研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
森本耕三	肺非結核性抗酸菌症の疫学	呼吸器内科	27	1–7	2015
Ohtsuka, K., Ohnishi, H., Nozaki, E., Pais Ramos, J., Tortoli, E., Yonetani, S., Matsushima, S., Tateishi, Y., Matsumoto, S. and Watanabe, T.	Whole-Genome Sequence of *Mycobacterium kyorinense*	Genome Announc.	2	e01062-01014	2014
Nishiuchi Y, Tamaru A, Suzuki Y, Kitada S, Maekura R, Tateishi Y, Niki M, Ogura H, Matsumoto S	Direct detection of *Mycobacterium avium* in environmental water and scale samples by loop-mediated isothermal amplification.	J Water Health	12(2)	211-219	2014
Nagi, S., Chadeka, E. A., Sunahara, T., Mutungi, F., Justin, Y. K., Kaneko, S., Ichinose, Y., Matsumoto, S., Njenga, S. M., Hashizume, M., Shimada, M. and Hamano, S.	Risk Factors and Spatial Distribution of *Schistosoma mansoni* Infection among Primary School Children in Mbita District, Western Kenya.	PLoS Negl Trop Dis.	8	e2991	2014
Morimoto, K., Ozawa, T., Awazu, K., Ito, N., Honda, N., Matsumoto, S. and Tsuruta, D.	Photodynamic Therapy Using Systemic Administration of 5-Aminolevulinic Acid and a 410-nm Wavelength Light-Emitting Diode for Methicillin-Resistant *Staphylococcus aureus* -Infected Ulcers in Mice.	PLoS One.	9	e105173	2014
Fujii, Y., Kaneko, S., Nzou, S. M., Mwau, M., Njenga, S. M., Tanigawa, C., Kimotho, J., Mwangi, A. W., Kiche, I., Matsumoto, S., Niki, M., Osada-Oka, M., Ichinose, Y., Inoue, M., Itoh, M., Tachibana, H., Ishii, K., Tsuboi, T., Yoshida, L. M., Mondal, D., Haque, R., Hamano, S., Changoma, M., Hoshi, T., Kamo, K., Karama, M., Miura, M. and Hirayama, K.	Serological surveillance development for tropical infectious diseases using simultaneous microsphere-based multiplex assays and finite mixture models.	PLoS Negl Trop Dis	8	e3040	2014
松本　壮吉、尾関　百合子	結核ワクチン開発の現状と新しい結核ワクチン開発に向けて	化学療法の領域	30	127-134	2014

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
西内　由紀子、松本　壮吉	抗酸菌の細菌学的特徴と病原性	感染症内科	2		2014
松本　壮吉	結核とその制圧を目指した研究	新潟県医師会報	766		2014
Hattori, Y., D. Morita, N. Fujiwara, D. Mori, T. Nakamura, H. Harashima, S. Yamasaki, and M. Sugita.	Glycerol monomycolate is a novel ligand for the human, but not mouse macrophage inducible C-type lectin, Mincle.	Journal of Biological Chemistry	289(22)	15405-15412	2014
吉岡佑弥、杉田昌彦	脂質免疫を基盤とした新しい遅延型アレルギー応答	臨床免疫・アレルギー科	62(6)	692-696	2014
瀬戸真太郎、永田　年、堀井俊伸、小出幸夫	結核菌ファゴソームの分子解剖	日本細菌学雑誌	69	513-525	2014

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
西山　晃史、松本　壮吉	抗酸菌と放線菌	荒川　宣親、神谷　茂、柳　雄介	病原微生物学	東京化学同人	東京	2014	120-129

特集　肺非結核性抗酸菌症の新たな展開

肺非結核性抗酸菌症の疫学*

森 本 耕 三**

Key Words: nontuberculous mycobacteria, pulmonary disease, epidemiology, incidence, prevalence

非結核性抗酸菌症の疫学調査

肺非結核性抗酸菌症(肺NTM症)は感染症としての報告義務がないこと，簡易で迅速な診断法が確立していないことから疫学調査を行うことは困難であり，そのバイアスからは逃れることはできない．故にNTMの疫学研究はさまざまな手法で行われてきた．手法としては大きく3つに分けられる．1つは皮膚反応試験を用いるもので発病しているかは不明であるがNTMの感染を把握できる．次に臨床検体における菌の分離数(頻度)を用いるもの．これには全陽性検体を扱う場合と複数回陽性となる場合を考慮して1菌/1患者/年とするものがある．3つ目は，診断基準を満たした患者数を用いるものである．これらには対象が施設，地域，国の各レベルからあり，その重要度は人口ベース(10万人あたり)で求められているか否かによる．しかし，人口ベースの値を導く手法・内容は異なり，罹患率(Incidence：一年間にNTMを発症した人の単位人口に対する割合)なのか有病率(Prevalence：一時点でNTMを発症している人の単位人口に対する割合)なのか，または分離頻度(Isolation incidence/prevalence)なのか違いがある．さらにデータの長期的な増減の有無，地理(海沿い，都市部など)，菌種の多様性，人種，性別，年齢，病型の違いがある．それらを把握しながら疫学報告を読む必要がある．手法としては診断症例を扱うのが最も困難であるが，保険データなどを用いて"菌の診断基準を満たす症例"を抽出する手法が報告されており主流となってきている．

本邦では7年ぶりとなる肺NTM症の全国疫学調査が行われており，その結果が注目されている．

本邦の疫学研究

1．皮膚反応試験を用いた大規模調査

本邦ではじめて行われたNTMの疫学調査は，皮膚反応検査を用いた1960年の岡田らによる「本邦における非定型抗酸菌の疫学的研究」が最初の報告である[1]．当時米国ではすでにいくつかの報告があり[2]，「わが国においてもこれらの菌による感染がどの程度起こっているかを検討する」ことを目的としている．対象は療養所入所中の結核患者，健康成人および学童などで，「対象中PPD-SよりPPD-YやPPD-Bがより著明な反応を呈した者は甚だ少なく，PPD-S陰性であってPPD-YまたはPPD-Bが陽性のものは結核患者，成人には甚だ少なく，学童においても数%を認めたにすぎなかった．それゆえに黄色菌やBattery菌の感

* The epidemiology of nontuberculous mycobacterial pulmonary disease.

** Kozo MORIMOTO, M.D.: 公益財団法人結核予防会複十字病院呼吸器センター〔〒204-8522 東京都清瀬市松山3-1-24〕; Respiratory Disease Center, Fukujuji Hospital, Japan Anti-Tuberculosis Association, Kiyose, Tokyo 204-8522, JAPAN

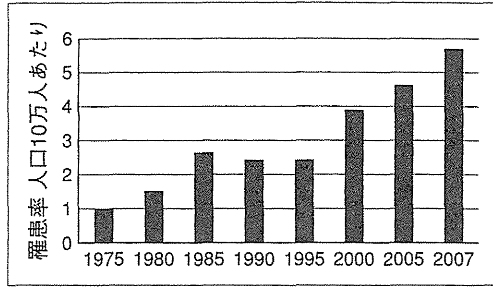

図1　肺非結核性抗酸菌症罹患率の推移

染はあるとしても僅少であることが考えられる」と結論している(PPD-YはM. *Kansasii*，PPD-BはBattery株から精製)．続いて全国11,561人を対象に行われたが[3]，nonphotochromogenが平均5.5%などで「中高年になるに従ってやや高くなる傾向が認められる」と予備調査とほぼ同様の結論に至っている．非定型抗酸菌ツベルクリンを用いた検討はその後抗原の供給がなかったことから，1993年に重籐，田坂らが独自に開発した精製ツベルクリンを用いた調査までなく，またこの後本邦では行われていない．

2. 国療研究班からはじまるわが国独自の罹患率調査(図1)

国立療養所非定型抗酸菌症共同研究班(国療研究班)により，非定型抗酸菌の「分離頻度，地理的分布ならびに肺感染症の地理的分布および菌種を調査研究する目的」で30年近く研究が行われた．基本データは，結核および非定型抗酸菌症に占める非定型抗酸菌症の割合とし，当初は6%前後であり太平洋南岸に多く東北部に少ないとした．肺感染症ではMAC 95%，*M. kansasii* 3.8%であり，*M. kansasii*は東京およびその周辺地域に多いと報告した．1980年に新登録活動性肺結核患者総数およびその人口10万の数値をとり，これに非定型抗酸菌症対肺結核比をかける手法を導入して本邦の罹患率データを示した(図1)[4]．1970年代の全国罹患率はほぼ横ばいであり，地域差では「大阪，高知で高く，東京，愛知が次いで東北部が低く北海道が最も低かった」，とした．

1978年頃からはそれまで東京周辺のみであった*M. kansasii*症が福岡，次いで1979年に大阪，1980年には福岡と高知でも認められ，のちに1987年度に北海道で同定され全国的な広がりが確認された[5]．1981年度報告では菌種の多様化と*M. kansasii*症が近畿中央病院で突出してきたことを明らかにし，1984年度報告で症例数が東京を上回ったことを報告した[6]．NTM全体の罹患率が2を超えたのは1984年で，当初は*M. kansasii*の増加が寄与しているものと考えられていたが，MACも1982年以後増加していることが確認され，以後漸増傾向を示した．国療研究班名での報告は1987年度および1988年度報告が最後となって

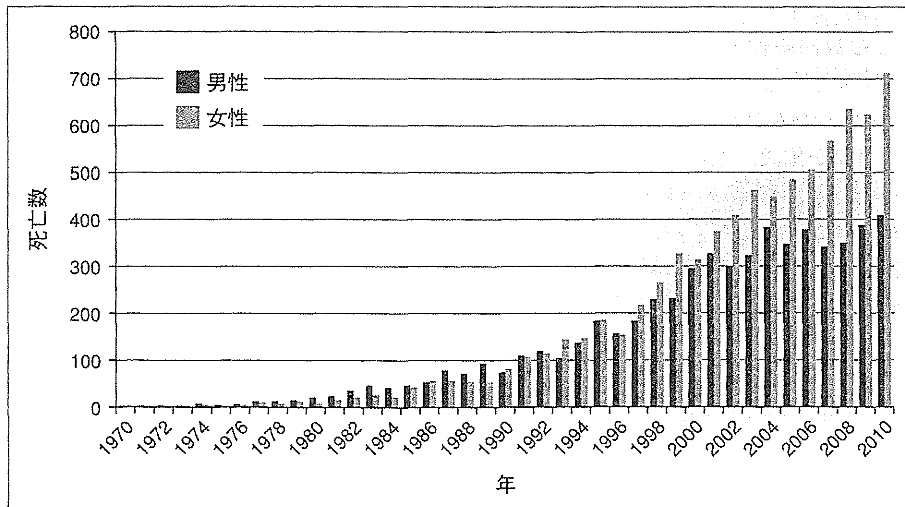

図2　本邦におけるNTM症死亡数の推移
2010年(男性409，女性712)(文献[9]より引用改変)

いる．期間は若干の重複があるがこの後の疫学データは，坂谷らによる1985年からのアンケート調査，続いて1990年代後半からは国の結核実態調査でのNTM症罹患率の成績，そして最近は非結核性抗酸菌症研究協議会の全国アンケート調査から知ることができる．

共同研究班をベースとした全国アンケート調査は，1985年から6年間国公私立の結核診療に実績のある211病院を選択して行われた[7]．罹患率は1991年で2.45とし，さらに他の報告と併せMAC症において*M. avium*は近畿以北で，*M. intracellulare*は中国以南で多いことを報告した．次いで2001年には非定型抗酸菌症協議会が全国200床以上の病院機関を対象にアンケートを行い，本症は全抗酸菌症中約29.2％の比率であり罹患率は5.9とした．また，*M. avium*と*M. intracellulare*の地域差は，中四国と九州がそれまで*M. intracellulare*優位であったのがほぼ同数になったとしている．2007年にも同様の調査が行われ，主要3菌種の罹患率は5.7であり，特に*M. avium*の増加が主である可能性を示唆している[8]．

3．結核の統計の一部として得られたNTM症の疫学データ

1995年から2003年までの期間は，結核の活動性分類で「非定型抗酸菌陽性」という項目が設けられ，結核の統計に詳細なデータをみることができる．しかし，NTM症の登録制を用いた報告は後述のオーストラリアを除いてこれまでになく，わが国は2003年を最後に本症の基本となるデータを知る手段を失ってしまったのである．

4．NTM症の死亡統計(図2, 3)と本邦の推定有病率

罹患率と同様に同症の死亡は本邦のNTM症の現状の側面を表すものといえる．

人口動態統計と国勢調査を基にした死亡統計調査が報告されている[9]．NTM症の死亡は1970年にはじめて3例が報告され以後漸増し，2000年頃から女性優位の傾向を示した(図2)．地域別では2010年の死亡数は東京が92例と最も多いものの，標準化死亡比では0.95と47都道府県中23番目であった．死亡比が1を上回っていたのは，南西部太平洋岸の県に多く，最も高かったのは高知県であった(1.92)(図3)．本邦は死亡統計が得られていることから，致死率のデータが得られれば有病率が推定できる．複十字病院で2004～2006年に肺MAC症と新規診断された症例309例の臨床追跡研究などから死亡率1～2％とした．これから2005年の本邦における有病率を33～66/10万(有病者数41,600～83,200)と推定している．この有病率は世界で最も高い数字であると思われる．

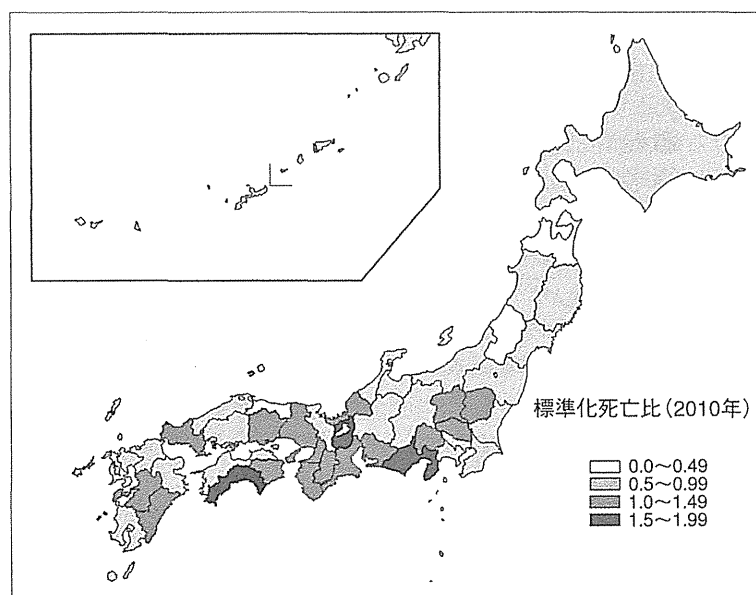

図3 県ごとの比較(標準化死亡比)(文献[9]より引用改変)

諸外国における疫学研究の動向（北米，オーストラリア）

1．皮膚反応調査

北米では古くは1943～49年[10]および1958～1965年に行われた調査がある[11]．米国出身の17～21歳の海軍兵に275,000人を対象に行ったところ南東部出身者の陽性率がきわめて高く，感染には地域差が大きいことを明らかにしている．前者は高容量PPD-Sを用いて反応をみる手法だが，同様に南東部の州出身者が多かったと結論している．北米では皮膚反応調査は断片的に行われており，Marrasらのグループは1971～1972年(1,490例)および1999～2000年(7,384例)に行われたNHANESの調査を比較し，陽性率は11.2%から16.6%へと上昇したことを明らかにした[12]．

2．菌分離頻度の調査

菌分離頻度を用いた手法は，検体データが集約されていれば比較的容易に行うことができるため，北米では古くから行われていた．1979～1980年にCDCは54施設ある全米抗酸菌検査室のうち42施設の解析から32%がNTMであり，うち58%がMAC，15%が*M. fortuitum*，10%が*M. kansasii*であったとしている[13]．ここで10万人あたりの感染者(分離頻度に近いデータ)はそれぞれ2.5，0.7，0.5であったとしている．1980年に同様の調査が行われており[14]，MACはAtlantic Ocean，Gulf of Mexico，南西部，カナダに接する州やKansas州に多く，*M. kansasii*は中部アメリカから北へ伸びる地域と南部に広がる地域(逆T字型：inverted T)で高いとしている．冒頭に記載したように1患者あたり複数の検体を出しているものも含まれるため過剰評価されていると思われる．

北米での大規模な菌調査は，NTM分離頻度の増加に加え各地域の検査施設でも抗酸菌検査が行われるようになったことから，州レベルでさえ行うことは困難になったと思われる．これはわが国と同様の経験といえる．この問題をクリアしているのにカナダのオンタリオ州がある．抗酸菌検査が行われている機関のデータをほぼ把握していることから信頼性の高いデータとして評価されている．オンタリオ州のデータ[15]は1患者あたり1検体として扱っていることから，複数検体が混在する問題はない．1997年に9.1/10万の分離頻度であったものが，2007年に14.1に上昇したと報告しており，さらにコホート研究から分離頻度に占める診断割合を求め，州レベルでの推定有病率を導いている．

3．診断症例による調査

診断例調査は，菌の基準を満たしさらに画像評価を含むカルテ調査が必要となることから大変な努力を要する．カナダのBritish Columbia州では1960～1972年の平均で0.37(肺NTM症は0.22)とした．菌種はMACが多く*M. kansasii*，*M. xenopi*が続いたとした．この州からは最近，1990～2006年の間で2000年から起こった同定数の増加やMACのコロナイゼーションの増加は，新しい検査法の導入によるアーチファクトとする報告をしている(罹患率1.6)[16]．治療を行っている症例を診断例(NTM症)としていることから過小評価の可能性はあるが，全経過でNTM症(治療導入例)が減少してきている．NTM症の減少傾向を示した数少ない報告である．

患者登録制のあるオーストラリアからは興味深い報告が続いている．Northern territoryでは1989～1997年の前期から後期で2.7から4.7へ上昇し(平均罹患率3.9，うち肺NTM症は2.1)，高齢男性が主で菌種はMACが多くを占めていたとしている[17]．Northern Territoryの東に位置するQueensland州からの報告では，肺NTM症の罹患率は1999年の4.85(肺NTM症：2.2)から2005年5.7(肺NTM症：3.25)へ上昇し，主な菌種は*M. intracellulare*であったとしている．さらにFCタイプに比べてNBタイプの上昇が顕著であったとしている[18]．

アメリカのオレゴン州からの報告は，細菌学的基準を満たす症例を抽出することで有病率を導いている(つまり画像や臨床所見調査を省く)[19]．NTM症の有病率は5.6，菌種ではMAC 74.2%，RGM 10%とした．冒頭に述べたように，この手法は注目を集めており有病率推定の主流となっている．前述のオンタリオ州のグループは州の分離頻度とコホート内診断率から有病率を求める手法を使っていたが，最近の報告で細菌学的診断基準を導入し，2008年肺MAC症の有病率を

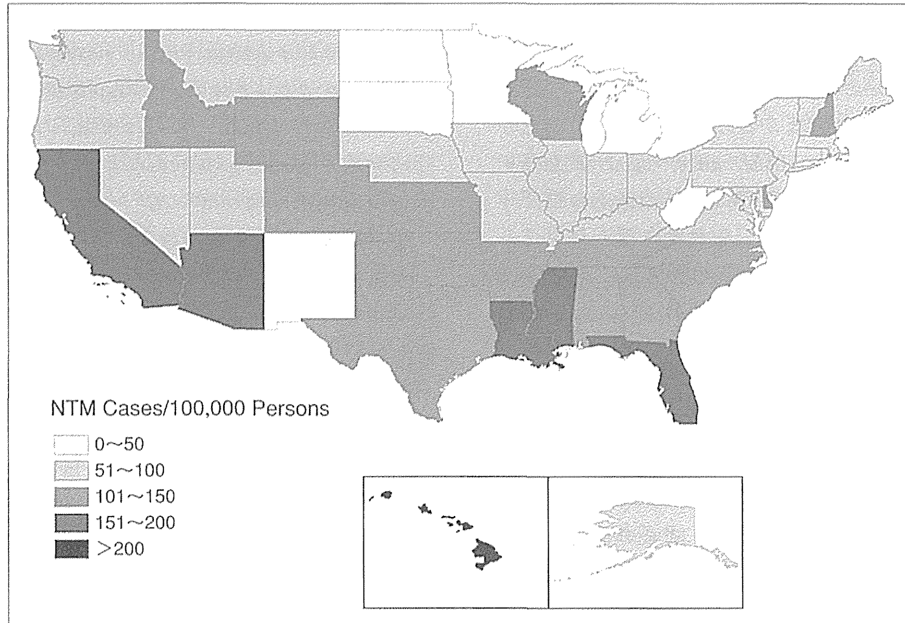

図4　米国における州ごとの有病率（文献[23]より引用）

6.81とした[20]．

以上は菌データが集約して把握されている，患者登録があるというアドバンテージを活かした手法であるが，近年は種々の医療システムのデータを用いた報告が相次いでいる．Billingerらは退院患者データの分析から，1998〜2005年でカルフォニア州では明らかな増加は認められないもののニューヨークとフロリダ州で増加があり，70歳以上の有病率は7.6であったとした[21]．NIAIDのグループは4つの保健提供システムの情報を使って細菌学的基準を満たす症例を抽出し，有病率を1.4〜6.7とし，菌種はMACが80%，*M. chelonae/M. abscessus*が12%，*M. kansasii* 5.5%とした[22]．また，同様にMedicareという65歳以上の高齢者を主体とする公的保険システムの5%抽出データを解析し，1997〜2007年の平均有病率は約30であったとし州ごとの有病率も明らかにした（図4）．さらに患者基本データから，Asian/Pacific islandersが白人よりも高い有病率であったとした[23]．

北米では古くから菌データを用いて分離頻度が報告されていたが，近年は細菌学的基準を用いた手法を取り入れたことにより，有病率を比較的正確に導くことができている．さらに医療保険システムのデータを用いることで，詳細な患者データの解析も加えている．まとめると高齢女性を中心とした有病率の増加を示しており，かつてから言われているようにGulf of Mexico（南東部），南西部に多いようである．菌種はオンタリオ州で*M. xenopi*が2割を占めるという例外があるが，本邦と同じくMACが8割前後で，*M. abscessus*を中心とした迅速発育菌，*M. kansasii*，*M. xenopi*が残りを分けている，というのがおおまかな傾向のようである．

4．ヨーロッパ，アジア

ヨーロッパのNTMは，BTSが行ったRCTが3菌種（MAC，*M. malmoense*，*M. xenopi*）を対象としたことから，その菌種の多様性を印象に持ちやすい[24]．近年の報告はオランダを筆頭に[25]，デンマーク[26]，フランス[27]，ギリシア[28]，イギリスのLeeds[29]，からがある．オランダでは専門機関により菌データの集約と分析が行われていることから，デンマークは国レベルで統一された医療システムを用いて，フランスは地域主幹病院の連携から診断した結核患者数と国の罹患率データからカバーする人口を求めるという独特の手法によりNTM罹患率・有病率を導いている．菌種をみるとこちらもMACが主体であるようだ

が，北米や日本ほど比率が高くなく50％前後で，その他*M. kansasii*，*M. fortuitum*，*M. xenopi*，*M. malmoense*，*M. abscessus*が種々の割合で占める，というのが大まかなところのようだ．しかし，Central Greeceは*M. malmoense*が最も多いなど全体像を把握するのは困難である．罹患率(有病率)が2を超えているとした報告はなく，一方で，高齢男性のCOPDを合併する*M. avium*症の増加が指摘されている．COPDは全世界的に増えているが，NTM罹患率に対する影響の可能性については本邦でも注視していくべきだろう．

アジアのNTM症についてはオランダのグループから2011年にreviewが出されている[30]．これによるとアジアからは人口ベースとなる罹患率(有病率)を求めているものはないとされている．さらに男性で結核の既往が多いという特徴と菌種はMAC主体だが，台湾や韓国の報告が引用され*M. abscessus*が目立つ点に注目されている．本邦でもMAC症の増加から2次感染，菌交代現象としての*M. abscessus*の存在について注目を集めていることは確かであるが，疫学的に明らかに増加しているという報告はこれまでにはないと思われる．今後いわゆる*M. massiliense*との区別を明確化したデータの集積が必要であろう．

文　献

1) 岡田　博，河盛勇造，重松逸造，ほか．本邦における非定型抗酸菌の疫学研究．日本医事新報社 1960；1909：14.
2) Edwards LB, Palmer CE. Epidemiologic studies of tuberculin sensitivity. I. Preliminary results with purified protein derivatives prepared from atypical acid-fast organisms. Am J Hyg 1958 ; 68 : 213.
3) 岡田　博．日本における非定型抗酸菌感染の疫学的研究．日本医事新報 1962；2007：22.
4) 束村道雄，下出久雄，喜多舒彦，ほか．日本における肺非定型抗酸菌症の疫学的・細菌学的研究．結核 1980；55：273.
5) 束村道雄，下出久雄，喜多舒彦，ほか．日本における非定型抗酸菌感染症の研究(国療非定型抗酸菌症共同研究班1980年度報告) *Mycobacterium kansasii*症の‘Endemic Status’から‘Epidemic Status’への変化．結核 1982；57：299.
6) 国立療養所非定型抗酸菌症共同研究班．日本における非定型抗酸菌感染症の研究（国療非定型抗酸菌症共同研究班1984年度報告）*M. kansasii*症の比較的多発を含め，感染菌種の多様化の時代が続いている．結核 1986；61：277.
7) 坂谷光則．第67回総会シンポジウム．III *Mycobacterium avium* Complex症の現状と将来．2. M. avium Comlex 症の疫学．結核 1993；68：43.
8) 佐藤滋樹．肺非結核性抗酸菌症の最近の話題．現代医学 2008；56：317.
9) Morimoto K, Iwai K, Uchimura K, et al. A steady increase in nontuberculous mycobacteriosis mortality and estimated prevalence in Japan. Ann Am Thorac Soc 2014 ; 11 : 1.
10) Palmer CE. Tuberculin sensitivity and contact with tuberculosis. Am Rev Tuberc 1953 ; 68 : 678.
11) Edwards LB, Acquaviva FA, Livesay VT, et al. An atlas of sensitivity to tuberculin, PPD-B, and histoplasmin in the United States. Am Rev Respir Dis 1969 ; 99(4) : Suppl : 1.
12) Khan K, Wang J, Marras TK. Nontuberculous mycobacterial sensitization in the United States : national trends over three decades. Am J Respir Crit Care Med 2007 ; 176 : 306.
13) Good RC. From the Center for Disease Control. Isolation of nontuberculous mycobacteria in the United States, 1979. J Infect Dis 1980 ; 142 : 779.
14) Good RC, Snider DE Jr. Isolation of nontuberculous Mycobacteria in the United States, 1980. J Infect Dis 1982 ; 146 : 829.
15) Marras TK, Chedore P, Ying AM, Jamieson F. Isolation prevalence of pulmonary non-tuberculous mycobacteria in Ontario, 1997-2003. Thorax 2007 ; 62 : 661.
16) Hernández-Garduño E, Rodrigues M, Elwood RK. The incidence of pulmonary non-tuberculous mycobacteria in British Columbia, Canada. Int J Tuberc Lung Dis 2009 ; 13 : 1086.
17) O'Brien DP, Currie BJ, Krause VL. Nontuberculous mycobacterial disease in northern Australia : a case series and review of the literature. Clin Infect Dis 2000 ; 31 : 958.
18) Thomson RM ; NTM working group at Queensland

TB Control Centre and Queensland Mycobacterial Reference Laboratory. Changing epidemiology of pulmonary nontuberculous mycobacteria infections. Emerg Infect Dis 2010 ; 16 : 1576.

19) Cassidy PM, Hedberg K, Saulson A, et al. Nontuberculous mycobacterial disease prevalence and risk factors : a changing epidemiology. Clin Infect Dis 2009 ; 49 : e124.

20) Al-Houqani M, Jamieson F, Mehta M, et al. Aging, COPD, and other risk factors do not explain the increased prevalence of pulmonary Mycobacterium avium complex in Ontario. Chest 2012 ; 141 : 190.

21) Billinger ME, Olivier KN, Viboud C, et al. Nontuberculous mycobacteria-associated lung disease in hospitalized persons, United States, 1998-2005. Emerg Infect Dis 2009 ; 15 : 1562.

22) Prevots DR, Shaw PA, Strickland D, et al. Nontuberculous mycobacterial lung disease prevalence at four integrated health care delivery systems. Am J Respir Crit Care Med 2010 ; 182 : 970.

23) Adjemian J, Olivier KN, Seitz AE, et al. Prevalence of nontuberculous mycobacterial lung disease in U.S. Medicare beneficiaries. Am J Respir Crit Care Med 2012 ; 185 : 881.

24) Jenkins PA, Campbell IA, Banks J, et al. Clarithromycin vs ciprofloxacin as adjuncts to rifampicin and ethambutol in treating opportunist mycobacterial lung diseases and an assessment of Mycobacterium vaccae immunotherapy. Thorax 2008 ; 63 : 627.

25) van Ingen J, Bendien SA, de Lange WC, et al. Clinical relevance of non-tuberculous mycobacteria isolated in the Nijmegen-Arnhem region, The Netherlands. Thorax 2009 ; 64 : 502.

26) Andréjak C, Thomsen VØ, Johansen IS, et al. Nontuberculous pulmonary mycobacteriosis in Denmark : incidence and prognostic factors. Am J Respir Crit Care Med 2010 ; 181 : 514.

27) Dailloux M, Abalain ML, Laurain C, et al. Respiratory infections associated with nontuberculous mycobacteria in non-HIV patients. Eur Respir J 2006 ; 28 : 1211.

28) Gerogianni I, Papala M, Kostikas K, et al. Epidemiology and clinical significance of mycobacterial respiratory infections in Central Greece. Int J Tuberc Lung Dis 2008 ; 12 : 807.

29) Henry MT, Inamdar L, O'Riordain D, et al. Nontuberculous mycobacteria in non-HIV patients : epidemiology, treatment and response. Eur Respir J 2004 ; 23 : 741.

30) Simons S, van Ingen J, Hsueh PR, et al. Nontuberculous mycobacteria in respiratory tract infections, eastern Asia. Emerg Infect Dis 2011 ; 17 : 343.

*　　*　　*

genomeA
Journals.ASM.org

CrossMark

Whole-Genome Sequence of *Mycobacterium kyorinense*

Kouki Ohtsuka,[a] Hiroaki Ohnishi,[a] Eriko Nozaki,[a] Jesus Pais Ramos,[b] Enrico Tortoli,[c] Shota Yonetani,[a] Satsuki Matsushima,[a] Yoshitaka Tateishi,[d] Sohkichi Matsumoto,[d] Takashi Watanabe[a]

Department of Laboratory Medicine, Kyorin University School of Medicine, Tokyo, Japan[a]; National Reference Laboratory for Tuberculosis, Centro de Referência Professor Hélio Fraga, Escola Nacional de Saúde Pública, Fiocruz, Rio de Janeiro, Brazil[b]; Emerging Bacterial Pathogens Unit, San Raffaele Scientific Institute, Milan, Italy[c]; Department of Bacteriology, Niigata University Graduate School of Medicine, Niigata, Japan[d]

We report here the first draft genome sequence of *Mycobacterium kyorinense*, which was described in 2009 and exhibits significant pathogenicity to humans.

Received 9 September 2014 **Accepted** 10 September 2014 **Published** 16 October 2014

Citation Ohtsuka K, Ohnishi H, Nozaki E, Pais Ramos J, Tortoli E, Yonetani S, Matsushima S, Tateishi Y, Matsumoto S, Watanabe T. 2014. Whole-genome sequence of *Mycobacterium kyorinense*. Genome Announc. 2(5):e01062-14. doi:10.1128/genomeA.01062-14.

Copyright © 2014 Ohtsuka et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 3.0 Unported license.

Address correspondence to Hiroaki Ohnishi, onishi@ks.kyorin-u.ac.jp.

Mycobacterium kyorinense is a slow-growing mycobacterium that was first described in 2009 (1). *M. kyorinense* is closely related to *M. celatum*, *M. branderi*, and *M. fragae*, and exhibits significant pathogenicity for humans, causing pneumonia, lymphadenitis, and arthritis (2–4). Antimicrobial susceptibility tests demonstrated that *M. kyorinense* is generally resistant to rifampin, isoniazid, and ethambutol (4). Further investigation is needed to clarify the genomics, biology, epidemiology, and pathogenicity of this species.

We sequenced the genomic DNA of the *M. kyorinense* type strain KUM060204^T on an Ion PGM system (Life Technologies) and assembled the reads using CLC Genomics Workbench 7.0. A total of 4,133,490 reads were generated, with an average read length of 203 bp, yielding a total sequence of 837,657,777 bp.

The assembled sequences of KUM060204^T comprised 453 contigs, with a combined length of 5,302,980 bp, with a G+C ratio of 66.9%. The average cover depth was 50×, the N_{50} contig size was 53,523, the average contig was 11,706 bp long, and the longest contig was 137,319 bp.

Genome annotation was performed using the RAST prokaryotic genome annotation server (http://www.nmpdr.org/FIG/wiki/view.cgi/Main/RAST). RAST predicted 5,405 putative open reading frames, including 5,351 coding sequences and 54 RNAs (46 tRNAs and 8 rRNAs). RAST functional analysis of the predicted protein-coding genes showed 78 genes involved in cell walls and capsules, 64 in membrane transport, 206 in protein metabolism, 93 in DNA metabolism, 141 in virulence and defense, 135 in respiration, 331 in fatty acids, lipids, and isoprenoids, 395 in cofactors, vitamins, prosthetic groups, and pigments, and 356 in amino acids and derivatives.

To explore the molecular mechanism underlying the resistance of *M. kyorinense* to anti-tuberculosis drugs, we selected several genes known to be responsible for resistance to rifampin (*rpoB*), ethambutol (*embB*), and isoniazid (*inhA*, *katG*, and *ahpC*). The sequences of these genes in *M. kyorinense* were compared with those in *M. tuberculosis* H37Rv to clarify whether they contain specific mutations associated with resistance to anti-tuberculosis drugs in *M. tuberculosis*. Analysis of the *rpoB* gene confirmed our previous finding that KUM060204^T harbors a Ser531Asp amino acid substitution, the most frequent mutation in rifampin-resistant *M. tuberculosis* (4, 5). In contrast, we did not detect a substitution at Met306 of *embB*, the major mutation in ethambutol-resistant *M. tuberculosis* (6). Nor did we find a Ser315Thr substitution of *katG*, a mutation in the regulatory region (nucleotides [nt] −48, −51, −54, −81, and −88) of *ahpC*, a Ser94Ala substitution in the *inhA* gene or a mutation in the regulatory region (nt −15 and −17) of *inhA*, which are common mutations in isoniazid-resistant *M. tuberculosis* (6). These results suggested that the mechanism underlying drug resistance in *M. kyorinense* is significantly different from that in *M. tuberculosis*.

In conclusion, we report the genome sequence of KUM060204^T which to the best of our knowledge is the first genome sequence of the species *M. kyorinense*.

Nucleotide sequence accession numbers. The whole genome sequence of KUM060204^T has been deposited in DDBJ/EMBL/GenBank under the accession numbers BBKA01000001 to BBKA01000453.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (23590688).

REFERENCES

1. Okazaki M, Ohkusu K, Hata H, Ohnishi H, Sugahara K, Kawamura C, Fujiwara N, Matsumoto S, Nishiuchi Y, Toyoda K, Saito H, Yonetani S, Fukugawa Y, Yamamoto M, Wada H, Sejimo A, Ebina A, Goto H, Ezaki T, Watanabe T. 2009. *Mycobacterium kyorinense* sp. nov., a novel, slow-growing species, related to *Mycobacterium celatum*, isolated from human clinical specimens. Int. J. Syst. Evol. Microbiol. 59:1336–1341. http://dx.doi.org/10.1099/ijs.0.000760-0.
2. Ramos JP, Campos CE, Caldas PC, Ferreira NV, da Silva MV, Redner P, Campelo CL, Vale SF, Barroso EC, Medeiros RF, Montes FC, Galvão TC, Tortoli E. 2013. *Mycobacterium fragae* sp. nov., a non-chromogenic species isolated from human respiratory specimens. Int. J. Syst. Evol. Microbiol. 63:2583–2587. http://dx.doi.org/10.1099/ijs.0.046862-0.
3. Campos CE, Caldas PC, Ohnishi H, Watanabe T, Ohtsuka K, Matsushima S, Ferreira NV, da Silva MV, Redner P, de Carvalho LD, Medeiros RF, Abbud Filho JA, Montes FC, Galvão TC, Ramos JP. 2012. First

isolation of *Mycobacterium kyorinense* from clinical specimens in Brazil. J. Clin. Microbiol. 50:2477–2478. http://dx.doi.org/10.1128/JCM.00023-12.

4. Ohnishi H, Yonetani S, Matsushima S, Wada H, Takeshita K, Kuramochi D, Caldas PC, Campos CE, da Costa BP, Ramos JP, Mikura S, Narisawa E, Fujita A, Funayama Y, Kobashi Y, Sakakibara Y, Ishiyama Y, Takakura S, Goto H, Watanabe T. 2013. *Mycobacterium kyorinense* infection. Emerg. Infect. Dis. 19: 508–510. http://dx.doi.org/10.3201/eid1903.120591.
5. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet 341:647–650. http://dx.doi.org/10.1016/0140-6736(93)90417-F.
6. Zhang Y, Telenti Y. 2000. Genetics of drug resistance in *Mycobacterium tuberculosis*, p 235–254. *In* Hatfull GF, Jacobs WR (ed), Molecular genetics of *Mycobacteria*. ASM Press, Washington, D.C.

Direct detection of *Mycobacterium avium* in environmental water and scale samples by loop-mediated isothermal amplification

Yukiko Nishiuchi, Aki Tamaru, Yasuhiko Suzuki, Seigo Kitada, Ryoji Maekura, Yoshitaka Tateishi, Mamiko Niki, Hisashi Ogura and Sohkichi Matsumoto

ABSTRACT

We previously demonstrated the colonization of *Mycobacterium avium* complex in bathrooms by the conventional culture method. In the present study, we aimed to directly detect *M. avium* organisms in the environment using loop-mediated isothermal amplification (LAMP), and to demonstrate the efficacy of LAMP by comparing the results with those obtained by culture. Our data showed that LAMP analysis has detection limits of 100 fg DNA/reaction for *M. avium*. Using an FTA® elute card, DNA templates were extracted from environmental samples from bathrooms in the residences of 29 patients with pulmonary *M. avium* disease. Of the 162 environmental samples examined, 143 (88%) showed identical results by both methods; 20 (12%) and 123 (76%) samples were positive and negative, respectively, for *M. avium.* Of the remaining 19 samples (12%), seven (5%) and 12 (7%) samples were positive by the LAMP and culture methods, respectively. All samples that contained over 20 colony forming units/primary isolation plate, as measured by the culture method, were also positive by the LAMP method. Our data demonstrate that the combination of the FTA elute card and LAMP can facilitate prompt detection of *M. avium* in the environment.

Key words | bathroom, direct detection, FTA elute card, loop-mediated isothermal amplification (LAMP), *Mycobacterium avium*

Yukiko Nishiuchi (corresponding author)
Toneyama Institute for Tuberculosis Research,
Osaka City University Medical School,
5-1-1 Toneyama, Toyonaka, Osaka 560-8552,
Japan
E-mail: *nishiuchi@med.osaka-cu.ac.jp*

Aki Tamaru
Department of Infectious Diseases,
The Osaka Prefectural Institute of Public Health,
1-3-69 Nakamichi, Higashinari-ku, Osaka 537-0025,
Japan

Yasuhiko Suzuki
Research Center for Zoonosis Control,
Hokkaido University, North 20, West 10 Kita-ku,
Sapporo, Hokkaido 001-0020,
Japan

Seigo Kitada
Ryoji Maekura
Yoshitaka Tateishi
National Hospital Organization Toneyama National Hospital, 5-1-1 Toneyama,
Toyonaka, Osaka 560-8552,
Japan

Yoshitaka Tateishi
Mamiko Niki
Sohkichi Matsumoto
Department of Bacteriology,
Osaka City University Graduate School of Medicine,
1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585,
Japan

Hisashi Ogura
Department of Virology,
Osaka City University Graduate School of Medicine,
1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585,
Japan

INTRODUCTION

The incidence of *Mycobacterium avium* complex (MAC) infection is gradually increasing all over the world, especially in developed countries (Falkinham 1996; Field *et al.* 2004; Griffith *et al.* 2007). MAC organisms inhabit the environment and are transferred to susceptible humans or farm animals, leading to infection and disease (Falkinham 2002; Field *et al.* 2004; Angenent *et al.* 2005). *M. avium* and other nontuberculous mycobacteria are widely distributed in natural and artificial environmental habitats, including natural water bodies, drinking water distribution systems, hot tubs, forest soils, peats, and potting soils (Falkinham 2009). We previously reported that MAC was frequently detected in

doi: 10.2166/wh.2013.007

samples from bathrooms in the residences of patients with pulmonary MAC disease, suggesting that the bathroom is the possible source of infection (Nishiuchi *et al.* 2007, 2009). Although MAC colonization in the human environment was polyclonal and displayed genetic diversity, some genotypes were identical or similar to the clinical isolates obtained from the corresponding patients (Nishiuchi *et al.* 2007, 2009). Moreover, the characteristics of MAC disease, such as multiple infections with genetically different strains (Wallace *et al.* 1998, 2002) and frequent relapse or reinfection (Kobashi & Matsushima 2003), could be attributable to the presence of a reservoir for MAC in the environment immediately surrounding the patients. It is important to break this cycle of infection by removing the infection source; identification of the source in the environment is thus the initial important step for controlling the disease.

In previous investigations, we isolated *M. avium* organisms by conventional culture. Although this method is basic and essential for the assessment of genetic diversity and drug susceptibility, the procedure is time consuming; it takes 3 weeks to obtain primary isolates and another 2 weeks to obtain pure cultures, followed by polymerase chain reaction (PCR) analysis for species identification (Nishiuchi *et al.* 2007, 2009). Thus, at least 5 weeks are usually required to detect *M. avium* organisms, underscoring the need for an alternative, rapid, and accurate method of *M. avium* detection in environmental specimens, which would in turn facilitate accelerated diagnosis. Nucleic acid amplification (NAA) tests are commonly used in hospitals to directly detect *Mycobacterium tuberculosis* and *M. avium* in clinical specimens because they require less time than culture. Several recent systematic investigations have confirmed the high specificity and sensitivity of NAA tests (Ichiyama *et al.* 1996; Soini & Musser 2001; Huggett *et al.* 2003; Park *et al.* 2006). A novel NAA method, termed loop-mediated isothermal amplification (LAMP), is commonly used to detect viruses, parasitic protozoans, and bacteria including *M. tuberculosis* complex (Iwamoto *et al.* 2003; Boehme *et al.* 2007; Pandey *et al.* 2008), *M. avium* (Iwamoto *et al.* 2003), *M. avium* subsp. *paratuberculosis* (Enosawa *et al.* 2003), *M. intracellulare* (Iwamoto *et al.* 2003), *M. kansasii* (Mukai *et al.* 2006) and *M. gastri* (Mukai *et al.* 2006). The LAMP method has been applied to detect mycobacteria in clinical samples (Iwamoto *et al.* 2003; Boehme *et al.* 2007; Pandey *et al.* 2008), but it has not been tested for environmental samples. In the present study, environmental samples obtained previously (Nishiuchi *et al.* 2009) were subjected to LAMP analysis for the direct detection of *M. avium* using novel primer sets targeting the *M. avium* 16S rRNA gene. The results were compared with those obtained previously by culture (Nishiuchi *et al.* 2009). We also employed FTA® elute cards for genomic DNA extraction; these cards allowed very easy recovery of DNA templates from the environmental samples without resorting to the use of any harmful reagent.

METHODS

Design of LAMP primers

Using conserved sequences of the 16S rRNA gene as a target, two inner primers, namely the forward inner primer (FIP) and backward inner primers (BIP), two outer primers (F3 and B3), and two loop primers (FL and BL) for *M. avium* were designed using PrimerExplorer V3 software (https://primerexplorer.jp; Eiken Chemical Co. Ltd, Tokyo, Japan). The primer sequences and other details are listed in Table 1.

LAMP reaction

LAMP was performed in 50 μl reaction volumes containing 4 μl of the extracted DNA template, 20 $\mu mol\ l^{-1}$ each of FIP and BIP, 25 $\mu mol\ l^{-1}$ each of F3 and B3, 30 $\mu mol\ l^{-1}$ each of FL and BL, 1.4 $mmol\ l^{-1}$ deoxynucleoside triphosphate mix, 0.8 $mol\ l^{-1}$ betaine (Sigma-Aldrich, St Louis, MO, USA), 20 $mmol\ l^{-1}$ Tris-HCl (pH 8.8), 10 $mmol\ l^{-1}$ KCl, 10 $mmol\ l^{-1}$ $(NH_4)_2SO_4$, 8 $mmol\ l^{-1}$ $MgSO_4$, and 6.4 U of *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA). The mixture was incubated at 64 °C for 60 min in a Loopamp® real-time turbidimeter (LA-200; Teramecs Co., Kyoto, Japan) and then heated to 80 °C for 2 min to terminate the reaction.

Analysis of LAMP products

The LAMP reaction causes turbidity in the reaction tube, which is proportional to the amount of amplified DNA. The reaction was considered positive when a turbidity of ≥ 0.1 was observed

Table 1 | Primers used for loop-mediated isothermal amplification

Primer type		Sequence	Location of the target sequence on the complete genome sequence[a]
F3	Forward outer	5′ – CTGGCTCAGGACGAACG – 3′	1,487,551 – 1,487,563
B3	Backward outer	5′ – GCCCATCCCACACCGC – 3′	1,487,759 – 1,487,746
FIP	Forward inner primer	5′ – TGCCCACGTGTTACTCATGCAAGTCGAACGGAAAGGCCT – 3′	1,487,654 – 1,487,638 + 1,487,588–1,487,609
BIP	Backward inner primer	5′ – TCGGGATAAGCCTGGACCAGAAGACATGCGTCTTGA –3′	1,487,669 – 1,487,683 + 1,487,732–1,487,712
FL	Loop forward	5′ – GTTCGCCACTCGAGTACCTCCG – 3′	1,487,634 – 1,487,613
BL	Loop backward	5′ – GAAACTGGGTCTAATACCGG – 3′	1,487,684 – 1,487,703

[a]*M. avium* 104 (GenBank accession no. CP000479.1).

within 50 min. For further confirmation, the amplified products were examined by restriction analysis using *Taq*I enzyme, which was selected on the basis of the restriction maps of the target sequences of the LAMP product. Following overnight digestion at 37 °C, the digested products were analyzed by agarose gel electrophoresis using a 2% agarose gel, followed by staining with ethidium bromide. For further confirmation that the correct LAMP product was obtained, melting curve analysis was performed as follows. The LAMP reaction was carried out after addition of SYBR Green I (1:50,000; Molecular Probes Inc., Eugene, OR, USA), and the melting curves of LAMP amplicons were obtained over a temperatures range of from 64–95 °C using an Applied Biosystems 7500 fast real-time PCR system. The ROX reference dye was not used.

Strains and environmental samples

The specificity of the selected primer sets was examined by performing the LAMP method for DNA extracted from various bacterial strains: *M. tuberculosis* ATCC 25618, *M. bovis* Ravenel, *M. bovis* BCG Tokyo, *M. africanum* ATCC 25420, *M. microti* TC 77, *M. kansasii* ATCC 12476, *M. avium* ATCC 15769, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. intracellulare* ATCC 13950, *M. marinum* ATCC 927, *M. simiae* ATCC 12476, *M. shimoidei* ATCC 27962, *M. nonchromogenicum* ATCC 19530, *M. xenopi* ATCC 19250, *M. scrofulaceum* ATCC 19981, *M. gordonae* ATCC 14470, *M. chelonae* subsp. *abscessus* ATCC 19977, *M. fortuitum* ATCC 6841, *M. austroafricanum* ATCC 33464, *M. pulveris* ATCC 35154, *M. asiaticum* ATCC 25276, *M. tokaiense* ATCC 27282, *M. malmoense* ATCC 29571, *Achromobacter xylosoxidans*, *Acinetobacter haemolyticus*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Shigella boydii*, *Staphylococcus aureus*, and *Streptococcus haemolyticus*. Genomic DNA was prepared from the bacterial strains by mechanical disruption, as described previously (Suzuki *et al.* 1995), and dissolved in 300 μl of TE buffer containing 10 $mmol\,l^{-1}$ Tris-HCl (pH 8.0) and 1 $mmol\,l^{-1}$ ethylenediaminetetraacetic acid (EDTA). All extracts were verified to contain DNA of $>10\,ng\,\mu l^{-1}$ concentration. Environmental samples collected by us previously (Nishiuchi *et al.* 2009) were also used in the present study. In brief, the samples collected on cotton swabs (scale and slime) were preincubated in 1 ml tryptic soy broth for 3 h at 25 °C and subjected to alkali treatment by the addition of 3 ml 2% sodium hydroxide solution and incubation for 10 min, followed by addition of 6 ml of phosphate buffer (PB) at pH 6.8 and centrifugation at $2270 \times g$ for 15 min. The pellets were resuspended in 0.5 ml of PB, and 0.2 ml of these suspensions was used for culture while the remaining samples were frozen until DNA extraction for use in the LAMP method. The water samples (200 ml) were centrifuged and subjected to alkali treatment, as described above.

Comparison of various methods of DNA extraction for LAMP analysis

Five methods of DNA extraction were used for comparing DNA detection limits obtained by the LAMP method. The methods included the conventional phenol/chloroform/isopropanol extraction method (Suzuki *et al.* 1995), the

Puregene Yeast and Gram-positive Bacteria Kit (Gentra, Tokyo, Japan), the QIAamp DNA Micro Kit (Qiagen, GmbH, Hilden, Germany) after overnight treatment of samples with 2 mg ml^{-1} of lysozyme solution (1 mol l^{-1} NaCl, 0.1 mol l^{-1} EDTA, 10 mmol l^{-1} Tris-HCl (pH 8.0), 0.5% Brij-58, 0.2% deoxycholate, and 0.5% sarkosyl), the silica-based method, which is capable of detecting 1–10 mycobacteria in samples (Bahador *et al.* 2004), and the FTA elute card method (Whatman Inc.), for which 40 µl of cell suspension containing 1.0×10^2–10^5 colony forming units (CFU) 100 μl^{-1} was used. For the other four methods, 100 µl of the cell suspension was used. Extractions using kits were performed according to the manufacturers' instructions, and extracts were eluted with 30 µl of TE buffer.

When punching FTA elute cards for recovery of DNA templates, precautions had to be taken to exclude the risk of contamination with carryover DNA. Therefore, every time an elute card was punched, the puncher was decontaminated by subsequently punching a wet Kimwipes® containing 1000 ppm of sodium hypochlorite, which is a well-known chemical decontaminant for DNA (Prince & Andrus 1992). We confirmed the effectiveness of this hypochlorite system for *M. avium* bacilli (up to 4×10^8 cells) and *M. avium* DNA (up to 1.2 µg) on FTA elute cards (data not shown). Subsequently, the decontaminant and damaged DNA remaining on the puncher were removed by punching a clean Kimwipes® twice.

Extraction by the silica-based method was performed as described previously (Bahador *et al.* 2004). In brief, 500 µl of lysis buffer (1.2% guanidine thiocyanate (Fluka Chemie AAG, Switzerland) in 0.1 mol l^{-1} Tris-HCl (pH 6.4), 36 mmol l^{-1} EDTA, and 2% Triton X-100) was added to 100 µl of the cell suspension, followed by 20 µl of acid-washed silica. The suspension was mixed vigorously and incubated for 30 min at 60 °C, followed by centrifugation at $13{,}800 \times g$ for 2 min. The pellet was washed twice with washing buffer containing 12% guanidine thiocyanate in 0.1 mol l^{-1} Tris-HCl (pH 6.4), twice with 70% ethanol, and once with acetone and then dissolved in 30 µl of TE buffer.

Extraction of DNA from environmental samples

The frozen samples were centrifuged at $13{,}800 \times g$ for 10 min, and cell pellets were resuspended in 80 µl of PB (pH 6.8). Forty microliters of the concentrated environmental samples was applied to FTA elute cards, and DNA was extracted in 30 µl of TE buffer as described above.

RESULTS

Sensitivity and specificity of the LAMP method

We first examined the sensitivity of this method by monitoring the detection of serially diluted DNA extracted from *M. avium* (Figure 1). The results indicated that the DNA detection limit was 100 fg/reaction as opposed to the detection limit of 1 pg DNA/reaction obtained using the previously reported primer sets that targeted *gyr*B (Iwamoto *et al.* 2003).

The method of DNA extraction is also known to influence sensitivity because the recovery rate of DNA generally depends on both the method used and the skills of the researcher. Although many methods have been proposed, some require numerous steps and the use of corrosive reagents, such as phenol and chloroform, while others require a large number of bacilli in the starting material because of their poor recovery. Thus, we next examined how many bacilli were required in the sample for the successful detection of *M. avium*, using five different methods of DNA extraction. Among these methods (Table 2), the FTA elute card method was the most sensitive and suitable for subsequent DNA detection using the LAMP method, as it detected *M. avium* when a minimum of 400 bacilli were present in the volume (40 µl) applied to the FTA elute card. Moreover, this method only required only 2–3 h because of the simple procedure.

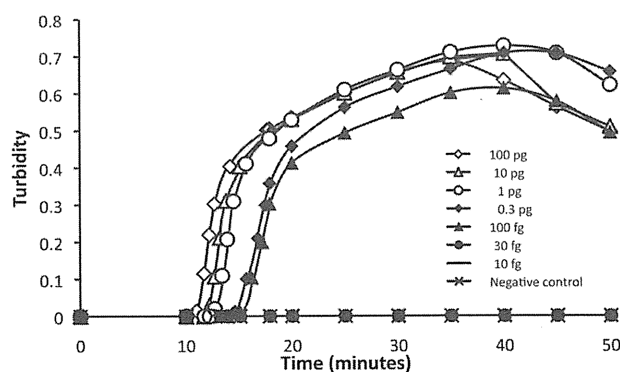

Figure 1 | Sensitivity of the LAMP method for *M. avium* detection.

Table 2 | Comparison of the DNA extraction methods[a]

Extraction method	Detection limit (CFU μl^{-1}) of sample	Processing time (h)	Use of corrosive reagent
Phenol/ chloroform/ isopropanol	$>10^5$ CFU 100 μl^{-1}	4	+
QIAamp DNA Micro Kit	10^3 CFU 100 μl^{-1}	25–26	–
Puregene yeast and Gram-Positive Bacteria Kit	10^5 CFU 100 μl^{-1}	2–3	–
Silica-based method	10^4 CFU 100 μl^{-1}	2–3	–
FTA elute card method	400 CFU 40 μl^{-1}	2–3	–

[a]DNA extracts were eluted with 30 μl of TE, and 4 μl of the extracted templates was used for the LAMP method.

The presence of contaminants such as dust, fungi, and other bacteria in the environmental samples necessitated the use of certain procedures, including pre-incubation for 3 h at 25 °C to bud fungi and spores, and the subsequent alkali-treatment to kill other microorganisms, to culture the organisms (Nishiuchi *et al.* 2009). We thus examined the effect of contaminants and the pretreatment procedures on the efficiency of DNA extraction. We used samples previously collected from the dust of air conditioners as a contaminant that contained many inorganic materials, bacteria, and fungi, but not mycobacteria (Nishiuchi *et al.* 2007). We pretreated the samples according to the previous method before extraction of DNA. Table 3 shows that both the presence of the dust and the pretreatment procedures hampered DNA extraction using the QIAamp DNA Micro Kit, but they had less effect on the efficiency of DNA extraction using the FTA elute card.

Table 3 | Effect of contaminants and pretreatment procedure on DNA extraction

	CFU of *M. avium* in sample ($\log_{10}$)								
	QIAamp DNA Micro Kit					FTA elute card method			
	6	5	4	3	2	4.6	3.6	2.6	1.6
Control	+	+	+	+	–	+	+	+	–
Added dust sample	+	+	–	–	–	+	+	–	–
Pretreatment[a]	+	+	–	–	–	+	+	+	–

[a]Pretreatment involved preculture for 3 h followed by alkali treatment.

We then evaluated the specificity of LAMP using genomic DNA from 23 different mycobacterial species and 10 other bacterial species. A successful LAMP reaction with species-specific primers caused turbidity in the reaction tubes. *M. avium* subsp. *paratuberculosis*, which causes Johne's disease, was also amplified using a *M. avium*-specific primer set and yielded a positive reaction. The specificity of amplification was further confirmed by restriction enzyme digestion of the LAMP products and melting curve analysis. As shown in Figure 2(a), restriction digestion yielded products that were in good agreement with the predicted sizes (171 and 163 bp). Furthermore, the peaks of the melting temperature curves were identical between *M. avium* genomic DNA and the environmental samples (Figure 2(b)).

Comparison between LAMP analysis and culture for the detection of *M. avium* in the environmental samples

In the present study, we used previously collected samples from bathrooms in the residences of 29 patients with pulmonary *M. avium* disease (Nishiuchi *et al.* 2009) and performed the DNA extraction followed by LAMP. The results were then compared with those obtained by culture (Table 4). Of a total

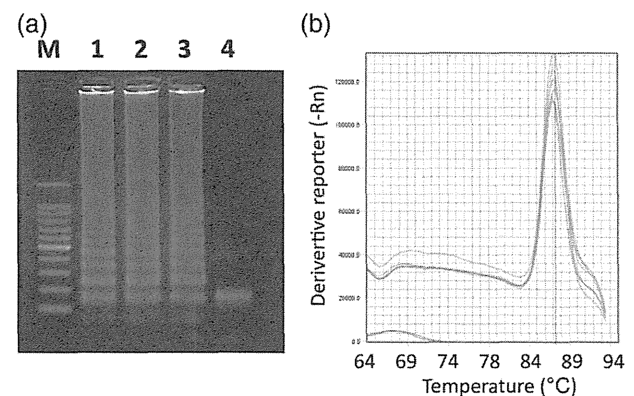

Figure 2 | The specificity of LAMP products. (a) LAMP products obtained from 10 pg of *M. avium* 104 DNA (Lane 1) and from nucleic acid extracts of pure cultures of *M. avium* 104 on the FTA elute cards (approximately 10^8 and 10^7 cells, Lanes 2 and 3, respectively), and restriction digestion of the products obtained from 10 pg of *M. avium* 104 DNA with *TaqI* (bands corresponding to 171 and 163 bp are expected, Lane 4). (b) The peaks of melting temperature curves of LAMP products amplified from control *M. avium* 104 DNA coincided with those from five isolates of environmental *M. avium*. Products from all isolates had a melting temperature of 87.3 °C.

Table 4 | Recovery and detection of *M. avium* from the residential bathrooms of patients with pulmonary *M. avium* disease using the culture and LAMP methods

	Number of samples						
Sampling site (sample type)	**Surface of the shower head (scale)**	**Inside the shower head (scale)**	**Shower (water)**	**Bathtub inlet (scale)**	**Bathtub (water)**	**Drain (slime)**	**Total no. of samples[a]**
No. of test samples	29	24	29	25	26	29	162 (29)
Culture positive	1	2	1	14	7	7	32 (15)
LAMP positive	3	0	3	13	6	6	27 (17)

[a]Numbers in parentheses represent the number of residences.
The result of the culture method is cited from the previous report (Nishiuchi *et al.* 2009).

of 162 samples *M. avium* was recovered from 32 samples (20%) by culture and detected in 28 samples (18%) by LAMP. Twenty samples (12%) were positive and 123 samples (76%) were negative for *M. avium* by both methods. The samples that tested positive by culture and/or the LAMP method are listed in Table 5. All samples that gave a *M. avium* recovery of >20 CFU/primary isolation plate by culture were also positive by LAMP.

DISCUSSION

Our data show that the combination of the FTA elute card (for DNA extraction) and the LAMP method is rapid and sensitive for detection of *M. avium* in environmental samples. It is advantageous over the culture method as it takes significantly less time and the entire procedure, from obtaining samples to *M. avium* detection, can be completed within 2–3 h. In general, the yields of DNA obtained from mycobacteria are low because of the presence of a robust, waxy cell wall that makes it difficult to lyse mycobacterial cells. Moreover, the samples examined in the present study, which were previously used for culturing the bacteria, had to be subjected to several procedures prior to DNA extraction, such as pre-culture to bud fungi, alkali-treatment to kill other microorganisms, freezing preservation, and concentration by centrifugation of the samples. These steps could decrease the recovery of DNA (Table 3) and the efficiency of NAA. Alkali-treatment may increase the concentration of alkali-soluble inhibitors such as humic substances that are widespread in the environment and are known to hamper PCR (Matheson *et al.* 2010). Although the presence of dust and pretreatment procedures hampered the efficiency of DNA extraction and the NAA method, they had minimal effect when the FTA elute card was employed. Therefore, use of the FTA elute card is likely to be suitable for examining the environmental samples containing mycobacteria, although it was originally developed to extract DNA from whole blood samples or buccal swabs (Tables 2 and 3).

Both the culture and LAMP methods yielded consistent results in 88% of the 162 examined environmental samples. In contrast, inconsistencies were observed with only 19 samples (12%; Table 5), which yielded <20 CFU/primary isolation plate by culture. It has been also reported that results obtained by NAA may show discrepancies with those obtained by culture (Iwamoto *et al.* 2003). This discrepancy is attributable to characteristic features of NAA, namely that it is capable of detecting DNA from dead cells. In addition, the NAA method is theoretically capable of detecting even a single copy of genomic DNA, but it is very susceptible to contamination with inhibitors and the efficiency of DNA extraction. Another possible reason for the discrepancy is the presence of viable but nonculturable (VNC) bacilli, although culture is theoretically capable of recovering a single viable bacterium. It has been recognized recently that the majority of bacteria in the environment enter a VNC state (Roszak & Colwell 1987). The pathogens in tap water (Moritz *et al.* 2010; Pawlowski *et al.* 2011) and in drinking water biofilms (Moritz *et al.* 2010) also enter the VNC state, as does *M. avium* in shower water or in the showerhead. This might also contribute to the discrepancy observed in the present study. However, whether *M. avium* can enter the VNC state remains uncertain. Further

Table 5 | List of all samples that tested positive by culture and/or LAMP methods

Participant no	Sampling site	Sample	Culture CFU/plate[a]	LAMP[b]
P-9	Bathtub inlet	Scale	>1,000	Positive
P-17	Bathtub inlet	Scale	>1,000	Positive
P-25	Bathtub inlet	Scale	>1,000	Positive
P-27	Bathtub inlet	Scale	>1,000	Positive
P-26	Bathtub inlet	Scale	>300	Positive
P-27	Bath drain	Slime	>300	Positive
P-8	Bathtub inlet	Scale	>100	Positive
P-12	Bath drain	Slime	>100	Positive
P-22	Bathtub inlet	Scale	>100	Positive
P-29	Bathtub inlet	Scale	>100	Positive
P-27	Bathtub	Water	47	Positive
P-12	Bathtub inlet	Scale	42	Positive
P-23	Bathtub inlet	Scale	20	Positive
P-29	Bath drain	Slime	13	–
P-17	Bath drain	Slime	9	Positive
P-8	Bath drain	Slime	6	Positive
P-28	Bathtub inlet	Scale	6	Positive
P-29	Bathtub	Water	6	Positive
P-9	Bathtub	Water	5	Positive
P-21	Bathtub inlet	Scale	4	Positive
P-26	Bathtub	Water	4	–
P-2	Bath drain	Slime	3	–
P-29	Showerhead inside	Scale	3	–
P-8	Bathtub	Water	1	–
P-9	Showerhead inside	Scale	1	–
P-9	Shower	Water	1	Positive
P-13	Bathtub inlet	Scale	1	–
P-15	Bathtub inlet	Scale	1	–
P-22	Bathtub	Water	1	–
P-23	Bath drain	Slime	1	–
P-25	Bathtub	Water	1	–
P-27	Showerhead surface	Scale	1	–
P-6	Shower	Water	–	Positive
P-9	Showerhead surface	Scale	–	Positive
P-9	Bath drain	Slime	–	Positive
P-11	Bathtub	Water	–	Positive
P-16	Showerhead surface	Scale	–	Positive
P-33	Showerhead surface	Scale	–	Positive
P-33	Shower	Water	–	Positive

[a]CFU/primary isolation plate where 200 μl of the sample was inoculated.
[b]The LAMP method was performed with 4 μl of the template in an assay mixture. The template was eluted from an FTA elute card with 30 μl of TE where 40 μl of the concentrated sample was originally applied to the FTA elute card.

studies are required to clarify this issue. In summary, all these facts should be taken into account when we assess environmental samples.

CONCLUSIONS

We demonstrated the utility of the LAMP method for the direct detection of *M. avium* in environmental samples by employing a novel set of six specific primers. Furthermore, we demonstrated that the FTA elute card is useful for DNA extraction from environmental *M. avium* without resorting to the use of harmful reagents. Thus, use of the LAMP method in combination with an FTA elute card for DNA extraction may facilitate the direct detection of environmental *M. avium* within a short period.

ACKNOWLEDGEMENTS

We are grateful to all participants who donated samples and to the hospital nurses who took part in our study. This research was supported by a Grant-in-Aid from the Institute for Fermentation, Osaka (IFO). It was also supported in part by the Global Center of Excellence (COE) Program; 'Establishment of International Collaboration Centers for Zoonosis Control', Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan; Grants-in-Aid for the Program of Founding Research Center for Emerging and Re-emerging Infectious Diseases from MEXT (Y.S.); the Japan Health Sciences Foundation; and the Ministry of Health, Labor and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research grants). We would like to thank Dr Tomotada Iwamoto for designing and providing us with the primer sets for *M. avium*. We would also like to thank Dr Eiichi Momodani for providing us with *M. avium* subsp. *paratuberculosis* samples.

REFERENCES

Angenent, L. T., Kelley, S. T., St Amand, A., Pace, N. R. & Hernandez, M. T. 2005 Molecular identification of potential pathogens in water and air of a hospital therapy pool. *Proc. Natl. Acad. Sci. USA* **102**, 4860–4865.

Bahador, A., Etemadi, H., Kazemi, B. & Ghorbanzadeh, T. 2004 Comparisons of five DNA extraction methods for detection of *Mycobacterium tuberculosis* by PCR. *J. Med. Sci.* **4**, 252–256.

Boehme, C. C., Nabeta, P., Henostroza, G., Raqib, R., Rahim, Z., Gerhardt, M., Sanga, E., Hoelscher, M., Notomi, T., Hase, T. & Perkins, M. D. 2007 Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *J. Clin. Microbiol.* **45**, 1936–1940.

Enosawa, M., Kageyama, S., Sawai, K., Watanabe, K., Notomi, T., Onoe, S., Mori, Y. & Yokomizo, Y. 2003 Use of loop-mediated isothermal amplification of the IS900 sequence for rapid detection of cultured *Mycobacterium avium* subsp. *paratuberculosis*. *J Clin. Microbiol.* **41**, 4359–4365.

Falkinham, III, J. O. 1996 Epidemiology of infection by nontuberculous mycobacteria. *Clin. Microbiol. Rev.* **9**, 177–215.

Falkinham, III, J. O. 2002 Nontuberculous mycobacteria in the environment. *Clin. Chest Med.* **23**, 529–551.

Falkinham, III, J. O. 2009 Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *J. Appl. Microbiol.* **107**, 356–367.

Field, S. K., Fisher, D. & Cowie, R. L. 2004 *Mycobacterium avium* complex pulmonary disease in patients without HIV infection. *Chest* **126**, 566–581.

Griffith, D. E., Aksamit, T., Brown-Elliott, B. A., Catanzaro, A., Daley, C., Gordin, F., Holland, S. M., Horsburgh, R., Huitt, G., Iademarco, M. F., Iseman, M., Olivier, K., Ruoss, S., von Reyn, C. F., Wallace Jr, R. J. & Winthrop, K. 2007 An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am. J. Respir. Crit. Care Med.* **175**, 367–416.

Huggett, J. F., McHugh, T. D. & Zumla, A. 2003 Tuberculosis: amplification-based clinical diagnostic techniques. *Int. J. Biochem. Cell Biol.* **35**, 1407–1412.

Ichiyama, S., Iinuma, Y., Tawada, Y., Yamori, S., Hasegawa, Y., Shimokata, K. & Nakashima, N. 1996 Evaluation of Gen-Probe amplified *Mycobacterium Tuberculosis* Direct Test and Roche PCR-microwell plate hybridization method (AMPLICOR MYCOBACTERIUM) for direct detection of mycobacteria. *J. Clin. Microbiol.* **34**, 130–133.

Iwamoto, T., Sonobe, T. & Hayashi, K. 2003 Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. *J. Clin. Microbiol.* **41**, 2616–2622.

Kobashi, Y. & Matsushima, T. 2003 The effect of combined therapy according to the guidelines for the treatment of *Mycobacterium avium* complex pulmonary disease. *Intern. Med.* **42**, 670–675.

Matheson, G. D., Giurney, C., Esau, N. & Lehto, R. 2010 Assessing PCR inhibition from humic substances. *Open Enz. Inhibit. J* **3**, 38–45.