yielded an intermediate QFT-GIT test result, but who was positive by QFT-G, although the IFN-level was just above the (lower) positive cut-off value in the latter test.

		QFT-GIT			
		(-)	( )	(+)	Total
QFT-G	(-)	67	16	11	94
		(71.2%)	(17.0%)	(11.7%)	(100%)
	( )	2	4	2	8
		(25%)	(50%)	(25%)	(100%)
	(+)	0	1	17	18
		(0%)	(6%)	(94%)	(100%)
	Total	69	21	30	120

(+): IFN- level > 0.35 IU/ml, (±): 0.1 ≤ IFN- level < 0.35, (-): IFN- level < 0.1 IU/ml

Table 1: Comparison of simultaneous QFT-G and QFT-GIT assays.

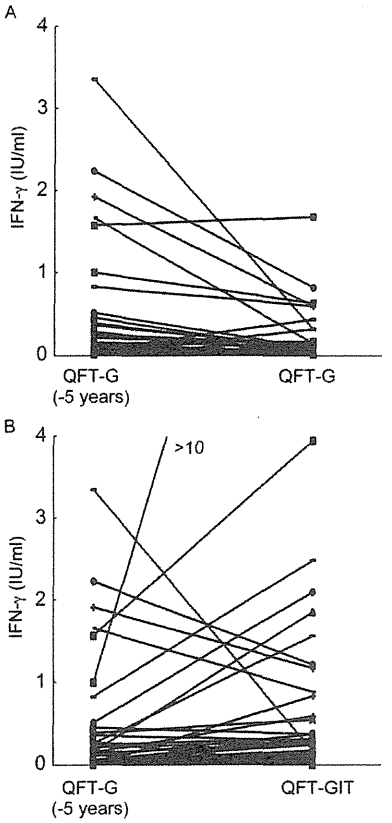

Figure 2: Longitudinal comparison of data obtained from the same HCW *recently and 5 years prior.* *GFT-G data obtained 5 years prior were compared* with recent results obtained using the QFT-G (A) and the QFT-GIT (B) tests. IFN- levels are plotted on a linear scale and changes in the same subjects are shown by lines. In both assays, an IFN- level less than 0.1 IU/ml was a negative result. IFN- levels falling in the shaded area (0.1 IU/ml ≤ IFN- level <0.35 IU/ml) are regarded as intermediate in Japan.

value (arrow in Figure 1). These results show that the QFT-GIT is more sensitive when used to detect latent Mtb infections and that the assays yield different results, with statistical significance.

Longitudinal analysis of HCW data

The QFT-GIT and QFT-G tests, performed simultaneously, yielded different results. It was thus important to conduct a longitudinal comparison of the extent of latent Mtb infections in HCW tested 5 years prior using the QFT-G test. Of 120 participants in the present study, results of QFT-G measured 5 years prior were available in 58 subjects (48.3%). Of these, 11 (19.0%) had been previously diagnosed as positive, 9 (15.5%) as intermediate, and 38 (65.5%) as negative. Upon re-testing, 6 (10.3%) were positive and 8 (13.8%) intermediate by QFT-G, and 14 (24.1%) positive and 14 (24.1%) intermediate by QFT-GIT. We compared these data with those of recent QFT-G testing (Figure 2A). Of 38 previously negative subjects, 2 (5.3%) became intermediate and 1 (2.3%) positive (IFN- level 0.43 IU/ml). Of the nine subjects who were previously intermediate, four (4.4%) remained intermediate and five (55.5%) became negative; no subject became positive. Five of 11 previously positive subjects (45.5%) remained positive on recent testing, but two (18.2%) became intermediate and four (36.4%) negative.

We further compared earlier QFT-G results with our QFT-GIT data (Figure 2B). Of 38 previously negative subjects, 10 (26.3%) were judged as intermediate and 1 (2.6%) was positive (0.84 IU/ml); this positive case was negative upon recent QFT-G testing. This subject was diagnosed as intermediate upon recent QFT-GIT testing. Of nine previously intermediate cases, four (44.4%) were positive and the others remained intermediate on recent QFT-GIT testing. Nine of 11 previously positive QFT-G cases remained positive, but two (18.2%) became intermediate upon recent QFT-GIT testing.

Discussion

In the present study, we simultaneously compared QFT-G and QFT-GIT test results and also performed a longitudinal comparison of recent data with earlier QFT-G results obtained 5 years prior, because such a comparison should be helpful in terms of early diagnosis of LTBI in HCW, especially staff of dedicated tuberculosis wards. We confirmed that the QFT-GIT test was more sensitive than the QFT-G test when used to screen HCW, similar to the results of a previous study in TB patients [20]. However, discrepancies were evident between the results of recent QFT-GIT and QFT-G tests and also when these data were compared with QFT-G results obtained 5 years prior. Differences between the results of the QFT-GIT and QFT-G tests may be attributable to differences in the antigens used to stimulate interferon secretion; such antigens may exert synergistic effects.

Differences between recent test results and older data may be attributable to functional regression of Mtb-specific T lymphocytes [22]. In BCG-vaccinated newborns, the levels of BCG-specific T lymphocytes peak 10 weeks after vaccination [23]. The levels of Mtb-specific IFN- -secreting T cells declined in both TB and LTBI patients during anti-TB treatment, suggesting that the IGRA results reflect the mycobacterial load [24,25]. In the present study, the QFT-G test values fell over the 5-year interval in most QFT-G-positive subjects (90.9%), and almost half became negative (54.5%). However, when the earlier results were compared with recent study QFT-GIT data, 10 of 38 (26.3%) previously negative subjects were of intermediate status and 1 (2.6%) was positive; this positive case was negative upon recent QFT-G testing. These data thus also suggested that the sensitivities of the two types of test differed, and that QFT-GIT testing was superior in terms of sensitivity. However, the results of the two tests conducted on the same subjects tended to differ, with statistical significance, suggesting that data from either test should not be compared.

Our present results using the QFT-G test are similar to those of a previous work with HCW of a Japanese tuberculosis referral hospital; approximately 10% were positive [26]. However, use of the more sensitive QFT-GIT test in the present study diagnosed 25% of subjects with LTBI; this proportion is very much higher than that of the general population [22]. A recent systematic review of the utility of IGRA assays used to screen HCW for TB showed that the initial positivity rate was 1.3-31.0%; the analysis included data from countries where the incidence of TB is high [7]. Such variation is attributable to differences in sample sizes and the backgrounds of HCW among the studies; the extent of involvement and contact with TB patients, administration of chemo-prophylaxis, and years of work with TB patients, will have varied significantly among the studies.

In conclusion, our simultaneous and longitudinal study of the same HCW including medical staff in TB-specific wards in a hospital in Japan suggests that QFT-GIT should be used for the screening of Mtb infection in HCW in Japan.

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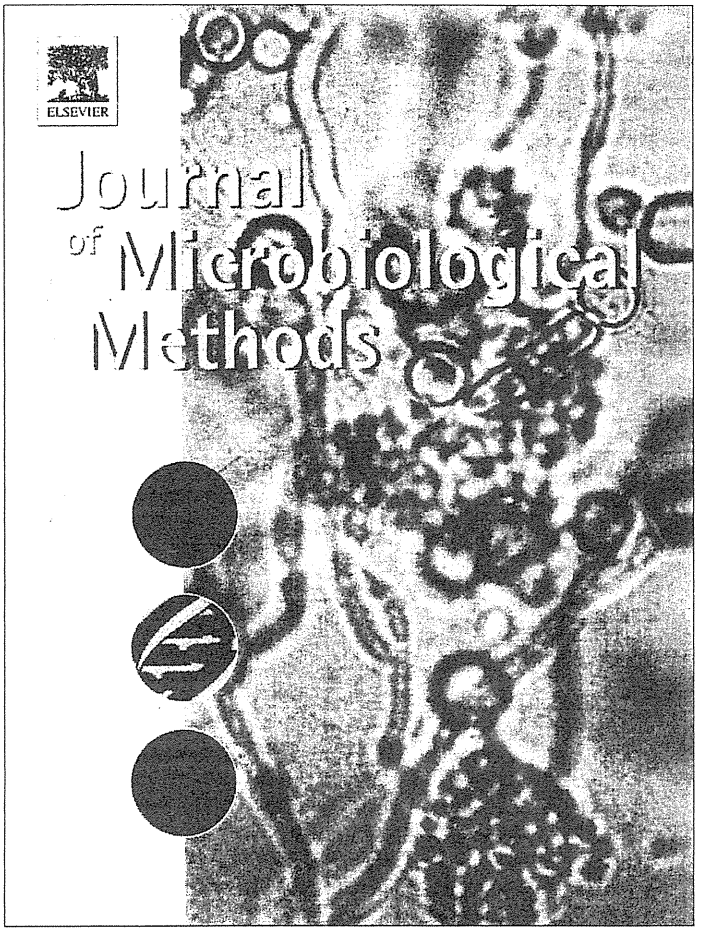

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Note

A novel approach to automated genotyping of *Mycobacterium tuberculosis* using a panel of 15 MIRU VNTRs

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ABSTRACT

In this study, we present a new approach to variable number tandem repeats (VNTR) analysis using the QIAxcel capillary electrophoresis system and a software-integrated peak calling function. Allelic ladders representing 15 mycobacterial interspersed repetitive units (MIRU)-VNTR loci were used to define peak calling tables thereby enabling high precision *Mycobacterium tuberculosis* strain identification.

Rapid and robust genotyping of long VNTRs in *Mycobacterium tuberculosis* is important in order to enable real-time intervention in control of transmission events. However, genotyping *M. tuberculosis* has up till now required a laborious procedure involving several methodological steps that may introduce errors.

Before the completion of the *M. tuberculosis* genome project in 1998, the IS6110 RFLP method made great contributions to understanding the molecular immunology of *M. tuberculosis* (Cave et al., 1991; Kremer et al., 1999), as it was extensively used for *M. tuberculosis* genotyping (Ano et al., 2006, 2008). However, although IS6110 RFLP fingerprinting yielded clear clustering and identification of the clinically isolated strains, the method was time-consuming and required over 1 μg of genomic DNA (Ano et al., 2006). In addition, the reproducibility between clinical laboratories was unsatisfactory.

The methodological weaknesses were partially overcome by whole genome sequencing, which enabled identification of various genomic markers and thereby unification of the framework for epidemiology and evolutionary analysis of the *M. tuberculosis* population. Variable number tandem repeats (VNTR) were found to be the most suitable and informative genetic markers for discriminating between *Mycobacterium abscessus* (Wong et al., 2012) and *M. tuberculosis* strains (Frothingham and Meeker-O'Connell, 1998; Supply et al., 2000, 2006). Compared to IS*6110* RFLP, VNTR-PCR is a rapid method that requires considerably less genomic DNA. Subsequently, a 15-loci MIRU-VNTR system has been proposed as the new standard for first-line routine epidemiological discrimination of *M. tuberculosis* isolates in Japan (Supply et al., 2006).

Traditionally, VNTR analysis has been performed using agarose gel electrophoresis, but this method is limited when it comes to accuracy in size estimation and digital-based data preparation. In recent years, capillary electrophoresis has been employed to improve the accuracy. In this study, we used the QIAxcel system which utilizes QX cartridges comprising an array of 12 capillaries prefilled with gel polymers, thus minimizing manual handling. These short capillaries cover the size range from 15 bp to 10 kb and provide a resolution of 3–5 bp up to 500 bp. In addition, the samples can be analyzed in a 96-well plate in one experiment without intervention. Results of the analysis were compared to the data obtained with i-chip SV1210 (Hitachi Electronics Engineering) that was previously used for the same analysis.

Allelic ladders for 15 MIRU-VNTR loci were prepared by combining samples with different numbers of repeats, so that each ladder contains all possible VNTR products of the respective target VNTR locus (see example in Fig. 1). All allele sizes in the allelic ladders were estimated using a commercial DNA ladder as reference. These sizes were used for preparing the peak calling tables for all loci of the 15-MIRU-VNTR panel. The ScreenGel software compares unknown samples to the allelic ladder markers.

M. tuberculosis strains were collected from culture-confirmed pulmonary tuberculosis (TB) patients. DNA purification was performed

Abbreviations: MIRU, mycobacterial interspersed repetitive units; VNTR, variable number of tandem repeats; RFLP, restriction fragment length polymorphism.

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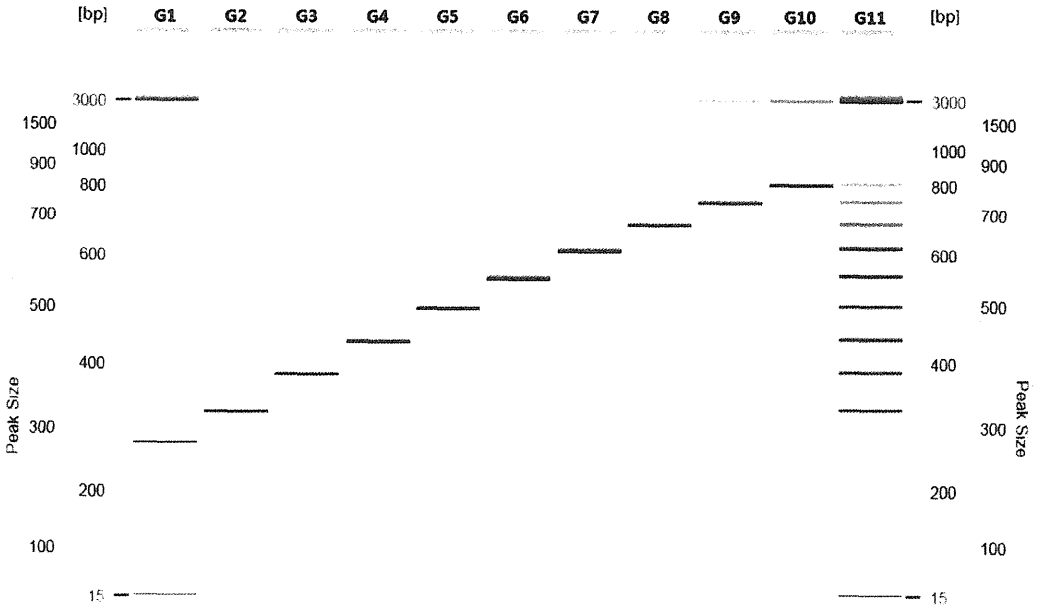

Fig. 1. Example of the allelic ladder for locus Mtub21. Lanes 1–10: individual alleles, Lane 11: allelic ladder.

according to the manufacturer's instructions (InstaGene Matrix BioRad). PCRs were performed according to an optimized protocol using AmpliTaqGold mastermix with addition of GC Enhancer and 0.2 μM primer in a final volume of 25 μl. After an initial denaturation time of 5 min at 94 °C, 35 cycles were run: at 94 °C for 30 s; 60 °C for 30 s and 72 °C for 3 min and a final extension time of 7 min at 72 °C.

The 130 samples were analyzed using two different systems: i-chip SV1210 (Hitachi Electronics Engineering) and QIAxcel Advanced (Qiagen Instruments AG). The i-chip PCR products were mixed with a 100 bp ladder (Takara) and run on the chip along with a 100 bp/1000 bp alignment marker. Alleles and size marker fragments longer than 1000 bp were analyzed manually due to system limitations. Sample analyses with the QIAxcel capillary electrophoresis system were performed in combination with QX DNA High Resolution kit and a customized method OM2100 with the following parameters: alignment marker injection at 4 kV for 10 s, sample injection at 5 kV for 10 s and separation at 2 kV for 2100 s. Alignment marker 15 bp/3 kb was run simultaneously with the samples. DNA size marker was analyzed and the data were saved as a Reference Marker Table. These data were used for size estimation of the allelic ladders. All samples were tested at 15 loci as listed in Table 1.

The peak calling instructions define the sizes of the alleles for every locus and the tolerance (in %) that compensates for possible migration time variations between the capillaries. These properties enable the QIAxcel ScreenGel software to identify the presence of specific alleles in the samples. The tolerance was calculated so that the sizes of neighboring alleles did not overlap. An example of a peak calling table is presented in Fig. 2.

A high correlation (99.2%) was found between the results of analysis of 130 samples at 15 loci using Hitachi i-chips and the QIAxcel system (Table 1). Since the i-chips cannot process data for fragments over 1000 bp, the calculations had to be done manually. This explains the discrepancies observed at some loci (see Table 1).

In conclusion, the technical characteristics of QIAxcel system make it suitable for the analysis of 15 MIRU-VNTR samples. Accurate peak calling results lead to positive strain identification in a streamlined procedure. This novel approach may prove useful in surveillance studies and monitoring of transmission events for *M. tuberculosis* and possibly other infectious pathogenic bacteria.

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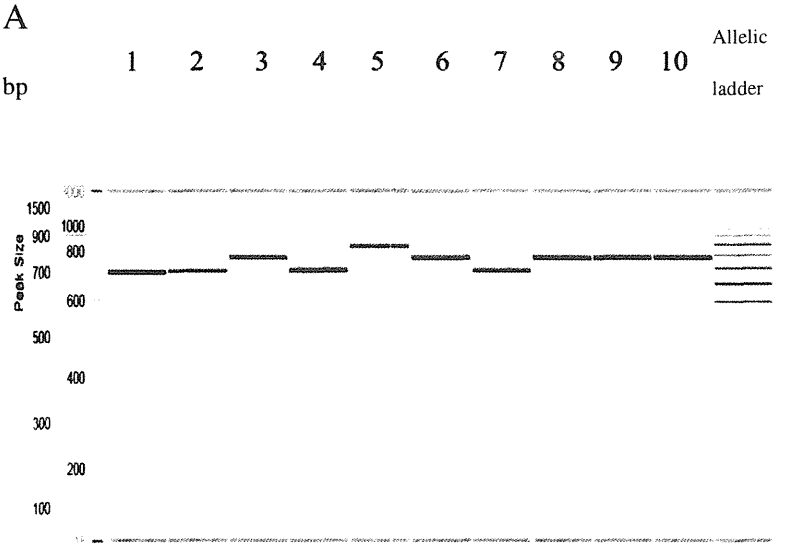
Table 1
List of 15 loci MIRU-VNTR analyzed at 130 samples. Correlation between the data provided by i-chip and QIAxcel systems.

Locus	Identified VNTR alleles in 130 samples	Copy number correlation between the QIAxcel and i-chip data (%)	Reference
ETR-A	1–7	100.0	Frothingham, R.,
ETR-C	2–7	97.6	Meeker-O'Connell,
ETR-D	1–3, 3.5, 4–7, 10	100.0	WA., Microbiology.
ETR-E	2–5	99.2	1998; 144: 1189–1196.
MIRU10	1–9, 11	99.2	Supply et al., J. Clin.
MIRU16	1–5	98.4	Microbiol. 2001; 39:
MIRU26	1–10, 12	100.0	3563–3571.
MIRU40	1–7	98.4	
Qub11b	2–9	97.5	Skuce, RA., et al.,
Qub26	2–10	98.0	Microbiology. 2002, 148: 519–28
QUB4156c	1–7	100.0	Skuce, RA. et al., J. Clin. Microbiol. 2002; 40: 2126–33
Mtub04	1–6	100.0	Le Flèche, P. et al.,
Mtub21	1–10	100.0	BMC Microbiol. 2002; 27:
Mtub30	1–4	100.0	
Mtub39	1–10, 15	100.0	

A



B

VNTR Repeat	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Allelic ladder
1	no	no	no	no	no	no	no	no	no	no	yes
2	no	no	no	no	no	no	no	no	no	no	yes
3	yes	yes	no	yes	no	no	yes	no	no	no	yes
4	no	no	yes	no	no	yes	no	yes	yes	yes	yes
5	no	no	no	no	yes	no	no	no	no	no	yes
6	no	no	no	no	no	no	no	no	no	no	yes
7	no	no	no	no	no	no	no	no	no	no	yes

Name	Position	Tolerance
Q4156-1	594 bp	5.10 %
Q4156-2	655 bp	4.50 %
Q4156-3	714 bp	4.10 %
Q4156-4	777 bp	3.70 %
Q4156-5	835 bp	3.50 %
Q4156-6	896 bp	3.40 %
Q4156-7	962 bp	3.40 %

Fig. 2. A. Gel image of the analysis of 10 samples on the Q4156 locus. B. The corresponding table with peak calling results is shown together with standard table for allele calling.

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ラインプローブ法による抗酸菌同定および結核菌薬剤感受性判定の臨床評価

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要旨：〔背景〕多剤耐性結核（MDR-TB）や超多剤耐性結核（XDR-TB）は治療が困難なことから問題とされているが，結核菌の薬剤感受性検査は培養株が得られてから3～4週間を要するため，迅速な検出方法が求められている。〔目的〕今回，菌種同定と薬剤感受性試験を同時に実施できる新規ラインプローブアッセイ（LiPA；ニプロ株式会社）の評価を行った。〔対象と方法〕臨床検体163例を収集し，LiPAにて菌種同定，結核菌群RFP，INH，PZA，LVFX感受性判定を行った。〔結果〕臨床検体から直接検出するうえで十分な感度で結核菌，*Mycobacterium avium*，*M.intracellulare*，*M.kansasii*の同定が可能であることが確認できた。また，結核菌における薬剤感受性についてはINH感受性では一部の検体で乖離した結果となったが，RFP，PZA，LVFXについては全て一致した結果であった。〔考察〕LiPAによる迅速な菌種同定と薬剤耐性判定は，適切な薬剤による治療の早期開始と感染リスクの予防に有用と考えられる。

キーワーズ：多剤耐性結核，迅速診断，ラインプローブアッセイ

はじめに

結核は，全世界の人口の3分の1が感染し，毎年920万人が発病し，毎年170万人の患者が亡くなる世界最大の感染症である[1]。日本において結核の発生者数は緩やかに減少しているが，世界的に見るとアジア，アフリカを中心にして増加している。その要因となっているのがHIV/AIDSと多剤耐性結核（MDR-TB）である。

MDR-TBとは，結核治療に重要なイソニアジド（isoniazid; INH）とリファンピシン（rifampicin; RFP）の2剤に少なくとも耐性である結核菌の総称である。最近，超多剤耐性結核（XDR-TB）が問題になっているが，XDR-TBとは，INH，RFPのほかにフルオロキノロン系（fluoroquinolones; FQ）ならびにアミノグリコシド系を中心とする注射剤に耐性のMDR-TBである。MDR-/XDR-TBは，治療の失敗によってつくられるのみならず，感染拡大もする。2011年3月に医療機関におけるXDR-TBの集団感染が国内で初めて報告された。従って，MDR-/XDR-TBを迅速に診断し感染対策を行うことが重要である。しかしながら現在，通常の結核菌の薬剤感受性検査は結果が判明するまでに早くても1カ月の期間を要しており，結核菌の薬剤耐性遺伝子変異を早急に検出し対応できる方法が必要になっている。また，抗酸菌排菌患者の結核症疑いに対する，結核感染対策としての隔離入院は，患者個人の貴重な時間を束縛するのみならず医療経済上も大きな負担である。迅速に結核症を否定し非結核性抗酸菌症と診断することにより，不必要な結核隔離入院を回避できることは医療経済上，そして患者の人権上有用である。

近年，結核菌の薬剤耐性に関与する遺伝子が明らかになってきており，RFP耐性結核菌では約95%が*rpoB*遺伝子に変異があることが知られている[2)~4)]。INH耐性に関与する遺伝子には複数の報告があり，なかでも*katG*遺伝子や*inhA*遺伝子のプロモーター領域の変異が高頻度

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でみられる[5)~7)]。ピラジナミド（pyrazinamide; PZA）耐性結核菌では*pncA*遺伝子に，FQ耐性結核菌では*gyrA*遺伝子に変異があることが知られている[8)~10)]。

これらの薬剤耐性遺伝子に加えて新たな遺伝子領域を追加したラインプローブアッセイ（LiPA）に基づいた新しい遺伝子変異検出キット（ニプロ株式会社）が開発され，今回，臨床上の有用性を確認するために臨床検体を用いた試験を行った。

方　法

（1）供試検体

2009年7月から2010年4月までに結核予防会複十字病院，国立病院機構（NHO）東京病院，NHO近畿中央胸部疾患センター，NHO茨城東病院，東京都立多摩総合医療センターならびに大阪府立呼吸器・アレルギー医療センターにて，結核患者94名，*Mycobacterium avium*症患者20名，*M.intracellulare*症患者14名，*M.kansasii*症患者11名，その他の非結核性抗酸菌症患者3名および陰性対照者21名の計163名から採取された臨床検体163例を収集した。収集した検体は全て結核予防会結核研究所に送付され，結核菌検査指針2007に従って塗抹検査実施後，N-アセチルL-システイン・水酸化ナトリウム（NALC-NaOH）処理を行った。

（2）同定と培養および薬剤感受性検査

結核菌群の検出としてコバス アンプリコア マイコバクテリウム ツベルクローシス（ロシュ・ダイアグノスティクス；以下，PCR法）を行い，コバス アンプリコア マイコバクテリウム アビウム・イントラセルラー（ロシュ・ダイアグノスティクス；以下，PCR法）にて*M.avium*および*M.intracellulare*の検出を行った。同定しえなかった検体はDDHマイコバクテリア '極東'（極東製薬工業）を用い，必要に応じて16S rRNAのシークエンス解析を行った[11)]。

また，培養は2%小川培地ならびにBDバクテックMGIT 960（日本ベクトン・ディッキンソン）を用い，培養陽性となった検体はキャピリアTB（日本ベクトン・ディッキンソン）にて結核菌の確認を行った。結核菌の薬剤感受性試験はRFP，INHおよびレボフロキサシン（Levofloxacin; LVFX）に対しては1%小川培地を用い，PZAに対してはMGITシリーズ ピラジナミド（日本ベクトン・ディッキンソン）を用いた。

（3）ラインプローブアッセイ

ラインプローブアッセイ（LiPA）はニプロ株式会社が開発した以下の4種のストリップ（Fig.）を用いた[12)]。すなわち，NTM/MDR-TBストリップでは*M.tuberculosis*，*M.avium*，*M.intracellulare*および*M.kansasii*の菌種同定，結核菌群*rpoB*遺伝子の検出および結核菌群*inhA*，*katG*遺伝子の検出を行い，INHストリップでは結核菌群*inhA*，*fabG1*，*furA*および*katG*遺伝子の検出を行い[13)]，PZAストリップでは結核菌群*pncA*遺伝子の検出を行い[14)]，FQストリップでは結核菌群*gyrA*遺伝子の検出を行った[15)]。

結　果

（1）菌種同定

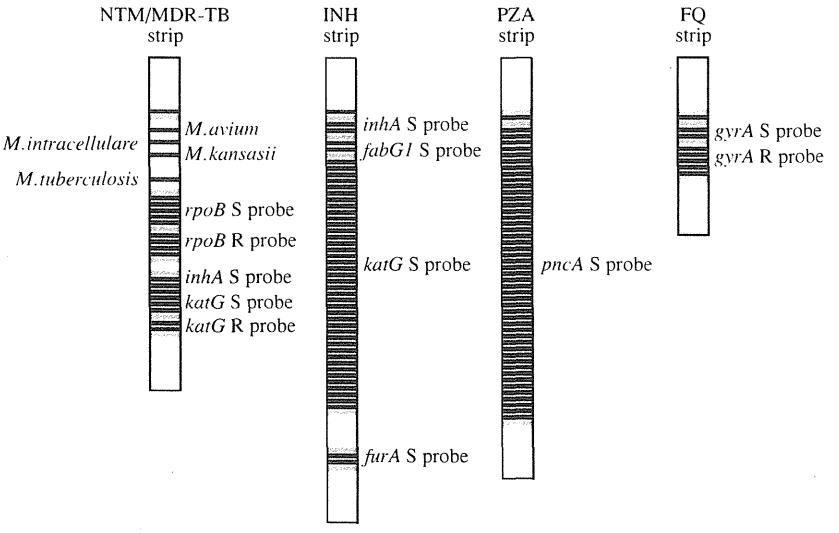

Fig. Design of NTM/MDR-TB strip, INH strip, PZA strip and FQ strip.
S probes are designed to detect the wild-type sequence of *M.tuberculosis*, whereas R probes are designed to detect the mutant sequence frequently found in drug-resistant *M.tuberculosis*.

NTM: nontuberculous mycobacteriosis　MDR-TB: multi-drug resistant tuberculosis
INH: isoniazid　PZA: pyrazinamide　FQ: fluoroquinolone

Table 1 LiPA results with NTM/MDR-TB strips for detection of TB, *M.avium*, *M.intracellulare* and *M.kansasii* compared to smear, culture and PCR tests

Smear result	n	LiPA		Culture		PCR	
		Positive	Negative	Positive	Negative	Positive	Negative
3+	18	18	0	18	0	18	0
2+	44	44	0	35	9	41	3
1+	17	11	6	8	9	13	4
±	15	10	5	6	9	6	9
Negative	45	26	19	20	25	18	27
Total	139	109	30	87	52	96	43

LiPA: line probe assay PCR: polymerase chain reaction

NTM/MDR-TBストリップでは*M.tuberculosis*, *M.avium*, *M.intracellulare*および*M.kansasii*を検出する。喀痰検体163例のうち検出対象となる菌種を含む139例について，塗抹検査成績と比較したものをTable 1に示す。塗抹検査2+以上の陽性例では全例検出されたが，1+，±ではそれぞれ17例中6例，15例中5例が陰性となった。また，塗抹陰性例では45例中19例が陰性となったが，26例は陽性となり，菌種を特定することが可能であった。全139例では109例（78.4%）が陽性となり，菌種も全て一致した。また，この139例で培養陽性およびPCR陽性となったのは87例（62.6%），96例（69.1%）であった。

（2）薬剤感受性検査

①リファンピシン（RFP）

結核患者から採取した臨床検体94例のうち，NTM/MDR-TBストリップを用いたLiPAによるRFP感受性判定がなされたのは81例であった。一方，培養陽性となり，RFP感受性検査結果が得られたのは59例であった。LiPAおよび培養の両者でRFP感受性結果が得られた55例の結果を比較すると，3例が*rpoB*変異型すなわちRFP耐性，52例が*rpoB*野生型すなわちRFP感受性と判定され，RFP感受性検査の結果と比較して感度・特異度共に100%であった（Table 2A）。

②イソニアジド（INH）

NTM/MDR-TBストリップを用いたLiPAでは75例がINH感受性の判定がなされ，このうちの52例については培養陽性となりINH感受性検査結果が得られた。INH耐性であった6例はLiPAでは3例が変異型すなわちINH耐性と判定されたが，3例は野生型すなわちINH感受性と判定された（感度50%）。INH感受性であった46例中45例はLiPAでINH感受性と判定されたが，1例はINH耐性と判定された（特異度97.8%）（Table 2A）。

また，INHストリップおよびINH感受性検査の両者で結果が得られた46例では，INH耐性であった4例についてLiPAでは3例がINH耐性と判定されたが，1例はINH感受性と判定された（感度75%）。INH感受性42例ではLiPAで39例がINH感受性と判定され，3例がINH耐性と判定された（特異度92.9%）（Table 2B）。

Table 2 Results of LiPA for drug susceptibility with NTM/MDR-TB strips (A), INH strips (B), PZA strips (C) and FQ strips (D)

(A)

Drug susceptibility test result		n	LiPA result with NTM/MDR-TB strip	
			Mutation	Wild type
RFP	Resistant	4	3	0
	Susceptible	55	0	52
INH	Resistant	7	3	3
	Susceptible	52	1	45

RFP: rifampicin

(B)

Drug susceptibility test result		n	LiPA result with INH strip	
			Mutation	Wild type
INH	Resistant	7	3	1
	Susceptible	52	3	39

(C)

Drug susceptibility test result		n	LiPA result with PZA strip	
			Mutation	Wild type
PZA	Resistant	5	4	0
	Susceptible	53	0	52

(D)

Drug susceptibility test result		n	LiPA result with FQ strip	
			Mutation	Wild type
LVFX	Resistant	7	7	0
	Susceptible	52	0	48

LVFX: levofloxacin

NTM/MDR-TBストリップはINH感受性判定プローブ8本であるのに対し，INHストリップはINH感受性判定プローブ46本を備えており，NTM/MDR-TBストリップでは検出しえない変異を検出することが可能である（Fig.）。それぞれのストリップおよびINH感受性検査の判定結果が一致しなかった乖離例をTable 3に示す。

③ピラジナミド（PZA）

PZAストリップを用いたLiPAによる感受性判定がなされたのは76例であった。一方，培養陽性となり，PZA

Table 3 Discrepant results between *in vitro* INH susceptibility test and LiPA with NTM/MDR-TB strips or INH strips

Sample No.	INH susceptibility test result	LiPA result for INH susceptibility		Mutation
		NTM/MDR-TB strip	INH strip	
10	Resistant	Wild type	Δ*katG*-39	*katG* T2093C
80	Resistant	Wild type	Δ*katG*-37	*katG* G1795T
148	Resistant	Wild type	Wild type	*katG* T571C, G1079A
8	Susceptible	Δ*inhA*-S6	Δ*inhA*-2	*inhA* C-15T
78	Susceptible	Wild type	Δ*fabG1*-1	*fabG1* G609A
144	Susceptible	Wild type	Δ*fabG1*-1	*fabG1* G609A

感受性検査結果が得られたのは58例であった。LiPAおよび培養の両者でPZA感受性結果が得られた56例の結果を比較すると，4例が*pncA*変異型すなわちPZA耐性，52例が*pncA*野生型すなわちPZA感受性と判定され，PZA感受性検査の結果と全て一致した（感度・特異度共に100%）（Table 2C）。

④レボフロキサシン（LVFX）

FQストリップを用いたLiPAによる感受性判定がなされたのは77例であった。一方，培養陽性となり，LVFX感受性検査結果が得られたのは59例であった。LiPAおよび培養の両者でLVFX感受性結果が得られた55例の結果を比較すると，7例が*gyrA*変異型すなわちFQ耐性，48例が*gyrA*野生型すなわちFQ感受性と判定され，LVFX感受性検査の結果と比較して感度・特異度共に100%であった（Table 2D）。

考　察

結核菌検査のゴールドスタンダードは，塗抹検鏡，培養検査であり，菌株から得られた薬剤感受性試験である。しかしながら抗酸菌，特に結核菌の場合は培養に日数を要し，そこから得られる薬剤感受性試験の結果は，固形培地で約2カ月，液体培地でも約1カ月を要する。XDR-TBの集団感染が報告された現在において迅速に感受性を判定できることは重要である。また，喀痰塗抹陽性患者の場合に非結核性抗酸菌症であるか結核症であるかの迅速な判断が必要とされる。

本邦で検出される非結核性抗酸菌では*M.avium*，*M. intracellulare*および*M.kansasii*の3菌種が主要なものであり，非結核性抗酸菌の約90%を占める[16]。結核菌を含めたこれらの4菌種139例について培養陽性87例（62.6%），PCR陽性96例（69.1%）に対し，LiPAでの陽性は78.4%（109/139）であった。LiPAでは死菌も検出されうるが，今回の培養検査については検体収集から保管・移送の影響があった可能性が考えられ，また，PCR法では*M.kansasii*が検出対象外であることから単純な比較はできないが，PCRおよび培養いずれかの方法で陽性となった検体は80.6%（112/139）（data not shown）であり，LiPAは臨床検体から直接検出するうえで十分な検出感度であった（Table 1）。

LiPAにより判定することができたRFP，PZAおよびLVFX薬剤感受性については，培養による感受性検査の結果と全例一致しており（Table 2），臨床検体からの直接検出であっても陽性であれば正しく薬剤感受性を判定できることが確認された。今回収集した臨床検体からは薬剤耐性結核菌数は少ないが，RFP耐性菌の約95%が*rpoB*遺伝子に[4)10)]，PZA耐性菌の72～95%が*pncA*遺伝子に[10)17)18)]，FQ耐性菌の75～94%が*gyrA*遺伝子に変異をもっていることが知られており[10)19)]，LiPAを用いることで同様の感度で臨床検体から迅速に薬剤感受性を判定できると考えられる。

INH耐性結核菌で高頻度にみられる変異は*katG*遺伝子の315番目のSerに該当する部位であり，次いで*inhA*遺伝子上流−15番目および−8番目の変異が知られている[10)20)21)]。この領域を標的としたNTM/MDR-TBストリップではINH耐性菌の3例は耐性と正しく判定されたが，3例では変異が検出されず感受性と判定された（Table 2A）。感受性と判定された3例はいずれも検出領域外の*katG*遺伝子に変異があることが確認されており，そのうち2例はINHストリップで変異が検出され，INH耐性と判定された（Table 3）。また，*inhA*遺伝子上流C-15Tの変異をもつ1例は，NTM/MDR-TBストリップ，INHストリップどちらも変異を検出しているが，感受性検査では感受性と判定された。*inhA*遺伝子上流域の変異をもつ場合は最小発育阻止濃度（MIC）が低いことが知られている[22)]。さらにINHストリップではINH感受性と判定された検体の中に*fabG1*遺伝子変異を2例で検出している（Table 3）。*fabG1*遺伝子G609A変異は*inhA*遺伝子上流域の変異と同程度にMICが低いと考えられる。

RFP耐性の場合，他の薬剤にも耐性である場合が多い[23)24)]。NTM/MDR-TBストリップは頻度の高いINH耐性変異を検出するが，RFP耐性・INH感受性と判定された場合，より多くの変異を検出するINHストリップを用いることでINH耐性変異を検出できる可能性がある。

MDR-TBでは治療が困難で予後は不良である。治療開