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ANDREA SANTOS LIMA

# FATORES E ESPÉCIES DE MICOBACTÉRIAS NÃO TUBERCULOSAS ASSOCIADAS AOS CASOS DE MICOBACTERIOSES PULMONAR E EXTRAPULMONAR NO ESTADO DE PERNAMBUCO.

RECIFE
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Tese apresentada ao curso de Doutorado Acadêmico em Saúde Pública do Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz para obtenção do título de doutora em ciências.

**Orientadores:** Drª Haiana Charifker Schindler

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“Há uma força motriz mais poderosa que
o vapor, a eletricidade e a energia
atômica: a vontade.”
Albert Einstein

LIMA, Andrea Santos. **Fatores e espécies de micobactérias não tuberculosas associadas aos casos de micobacterioses pulmonar e extrapulmonar no estado de Pernambuco**. 2013. Tese (Doutorado Acadêmico em Saúde Pública) - Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, 2013.

## RESUMO

Esta Tese aborda as infecções pulmonares e extrapulmonares, os fatores associados para o adoecimento por micobactérias não tuberculosas (MNT), a diversidade e a frequência das espécies de MNT, além do diagnóstico das micobacterioses no estado de Pernambuco, região nordeste brasileira. Os resultados são apresentados em três artigos científicos que respondem a seus objetivos. O primeiro artigo destaca as lacunas no diagnóstico convencional das micobacterioses e avalia uma PCR Multiplex na diferenciação de espécies de MNT como método auxiliar no diagnóstico diferencial entre as micobacterioses e a tuberculose. O segundo trás o primeiro relato no Brasil de infecção extrapulmonar por *Mycobacterium wolinskyi* diagnosticada após procedimento cirúrgico invasivo em serviço privado do estado. O terceiro artigo descreve o perfil clínico, epidemiológico, laboratorial e fatores associados aos casos de micobacterioses pulmonar por MNT, como também a frequência, diversidade e perfil de resistência aos antimicrobianos das espécies de MNT no estado de Pernambuco que é sabidamente uma região endêmica para tuberculose. Conclui-se que as técnicas de biologia molecular (PCR multiplex, PRA-*hsp65* e sequenciamento de genes específicos) podem ser ferramentas valiosas para identificação das espécies de micobactérias reduzindo o tempo necessário para diagnóstico correto e início do tratamento adequado. Os casos de micobacteriose extrapulmonar, no estado de Pernambuco, foram identificados em mulheres submetidas a procedimentos cirúrgicos invasivos (mamoplastia e abdominoplastia) e as espécies associadas a estes casos foram as micobactérias de crescimento rápido (MCR). Os casos de infecção pulmonar foram a maioria (84%) entre as infecções por MNT no estado de Pernambuco. As principais características encontradas nestes casos foram: Sexo masculino, idade superior a 50 anos e estória de infecção prévia por tuberculose. Neste contexto, faz-se necessário o diagnóstico diferencial entre tuberculose e doença pulmonar por MNT, sobretudo em indivíduos com relato de tuberculose anterior ou que falharam o tratamento para TB.

**Palavras-chaves: Micobactérias não tuberculosas, diagnóstico molecular, fatores de risco, micobacterioses pulmonar e extrapulmonar.**

LIMA, Andrea Santos. **Factors and species of nontuberculous mycobacteria associated with cases of pulmonary and extrapulmonary mycobacteriosis in the state of Pernambuco.** 2013. Thesis (Doctor of Public Health) - Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Recife, 2013.

## ABSTRACT

This work addresses pulmonary and extrapulmonary infections, associated factors to the disease caused by nontuberculous mycobacteria (NTM), the diversity and frequency of NTM species, as well as the diagnosis of mycobacteriosis in the state of Pernambuco Brazilian northeast. The results are presented in three papers that addresses our goals. The first article highlights the gaps in the conventional diagnosis of mycobacteriosis and evaluates a multiplex PCR for differentiation of NTM species as aid in the differential diagnosis between tuberculosis and mycobacteriosis method. The second paper addresses the first report in Brazil of extrapulmonary infection with *Mycobacterium wolinskyi* diagnosed after invasive surgical procedure in a private service of the state. The third article describes the clinical, epidemiological and laboratory factors associated with mycobacteriosis cases pulmonary exerted by NTM, as well as the frequency diversity and antimicrobial resistance profile of NTM species, in the state of Pernambuco, which is known to be an endemic area for tuberculosis. We conclude that molecular biology techniques (PCR multiplex, PRA- hsp65 and sequencing of specific genes) can be valuable tools for the identification of mycobacterial species by reducing the time required for accurate diagnosis and adequate treatment. Cases of extrapulmonary mycobacteriosis in the state of Pernambuco, were identified in women undergoing invasive surgical procedures (breast lift and tummy tuck) and species associated with these cases were rapidly growing mycobacteria (RGM). Cases of pulmonary infection were the majority (84 %) between NTM infections in the state of Pernambuco. The main characteristics found in these cases were: male gender, age over 50 years, and history of previous tuberculosis, this context, it is necessary the differential diagnosis between tuberculosis and NTM lung disease, especially in individuals with a history of tuberculosis or who have failed previous treatment for tuberculosis.

**Keywords:** Nontuberculous mycobacteria, molecular diagnosis, risk factors, pulmonary and extrapulmonary mycobacteriosis.

## LISTA DE ILUSTRAÇÕES

## LISTA DE TABELAS

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| ANVISA | Agência Nacional de Vigilância Sanitária |
| ATS | American Thoracic Society |
| BCG | Bacilo Calmette-Guerin |
| BAAR | Bacilo Álcool-Ácido Resistente |
| CD4 | Cluster of Differentiation 4 |
| CMTB | Complexo *Mycobacterium tuberculosis* |
| CMI | Concentração Mínima Inibitória |
| CLSI | Clinical and Laboratory Standards Institute |
| DNA | Ácido desoxirribonucleico |
| DPOC | Doença pulmonar obstrutiva crônica |
| FC | Fibrose cística |
| EUA | Estados Unidos da América |
| HIV | Vírus da imunodeficiência adquirida |
| IFN- γ | Interferon- γ |
| IL-2 | Interleucina-2 |
| IS*6110* | Insertion sequence *6110* |
| LJ | Löwenstein-Jensen |
| MAC | Complexo *Mycobacterium avium* |
| MCL | Micobactérias de crescimento lento |
| MCR | Micobactérias de crescimento rápido |
| MNT | Micobactérias não tuberculosas |
| NK | Natural killer |
| PNB | Ácido p-nitrobenzóico |
| PCR | Reação em cadeia da polimerase |
| PRA- *hsp65* | Restriction enzyme analysis of the *hsp65gene* |
| *rpoB* | Gene *rpoB* |
| TB | Tuberculose |
| TS | Teste de sensibilidade |
| TNF-α | factor de necrose tumoral-α |

## SUMÁRIO

# 1 INRODUÇÃO

## 1.1 Classificação das micobactérias

Em 1896, Lehmann e Neumann propuseram a criação do gênero *Mycobacterium*, visando à inclusão dos bacilos da tuberculose e da hanseníase, até então classificados como *Bacterium tuberculosis* e *Bacterium leprae*. Este gênero faz parte da família *Mycobacteriaceae* e está posicionado taxonomicamente na subordem *Corynebacterineae*, que pertence à ordem *Actinomycetales*, da subclasse *Actinobacteridae*, da classe e do filo *Actinobacteria*, do domínio *Bacteria.* Fazem parte do gênero *Mycobacterium* as espécies que apresentam no mínimo três características: (i) estrutura de ácidos micólicos, (ii) álcool-ácido resistência dos bacilos e (iii) o conteúdo de guanina e citosina no DNA (ácido desoxirribonucléico) na ordem dos 62-70%, com exceção do *Mycobacterium leprae* que tem cerca de 58% (BARRERA, 2007; LEÃO et al., 2004; RASTOGI et al., 2001).

A partir da década de 1990, com os avanços das técnicas moleculares nos estudos taxonômicos, o número de novas espécies de micobactérias descritas vem aumentando. Atualmente o gênero *Mycobacterium* é composto por 165 espécies e 13 subespécies (TORTOLI, 2006; EUZÉBY, 2013). O gênero *Mycobacterium* é composto pelo complexo *Mycobacterium tuberculosis* (CMTB) *(M. bovis, M. bovis-BCG, M. africanum, M. microti, M. caprae, M. canettii, M. pinnipedii ), M. leprae* e outras espécies denominadas micobactérias não tuberculosas (MNT) ou micobactérias atípicas, estas com diferentes características fenotípicas, genéticas e patogênicas (BRASIL, 2008; TORTOLI, 2003; ZAMARIOLI et al., 2008).

Runyon (1959) propôs uma classificação das MNT em quatro grupos baseados na pigmentação e tempo de crescimento das colônias. As espécies que apresentam crescimento em meio sólido após sete dias são classificadas como micobactérias de crescimento lento (MCL) e aquelas que apresentam crescimento em menos de sete dias, micobactérias de crescimento rápido (MCR). Em relação à produção de pigmentação, essas bactérias podem ser divididas em três grupos: acromógenas, fotocromógenas e escotocromógenas. Estas características associadas (tempo de crescimento e pigmentação) possibilitam a classificação das micobactérias em quatro grupos, chamados grupos de Runyon: I, II, III e IV (Quadro 1). Essa classificação é, ainda, utilizada para identificação das MNT juntamente com outros testes (BRASIL, 2008).

**Quadro 1-** Classificação das micobactérias de acordo com o tempo de crescimento e produção de pigmento.

| **Grupos** | **Pigmentação** | **Tempo de crescimento** |
|---|---|---|
| Grupo I | Fotocromógenas (colônias adquirem pigmentação quando expostas à luz) | Lento |
| Grupo II | Escotocromógenas (colônias produzem pigmentação mesmo quando crescem na ausência de luz) | Lento |
| Grupo III | Acromógenas (não apresentam pigmentação) | Lento |
| Grupo IV | Produtoras ou não de pigmento | Rápido |

Fonte: Runyon (1959).

A classificação atual fundamenta-se ainda em Runyon (1959), baseada, sobretudo em características morfológicas, fisiológicas e bioquímicas das micobactérias, mas enriquecida por evidências antigênicas e informações genômicas, obtidas com técnicas de biologia molecular. Além disso, houve também o desenvolvimento de técnicas quimiotaxonômicas, com importantes resultados relacionados à análise da carga lipídica da parede celular das micobactérias, que inclui moléculas únicas, como os ácidos micólicos (MACEDO et al., 2009; TORTOLI, 2003).

As MNT são também classificadas conforme sua capacidade de causar doença no homem como potencialmente patogênicas e não patogênicas (Quadro 2).

**Quadro 2**- Classificação das espécies de MNT de acordo com a patogenicidade.

| MNT potencialmente patogênicas | MNT raramente patogênicas |
|---|---|
| *M. abscessus* | *M. agri* |
| *M. asiaticum* | *M. aurum* |
| *M. avium* | *M. branderi* |
| *M. avium subsp. Paratuberculosis* | *M. chitae* |
| *M. celatum* | *M. duvalli* |
| *M. chelonae* | *M. fallax* |
| *M. fortuitum* | *M. flavescens* |
| *M. genavense* | *M. gastri* |
| *M. haemophilum* | *M. gordonae* |
| *M. immunogenum* | *M. hassiacum* |
| *M. intracellulare* | *M. mageritense* |
| *M. kansasii* | *M. neoaurum* |
| *M. lentiflavum* | *M. nonchromogenicum* |
| *M. malmoense* | *M. phlei* |
| *M. marinum* | *M. porcinum* |
| *M. mucogenicum* | *M. pulveris* |
| *M. peregrinum* | *M. smegmatis* |
| *M. scrofulaceum* | *M. terrae* |
| *M. shimoidei* | *M. triviale* |
| *M. simiae* | |
| | *M. vaccae* |
| *M. szulgai* | |
| *M. ulcerans* | |
| *M. xenopi* | |

Fonte: Leão et al.( 2004).

## 1.2 Epidemiologia das doenças causadas por micobactérias não tuberculosas

As MNT estão amplamente distribuídas no meio ambiente, tendo sido isoladas na água, incluindo água canalizada, solo, animais, equipamentos cirúrgicos e, inclusive em soluções desinfetantes. A infecção ocorre por inalação, inoculação ou ingestão de material contaminado por micobactérias, podendo causar doenças pulmonares e infecções de feridas cirúrgicas em diferentes tecidos, porém parece não ocorrer à transmissão de pessoa a pessoa (COWMAN et al., 2012; GÓMEZ, 2009).

Ao contrário das espécies do complexo *M. tuberculosis*, estas micobactérias apresentam patogenicidade variável (ZAMARIOLI et al., 2008). A capacidade das MNT em causar doença está claramente documentada na literatura. Os primeiros quadros clínicos foram descritos na década de 50 e por muitos anos foram considerados ocasionais e quase sempre ligados a situações de imunodeficiência.

Nos últimos 20 anos tem se tornado uma infecção mais frequente e estudada, podendo ou não estar relacionada com deficiência do sistema imunológico (GÓMEZ, 2009). Dessa

forma a prevalência da infecção MNT está crescendo e se tornando cada vez mais comum na prática clínica (COWMAN et al., 2012).

Há uma distribuição geográfica variável das espécies de MNT, associadas à doença, nos diferentes continentes (Quadro 3).

**Quadro 3**- Formas clínicas, distribuição geográfica e espécies de micobactérias não tuberculosas.

| **Doença** | **Distribuição geográfica** | **Espécies comuns** | **Espécies incomuns** |
|---|---|---|---|
| Pulmonar | Mundial | *M. abscessus e* MAC | *M. asiaticum* |
| | EUA, Europa, África do Sul | *M. kansasii* | *M. haemophilum* |
| Linfadenite | Mundial | MAC, *M. scrofulaceum* | *M. kansasii, M. szulgai* |
| | Reino Unido e Escandinávia | *M. malmoense* | *M. abscessus* |
| Disseminada | Mundial | MAC | *M. abscessus* |
| | EUA e África do Sul | *M. kansasii* | *M. immunogenum* |
| Cutânea | Mundial | *M. marinum, M. abscessus* | MAC |
| | África, Ásia e Austrália | *M. ulcerans* | *M. szulgai* |

Fonte: Griffith (2007).
Nota: Complexo *Mycobacterium avium* (MAC)

A doença pulmonar crônica é a manifestação clínica mais comum das MNT (FALKINHAM, 1996; O'BRIEN et al., 1987 ; WOLINSKY, 1979). As espécies de MNT que mais causam doença pulmonar nos Estados Unidos são as do Complexo *Mycobacterium avium* (MAC), seguidas *pelo M. chelonae/abscessus, M. fortuitum e M. kansassi* (PREVOTS, 2010). No Reino Unido os principais patógenos são os do *Complexo M. avium* (MAC), *M. kansasii, Mycobacterium malmoense e Mycobacterium xenopi* (COWMAN et al., 2012; HENRY et al., 2004). Quando se trata de doença extrapulmonar, as infecções de pele e tecidos subcutâneos são comumente causadas por *M. fortuitum*, *M. abscessus*, *M. chelonae*, *M. marinum* e *M. ulcerans* (GRIFFITH, 2007).

No Brasil, a importância das MNT na patologia humana foi reconhecida na década de 30 com a identificação do primeiro caso de infecção por *M. fortuitum* no Rio de Janeiro (COSTA, 1938) e a partir daí, sua relevância clínica foi sendo considerada (ANDRADE et al, 1976; AZULAY et al., 1974; CARRIJO et al., 1973; CRUZ et al., 1967; MAGALHÃES, 1965; TEIXEIRA et al., 1970; ZANON et al., 1966). Atualmente, mesmo com a alta prevalência de tuberculose, o estudo das MNT vem despertando interesse, sobretudo devido ao aumento na detecção de casos de micobacterioses em indivíduos com HIV ou que se submeteram a procedimentos cirúrgicos. Por não serem transmissíveis, as doenças causadas

pelas MNT, não são de notificação obrigatória, a não ser em casos de infecção após procedimentos cirúrgicos realizados em serviços de saúde, levando a falta de registros oficiais para que se possa estimar sua prevalência (BRASIL, 2008; UEKI et al., 2005).

Nas diferentes regiões brasileiras também observamos uma diversidade de espécies de MNT associadas a casos de doença pulmonar e extrapulmonar, que ocorre, possivelmente, devido a condições ambientais que podem favorecer seu predomínio. Diferentes estudos epidemiológicos vêm demonstrando um aumento na frequência de isolamentos de MNT em amostras clínicas pulmonares e extrapulmonares, além de um número maior de pesquisas relacionadas com a identificação de espécies de MNT e de casos confirmados da doença (COSTA et al., 2012; MARTÍN-CASABONA, 2004; PASQUALOTTO, 2003 PEDRO, 2008; UEKI et al., 2005; ZAMAROLI, 2008).

Em estudo realizado por Barreto e Campos (2000) utilizando 590 isolados de micobactérias obtidas de diferentes regiões brasileiras durante o período de 1994 a 1999 foi observada uma preponderância de *M avium-intracellulare* (44,4%), seguido de *M. kansasii* (13,7%) e *M. fortuitum* (10,8%). Em relação aos casos confirmados de micobacterioses por região brasileira foi demonstrad**o** a maior participação das regiões Sudeste e Sul, com 57,6% do total de casos no país, sendo 17,86%,16,4% e 8,12%, no Centro Oeste, Nordeste e Norte, respectivamente. Dentre os 431 pacientes estudados, 106 foram considerados casos de micobacteriose, sendo a forma mais frequente a pulmonar (60,3%). Em São Paulo, *M. kansasii* e *complexo M. avium* foram as espécies mais frequentemente associadas à forma pulmonar da doença, já nos casos extrapulmonar e disseminado foram respectivamente *M. marinum* e complexo *M. avium* (UEKI, 2005).

Em estudo desenvolvido na Bahia por Matos et al. (2004) com pacientes em tratamento para tuberculose multirresistente, no período de 1998 a 2003, foram isoladas micobactérias não tuberculosas em espécimes biológicos de 19 (8,2%) dos 231 pacientes estudados. Sendo identificados os complexos *M. chelonae/M. abscessus* (58%), *M. avium/intracelulare* (16%) e *M. fortuitum* (11%). No Pará, Costa et al. (2012) descreveram que as espécies mais isoladas nos casos pulmonares foram *M. massiliense, M. simiae complex, M. intracellulare e M. avium*, no período de 1999 a 2010, e a maioria destes casos havia feito tratamento anterior para tuberculose.

A Agência Nacional de Vigilância Sanitária (2011) publicou o número de casos de infecções por micobactérias de crescimento rápido (MCR) obtido nos últimos onze anos no Brasil, demonstrando a ocorrência desta doença em indivíduos submetidos a procedimentos invasivos, em sua maioria cirurgias guiadas por vídeo, cujos instrumentos foram submetidos à

esterilização de alto nível em solução de glutaraldeído. De 1998 a 2009, foram notificados 2520 casos de infecções pós-cirúrgicas devido à MCR incluindo *M. fortuitum, M. chelonae, M. abscessus e M. massiliense,* distribuídas predominantemente em hospitais privados do país. Os casos notificados ocorreram em 23 estados, dez desses concentrando 97,8% dos casos (AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA, 2011) (Tabela 1).

**Tabela 1-** Distribuição dos casos notificados de infecção por MCR associadas a procedimentos invasivos, segundo o estado de notificação. Brasil, 1998-2009

| Estado de notificação | nº casos | % | % Acumulado |
|---|---|---|---|
| RJ | 1.107 | 43,9 | 43,9 |
| ES | 363 | 14,4 | 58,3 |
| PA | 327 | 13,0 | 71,3 |
| SP | 193 | 7,7 | 79,0 |
| PR | 149 | 5,9 | 84,9 |
| RS | 115 | 4,6 | 89,5 |
| GO | 95 | 3,8 | 93,3 |
| MT | 50 | 2,0 | 95,3 |
| DF | 33 | 1,3 | 96,6 |
| MG | 31 | 1,2 | 97,8 |
| PI | 11 | 0,4 | 98,2 |
| BA | 11 | 0,4 | 98,6 |
| CE | 9 | 0,4 | 99,0 |
| SC | 6 | 0,2 | 99,2 |
| SE | 5 | 0,2 | 99,4 |
| PE | 5 | 0,2 | 99,6 |
| AL | 3 | 0,1 | 99,7 |
| TO | 2 | 0,1 | 99,8 |
| RR | 1 | 0,0 | 99,8 |
| RN | 1 | 0,0 | 99,8 |
| PB | 1 | 0,0 | 99,8 |
| AM | 1 | 0,0 | 99,8 |
| Total | 2.520 | | 100 |

Fonte: Agência Nacional de Vigilância Sanitária (2011).

Verifica-se que as MCR mais incidentes nos casos de infecções pós-cirúrgicas foram as espécies *M. abscessus* (31,3%), *M. abscessus* subspécie *bolletii* (30,4%); *M. fortuitum* (13,8%) e *M. chelonae* (1,5%). Outras espécies corresponderam a 2,7% dos casos (AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA, 2011).

Diferentes estudos revelam que na maioria das infecções hospitalares causadas por MCR houve falha na esterilização dos instrumentos cirúrgicos ou dispositivos médicos (COSTA, 1938; DE GROOTE, 2006; WALLACE et al., 1998). O crescente número de casos notificados de infecções pós-cirúrgicas, sobretudo mamoplastia e procedimentos vídeo assistidos pode ser devido, ou pelo menos em parte, a tolerância ao glutaraldeído ou a baixa suscetibilidade aos saneantes de alto nível entre algumas espécies de MCR. (BRICKMAN et

al., 2005; CARDOSO et al., 2008; MANZOOR et al., 1999; NOMURA et al., 2006; PADOVEZE et al., 2007; RAHAV et al., 2006; VAN KLINGEREN et al., 1993; VIANA-NIERO et al., 2008; VIJAYARAGHAVAN et al., 2006; VINH et al., 2006).

### 1.3 Formas clínicas da doença

No homem, foram descritas diversas formas de doença causada por MNT, acometendo pulmões, gânglios e pele, como também a forma disseminada (GRIFFITH, 2007).

A doença pulmonar por MNT geralmente ocorre em pacientes com doença pulmonar crônica como pneumoconiose, doença pulmonar obstrutiva crônica, tuberculose pré-existente, bronquite crônica, bronquectasia e doença esofágica associada à aspiração crônica de material alimentar pelas vias aéreas. A avaliação clínica é frequentemente complicada devido à similaridade da sintomatologia com as doenças pulmonares pré-existentes. Os sinais e sintomas das doenças causadas pelas MNT são variáveis e inespecíficos. Na maioria das vezes, a sintomatologia clínica se assemelha à evolução crônica da tuberculose (GRIFFITH, 2007; HADAD et al., 2005). Os sinais e sintomas do comprometimento pulmonar são inespecíficos, incluindo tosse crônica, expectoração e fadiga. Nas formas mais avançadas da doença o mal-estar, dispneia, febre, hemoptise e perda de peso podem surgir (CAMPOS, 2000; GRIFFITH, 2007; HADAD et al., 2005). As imagens radiológicas podem demonstrar imagens fibrocavitárias (semelhante a TB) ou caracterizadas por nódulos e bronquiectasias (doença nodular/bronquiectasia). Em comparação com os resultados radiográficos na tuberculose, pacientes com micobacteriose pulmonar usualmente tem as seguintes características: cavidades de paredes finas e menos infiltrado parenquimatoso em volta, a disseminação é mais por contiguidade do que broncogênica e um envolvimento marcante da pleura nas áreas pulmonares envolvidas. (CAMPOS, 2000; EVANS et al., 1996; WALLACE et al., 1997; WOLINSKY, 1979). Nenhuma dessas diferenças nos achados radiológicos, no entanto, é suficientemente específica para excluir o diagnóstico de tuberculose (GRIFFITH, 2007).

As doenças causadas por MNT na pele ou tecidos moles, geralmente apresentam sinais e sintomas de inflamação como dor, aumento de temperatura, eritema, nódulos e ou abscessos, podendo evoluir com drenagem de secreção, fístulas ou deiscências de suturas. O período de incubação pode variar de uma semana a dois anos (HADAD et al., 2005). As lesões dermatológicas após perfuração e trauma, comumente, são causadas por MCR como, *M. fortuitum, M. abscessus, ou M. chelonae* (DUARTE et al., 2009; WALLACE et al., 1983).

As MCR também são as espécies frequentemente encontradas nas infecções nosocomiais de pele e tecidos moles, incluíndo infecções por via intravenosa, catéteres peritoneal, abscessos pós injeção, infecção após lipoaspiração, mamoplastias, cirurgias cardíacas ou oftamológicas (CARDOSO et al., 2008; CHANDRA et al., 2001; DE GROOTE, 2006; NOMURA et al., 2006; PADOVEZE et al., 2007; RAHAV et al., 2006; VIANA-NIERO et al., 2008; VIJAYARAGHAVAN et al., 2006; VINH et al., 2006).

A linfadenite submandibular, submaxilar, cervical ou pré–auricular em crianças de 1 a 5 anos é a apresentação clínica mais comum nas formas ganglionares causadas por MNT. (CAMPOS, 2000; GRIFFITH, 2007; HORSBURGH; SLIK, 1889). Os linfonodos frequentemente mais atingidos são os do pescoço, principalmente os submandibulares. Os linfonodos inguinais, axilares e epitrocleares são acometidos mais raramente, sendo geralmente unilaterais (fato que também ocorre quando há acometimento de gânglios cervicais) (HADAD et al., 2005) . A doença ocorre de forma insidiosa, e é raramente associada com sintomas sistêmicos. É importante a realização do diagnóstico diferencial entre a linfadenopatia causada pelo *M. tuberculosis* ou por complicações da vacina BCG intradérmica e por outras MNT. O diagnóstico definitivo requer cultura com identificação da micobactéria. O teste tuberculínico negativo e radiografia de tórax normal podem sugerir infecção por MNT (GRIFFITH, 2007).

A doença disseminada devido a MNT está entre as infecções mais comuns e graves em pessoas com HIV que estejam em estágio avançado de imunossupressão e mais raramente têm sido relatada em pacientes imunodeprimidos com insuficiência renal crônica, transplantes cardíaco, uso de corticoide crônico e leucemias (GRIFFITH, 2007). A apresentação clínica em pacientes com infecção avançada por HIV pode ser confundida com uma série de outras infecções. Reclamações clássicas em pessoas com doença disseminada causada por MAC são febre, suores noturnos, e perda de peso, além de dor abdominal e diarréia (NIGHTINGALE et al., 1992). As alterações laboratoriais, nestes casos, podem incluir anemia grave, com um hematócrito inferior a 25%, fosfatase alcalina e lactato desidrogenase elevados (GORDIN et al., 1997; HORSBURGH et al., 2001).

## 1.4 Patogênese

As MNT podem ser inaladas pelo trato respiratório a partir da água, poeira ou outros aerossóis e a maior parte das vezes são retidas pelos cílios nasais ou pela tosse e não causam doença. Caso consigam atingir o espaço alveolar, os bacilos infecciosos são fagocitados pelos

macrófagos alveolares, mas impedem a fusão do fagossoma com o lisossoma. Deste modo, não se gera um ambiente hostil de pH ácido, permitindo-lhes sobreviver e replicar (MCGARVEY, 2002).

Em hospedeiros imunocompetentes, os linfócitos CD4 e células natural killer (NK) podem interagir com as células mononucleares infectadas e matar os microorganismos. O papel dos neutrófilos e anticorpos é controverso e parece não ser substancial (MCGARVEY, 2002).

Ao tentar explicar alguns fatores do indivíduo que podem levar ao adoecimento por MNT, ao longo das ultimas décadas, três observações foram feitas em relação à patogênese destas infecções: nos casos de doenças disseminadas por MNT os pacientes com HIV e níveis linfócitos T CD4 inferiores a 50/l são os mais susceptíveis, reafirmando a participação destas células na resistência as micobactérias (AMERICAN THORACIC SOCIETY, 1987; HORSBURGH, 1996). Nos indivíduos soro negativos para o vírus HIV as infecções por MNT disseminadas estão associadas a síndromes genéticas como: mutações específicas nos genes que codificam o interferon- **γ** (IFN- γ) e interleucina (IL-12) (CASANOVA; ABEL, 2002; DORMAN; HOLLAND, 2000), no receptor 1 do IFN-γ (IFN-γ R1), receptor 2 do IFN-γ (IFN- γ R2), receptor da subunidade β 1 da IL-12 (IL12R β 1), na subunidade p40 da IL-12 ( IL12p40), no transdutor de sinal e ativador da transcrição (STAT1), e no fator nuclear-κβ modulador essencial (NEMO) (GRIFFTH, 2007). Para o combate das micobactérias o indivíduo requer uma eficaz resposta imune mediada por células Th1 (HILL, 1998) mediada pelo IFN- **γ** e conduzido por IL-12 e factor de necrose tumoral (TNF)-α (OTTENHOFF, 2005). Diferentes estudos demostram que a doença pulmonar por MNT ocorre com maior frequencia nos indivíduos com problemas estruturais no pulmão como a doença pulmonar obstrutiva crônica (DPOC), bronquiectasia, fibrose cística (FC), pneumoconiose, TB prévia, proteinose alveolar pulmonar e distúrbios da motilidade esofágica (GRIFFITH et al., 1993; OLIVIER et al., 2003a, 2003b). As mulheres sem fatores predisponentes claramente reconhecidos também podem sofrer com doença pulmonar por MNT (JARZEMBOWSKI ; YOUNG, 2008 ; PRINCE et al, 1998; WALLACE et al., 1998b).

Além da inalação de aerossóis, outra forma de transmissão das MNT é a inoculação pós-traumática. Com isso, pode ocorrer infecção por quebra da barreira corneana, cutânea ou mucosa, decorrente de procedimentos médicos para fins terapêuticos (cirurgias laparoscópicas e endoscopia, por exemplo) ou para fins estéticos (cirurgias plásticas e implantes). Este tipo de infecção acomete a pele e outros tecidos moles de indivíduos sem imunossupressão e está normalmente associado às MCR (COOK, 2010; DUARTE et al., 2009; GENTRY, 2005).

## 1.5 Diagnóstico laboratorial das micobacterioses

O diagnóstico diferencial entre a tuberculose e as micobacterioses é importante porque as duas doenças apresentam diferenças na epidemiologia, prognóstico e tratamento. Pelo fato de clinicamente serem semelhantes apenas o diagnóstico bacteriológico é confirmatório. Para isso, se faz necessária a participação de um laboratório especializado com infraestrutura adequada para o isolamento e identificação das espécies (BRASIL, 2005a). As micobacterioses exigem muita cautela para seu diagnóstico, pois o isolamento de MNT a partir de espécimes clínicos não estéreis pode significar colonização transitória ou contaminação. A *American Thoracic Society* recomenda que o diagnóstico das micobacterioses seja feito com base em uma série de critérios bacteriológicos, clínicos e radiológicos (BRASIL, 2005b; BRUNELLO et al., 2001; GRIFFITH, 2007).

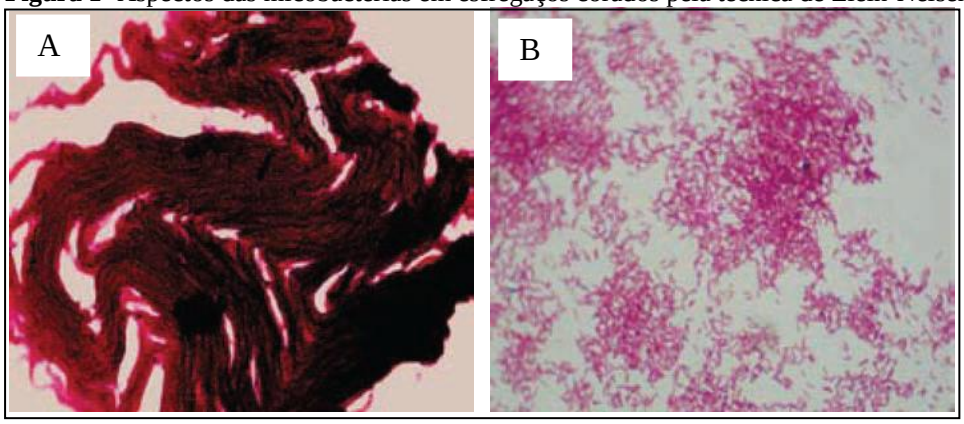
A baciloscopia é um exame básico para a pesquisa das micobactérias em espécimes biológicos e que consiste na pesquisa direta de Bacilo Álcool-Ácido Resistente (BAAR) em um esfregaço de amostra clínica preparado e corado com metodologia padronizada (BRASIL, 2001). É uma metodologia de execução rápida, fácil, de baixo custo e que permite estimar o número de bacilos presentes na amostra, porém possui sensibilidade limitada, sendo necessários entre 5000 a 10000 bacilos por mililitros, para se obter um resultado positivo (ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2007). Além de não ser capaz de diferenciar morfologicamente os bacilos ácido-álcool-resistentes, podendo levar a um diagnóstico equivocado das doenças causadas pelas MNT, sendo muitas vezes o paciente notificado erroneamente como portador de tuberculose (GRIFFITH, 2007).

A cultura é o exame laboratorial que permite a multiplicação e o isolamento de BAAR a partir da semeadura da amostra clínica, sendo um método sensível e específico para as doenças causadas por micobactérias (BRASIL, 2008). O limite de detecção de bacilos é de 10 a 100 bacilos cultiváveis por milímetros da amostra (ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2004; RIEDER, 2007). Além disso, permite a posterior identificação da espécie de micobactéria isolada e o teste de sensibilidade aos antimicrobianos. Como o tempo de crescimento bacilar varia de duas a seis semanas, a cultura apresenta como desvantagem o tempo necessário para a liberação do resultado (BRASIL, 2008).

Os testes fenotípicos e bioquímicos para identificação das espécies de micobactérias são realizados a partir de culturas puras e para obtenção dos resultados são necessários de três a seis semanas. A diferenciação dos bacilos do CMTB e as MNT tradicionalmente iniciam-se com a análise microscópica da colônia isolada em meio de cultura para avaliar a formação de corda.

As espécies do CMTB apresentam a formação de aglomerados lineares com aspecto de corda, que podem ser observados em esfregaços corados pelo método de Ziehl-Neelsen. Enquanto que a maioria das MNT não forma corda, exceto algumas espécies como, *M. kansassi*, *M. fortuitum* e *M. chelonae* (BRASIL, 2008) (Figura 1).

**Figura 1**- Aspectos das micobactérias em esfregaços corados pela técnica de Zielh-Nelsen.




Fonte: Brasil (2008).
A- Complexo *M. tuberculosis*, B- Micobactérias não tuberculosas.

A análise macroscópica da cultura é feita na cultura original em meio sólido. As colônias de micobactérias podem apresentar diferentes morfologias e pigmentação. As colônias do CMTB são acromógenas, geralmente de cor creme e rugosas; já as colônias das MNT são pigmentadas ou acromógenas, lisas ou rugosas. O teste de inibição de crescimento em meio com ácido p-nitrobenzóico (PNB) separa os membros do CMTB, pois ao contrário da maioria das MNT, todas as espécies do CMTB não crescem neste meio. Algumas espécies de MNT como *M. kansasii*, *M. gastri*, *M. xenopi* podem eventualmente não crescer no meio contendo PNB. O teste da niacina baseia-se na detecção visual da produção de niacina pelas bactérias. Embora seja produzida por todas as micobactérias, somente algumas espécies do CMTB, como *M. tuberculosis* e *M. africanum* e raras espécies de MNT (*M. simiae, M. chelonae* e *M. marinum*) produzem quantidades detectáveis por meio deste teste (BRASIL, 2008). Por isso estes testes podem auxiliar na diferenciação entre o CMTB e as MNT.

A identificação das espécies que constituem o grupo das MNT pode ser feita pelos testes de produção de pigmento, crescimento em meio de cultura a 45°C e a 25°C, determinação do tempo de crescimento em Löwenstein-Jensen (LJ), meio Sauton com ácido pícrico e em ágar comum, inibição de crescimento em meio com NaCl 5%, arilsulfatase, hidrólise do tween 20, β-galactosidase, redução do telurito de potássio, inibição do crescimento em meio contendo PNB, redução do nitrato, urease e pirazinamidase. Ainda, para

MCR, são realizados os testes de captação do ferro, inibição de crescimento em meio contendo citrato de sódio, manitol e inositol (BRASIL, 2008). As principais limitações do método fenotípico consistem na demora na obtenção de resultados e a dificuldade de diferenciar diversas espécies, além de resultados duvidosos que podem ser apresentados pelos testes bioquímicos (BRASIL, 2008; NEONAKIS et al., 2008; NGAN et al., 2011). No Brasil, os laboratórios que realizam cultura para micobactérias devem ser capazes de separar espécies do CMTB das MNT, ou então deverão encaminhar a cultura a um laboratório de referência que possua condições técnicas para fazê-lo (BRASIL, 2008). Os isolados de micobactérias do estado de Pernambuco são encaminhados para o Laboratório de Referência Nacional, Centro de Referência Professor Hélio Fraga, no Rio de Janeiro, que utiliza métodos moleculares para identificação das espécies de micobactérias não tuberculosas.

Os métodos moleculares têm proporcionado melhoria considerável na velocidade de identificação das MNT devido ao maior poder discriminatório e precisão destas ferramentas. Além do que possibilita a detecção de novas espécies de MNT, que não foram fenotipicamente caracterizadas (IOANNIS et al., 2008, PIERSIMON; SCARPARO, 2008; UEKI et al., 2005).

Dentre as técnicas moleculares o sequenciamento dos genes específicos *rpoB e hsp65* tornou-se o método padrão ouro para a identificação de micobactérias (CHEMLAL; PORTAELS, 2003; HERRERA et al., 2009). Devido ao seu elevado poder discriminatório, esta ferramenta vem sendo utilizada por vários grupos em estudos epidemiológicos, na identificação de espécies envolvidas em doenças humanas, em casos de surtos ou na reclassificação taxonômica (COSTA et al., 2008, DUARTE et al., 2009).

Um dos alvos moleculares mais comumente utilizados é o elemento de inserção IS*6110* (GARCIA - QUINTANILLA et al., 2002; KOX et al., 1994; SPRINGER et al., 1996; TORTOLÌ, 2003). O IS*6110* é encontrado apenas nas espécies do complexo *M. tuberculosis*, sendo usado para diferenciá-lo das MNT. A principal vantagem do IS6110 é que está presente em múltiplas cópias no genoma do *M. tuberculosis* (KURABACHEW et al., 2004). O gene *dna*J codifica uma proteína de estresse e é altamente conservado entre as bactérias. Os membros da família *Mycobacteriaceae* possuem o *dna*J e esta sequência demonstrou ser útil na identificação destas espécies (MORITA et. al, 2004; PAO et al., 1990 TAKEWAKI et al., 1993). Outro gene de interesse que vem sendo estudado por diferentes grupos de pesquisa é o *hsp*65, comum a todas as bactérias do gênero *Mycobacterium* que codifica o antígeno de 65kDa sendo bastante utilizado na diferenciação de espécies de MNT (IOANNIS et al., 2008, MUN et al., 2007; NEONAKIS, 2008; RINGUET et al., 1999; TELENTI et al., 1993). O

gene *rpoB* está presente em todas as bactérias e devido a presença de regiões de hipervariabilidade vem sendo utilizado na detecção de resistência a rifampicina para cepas do complexo *M. tuberculosis* e diferenciação de micobactérias (ADEKAMBI et al., 2006; DUARTE et al., 2009).

No Brasil, a PCR seguida de análise de restrição (PCR PRA-*hsp65*), proposta por Telenti et al.(1993) e Devallois et al.(1997), têm possibilitado também a identificação rápida de diversas espécies de micobactérias apresentando boa correlação com os resultados de identificação fenotípica e bioquímica no reconhecimento de espécies não caracterizadas anteriormente através de métodos convencionais (CHIMARA et al., 2008; SILVA et al., 2001, 2002). Este método apresenta como principais vantagens sua alta especificidade, a obtenção rápida do resultado e o fato de requerer somente equipamentos básicos de PCR e de eletroforese em gel de agarose. Entretanto, não é capaz de distinguir as espécies do CMTB, algumas espécies de MNT apresentam perfil compartilhado por mais de uma espécie e, ainda, é possível verificar que uma mesma espécie pode apresentar mais de um perfil de restrição. Pode-se encontrar, também, perfis que ainda não foram descritos na literatura (BRASIL, 2008; CHIMARA et al., 2008).

### 1.6 Teste de sensibilidade a antimicrobiano e tratamento das micobacterioses

O teste de sensibilidade (TS) é o exame laboratorial realizado para detectar a resistência/ sensibilidade das micobactérias aos antimicrobianos (BRASIL, 2008). De acordo com as recomendações do CLSI (2011), o método mais aceito é o que determina a Concentração Mínima Inibitória (CMI), que é definida como a menor concentração da droga capaz de impedir o crescimento microbiano e está validado para ser realizado em algumas espécies de MNT (CLINICAL AND SUSCEPTIBILITY LABORATORY STANDARS INSTITUTE, 2011).

A identificação das espécies de MNT exerce papel fundamental no esquema terapêutico do paciente, pois fornece a primeira indicação em relação à suscetibilidade antimicrobiana (BROWN-ELLIOTT; WALLACE, 2002; WILSON et al., 2001). O tratamento para estes agentes infecciosos deve ser individualizado e levar em consideração os estudos de sensibilidade a drogas das cepas causadoras da doença. Embora algumas espécies possam ter um padrão de sensibilidade comum, existem as diferenças de susceptibilidade a drogas intra-espécies (ALCAIDE; ESTEBAN, 2010). As MNT são, muitas vezes, resistentes aos antibióticos, mas em testes de sensibilidade *in vitro* correlaciona-se mal com o resultado

do tratamento, com exceção da resistência a macrolídeos que confere um pior prognóstico (RESEARCH COMMITTEE OF THE BRITISH THORACIC SOCIETY, 2001; TANAKA et al., 1999 ).

Não há um consenso sobre o tratamento mais adequado para as MNT, podendo diferir em função da espécie envolvida, visto que a sensibilidade *in vitro* às drogas e a eficácia clínica são diferentes (GRIFFITH et al., 2007; JENKINS et al., 2008 ).

As reações adversas são comuns devido ao grande número de antibióticos utilizados no tratamento (JENKINS et al., 2008). Caso seja possível, regimes mais simples são recomendados como a administração de antimicrobianos três vezes por semana na tentativa de minimizar estes efeitos (COWMAN et al., 2012).

Alguns MNT (exemplo: *Mycobacterium abscessus*) são intrisicamente resistentes a diversos fármacos, e a cura pode não ser possível. Procedimentos cirúrgicos podem ser considerados em pacientes que apresentem resistência a antimicrobianos ou aqueles que não respondem ao tratamento. Em pacientes debilitados, com múltiplas co-morbidades, uma abordagem mais conservadora pode ser apropriada (COWMAN et al., 2012)

O tratamento das infecções por MNT necessita de longos períodos de multidrogaterapia, com ou sem intervenção cirúrgica (BRASIL, 2008; GRIFFITH; AKSAMIT, 2012).

## 2 JUSTIFICATIVA

O estudo e monitoramento da dinâmica e magnitude das micobacterioses em diferentes regiões no país são de grande importância em saúde pública visto que nas últimas décadas diversas espécies de MNT vêm sendo relacionadas como causadoras de doença. Nos países endêmicos para TB há uma carência de estudos regionais para avaliar a verdadeira prevalência das infecções por MNT. No Nordeste do Brasil não há um serviço de referência na identificação e caracterização de MNT tornando ainda mais árduo e prolongado o diagnóstico desta patologia, além da carência de informações epidemiológicas, tanto no que diz respeito às espécies envolvidas quanto ao número de casos e formas clínicas.

Apesar da detecção das micobactérias na fase inicial da doença ser de vital importância para o tratamento precoce e efetivo, os exames convencionais existentes deixam lacunas no que se refere à determinação de infecções por *Mycobacterium spp*.

Diante da escassez de dados epidemiológicos e dos problemas existentes no diagnóstico das micobacterioses, sobretudo no Nordeste do Brasil, esta pesquisa pretende determinar quais fatores podem estar associados aos casos de micobacterioses pulmonar e extrapulmonar no estado de Pernambuco, além de elucidar quais as principais espécies de micobactérias estão envolvidas com esta patologia, através de um diagnóstico mais preciso que possa auxiliar no manejo clínico e terapêutico do paciente, além de contribuir para as medidas de controle da doença.

## 3 PERGUNTA CONDUTORA

Quais fatores podem estar associados com os casos de infecção por micobactérias não tuberculosas no estado de Pernambuco?

## 4 HIPÓTESE

Existem fatores associados com os casos de micobatérias não tuberculosas no estado de Pernambuco.

# 5 OBJETIVOS

## 5.1 Objetivo Geral

Identificar fatores associados à ocorrência de micobacterioses pulmonar e extrapulmonar, além das espécies de *Mycobacterium spp* e o padrão de resistência aos principais antimicrobianos utilizados.

## 5.2 Objetivos Específicos

a) Identificar as cepas de *Mycobacterium spp*. através dos métodos fenotípicos, bioquímicos e técnicas de biologia molecular (sequenciamento de genes específicos e multiplex PCR)**;**
b) Caracterizar a amostra quanto ao perfil clínico, epidemiológico, laboratorial, hábitos de vida e tipo de serviço de saúde;
c) Identificar fatores associados com a ocorrência de micobacterioses pulmonar e extrapulmonar;
d) Descrever o padrão de resistência das cepas de *Mycobacterium spp*. aos principais antimicrobianos.

# 6 PERCURSO METODOLÓGICO

A pesquisa trata-se de um corte transversal do tipo seccional de natureza descritiva. A apresentação dos resultados foi feita em três publicações que respondem aos seus objetivos e compõem a Tese de Doutorado no formato coletânea de artigos científicos.

As publicações abordam o tema epidemiologia e diagnóstico das doenças causadas por micobactérias não tuberculosas, no que se refere especificamente ao diagnóstico diferencial entre a micobacterioses pulmonar e extrapulmonar e a tuberculose, os fatores associados ao adoecimento pelas MNT, além da diversidade, frequência e perfil de resistência aos antimicrobianos das espécies de MNT associadas aos casos de doença no estado de Pernambuco, Brasil.

## 7 ARTIGOS CIENTÍFICOS

Nessa seção são apresentados os artigos científicos contendo os resultados da pesquisa. Nelas são apontadas questões que procuram, a partir dos pressupostos do estudo, dar conta do tema da tese, respondendo as perguntas condutoras e a seus objetivos.

## 7.1 Artigo 1- Rapid detection and differentiation of mycobacterial species using a multiplex PCR system

Este primeiro artigo destaca as lacunas no diagnóstico convencional das micobacterioses e a avaliação de uma PCR Multiplex para diferenciação de espécies de MNT como método auxiliar no diagnóstico diferencial entre as micobacterioses e a tuberculose.

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Major Article

# Rapid detection and differentiation of mycobacterial species using a multiplex PCR system

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## ABSTRACT

**Introduction**: The early diagnosis of mycobacterial infections is a critical step for initiating treatment and curing the patient. Molecular analytical methods have led to considerable improvements in the speed and accuracy of mycobacteria detection. **Methods:** The purpose of this study was to evaluate a multiplex polymerase chain reaction system using mycobacterial strains as an auxiliary tool in the differential diagnosis of tuberculosis and diseases caused by nontuberculous mycobacteria (NTM) **Results:** Forty mycobacterial strains isolated from pulmonary and extrapulmonary origin specimens from 37 patients diagnosed with tuberculosis were processed. Using phenotypic and biochemical characteristics of the 40 mycobacteria isolated in LJ medium, 57.5% (n=23) were characterized as the *Mycobacterium tuberculosis* complex (MTBC) and 20% (n=8) as nontuberculous mycobacteria (NTM), with 22.5% (n=9) of the results being inconclusive. When the results of the phenotypic and biochemical tests in 30 strains of mycobacteria were compared with the results of the multiplex PCR, there was 100% concordance in the identification of the MTBC and NTM species, respectively. A total of 32.5% (n=13) of the samples in multiplex PCR exhibited a molecular pattern consistent with NTM, thus disagreeing with the final diagnosis from the attending physician. **Conclusions**: Multiplex PCR can be used as a differential method for determining TB infections caused by NTM a valuable tool in reducing the time necessary to make clinical diagnoses and begin treatment. It is also useful for identifying species that were previously not identifiable using conventional biochemical and phenotypic techniques.

**Keywords:** *Mycobacterium tuberculosis* complex. Nontuberculous mycobacteria. Diagnosis. Polymerase chain reaction. Multiplex.

## INTRODUCTION

The ability of nontuberculous mycobacteria (NTM) to cause disease is clearly described in the literature, and its importance is increasing progressively, with an increasing amount of various species being cultured and isolated in laboratories[1-3].

In Brazil, the NTM species most frequently associated with lung disease are *Mycobacterium kasasii* and *Mycobacterium avium*. Other species, such as *Mycobacterium xenopi, Mycobacterium malmoense, Mycobacterium lentiflavum, Mycobacterium abscessus* and *Mycobacterium szulgai*, have occasionally been isolated in cultures[4,5]. In 2011 the National Agency for Sanitary Surveillance (ANVISA) published the number of cases of infections by rapidly growing mycobacteria (RGMs) obtained in the last eleven years in Brazil.

Data also suggest that this disease occurs in subjects undergoing invasive procedures, mostly surgical cases guided by video, where the instruments are subjected to high levels of sterilization in glutaraldehyde solution. From 1998 to 2009, there were 2,520 reported cases of post-surgical infections related to rapidly growing mycobacteria, including *Mycobacterium fortuitum, Mycobacterium chelonae, M. abscessus* and *Mycobacterium massiliense*, which were distributed predominantly in private hospitals throughout the country. Cases have been reported in 23 states of Brazil, with 97.8% of the cases concentrated within 10 states[6]. The diseases caused by NTMs are not compulsorily notifiable, except where the infection occurred during surgical procedures; however, several studies in Brazil have noted isolated strains of nontuberculous mycobacteria in pulmonary and extrapulmonary clinical samples from patients with suspected tuberculosis[4,7,8].

The differential diagnosis between tuberculosis (TB) and diseases caused by NTMs is of great importance because the epidemiology, treatment and prognosis are different. Conventional diagnostics present numerous difficulties as clinical symptoms are generally similar and non-specific smear testing has limited sensitivity and cannot differentiate the species of mycobacteria.

For many years, the identification of mycobacteria was undertaken by gathering phenotypic results and biochemical test results of isolated species in culture medium[9,10]. In the last 20 or 30 years, with the increasing need to identify a greater number of species and also for early diagnosis, new methods

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have been developed to enable rapid and accurate identification of mycobacterial species[10-12]. In Brazil, the PRA-hsp65 technique proposed by Telenti et al.[13], and Devallois et al.[14] has enabled the identification of several species of NTM, the results of which correlated well with those from biochemical identification[13-17]. Beyond the molecular techniques, the sequencing of the specific genes *rpoB* and *hsp65* has become the gold standard for identifying mycobacteria[18,19]. Because of its high discriminatory power, this tool has been used by several groups in epidemiological studies to identify the species involved in human diseases and in outbreaks, and also for taxonomic reclassification[20,21].

Multiplex polymerase chain reaction (PCR) is a fast tool that allows the simultaneous amplification of more than one sequence of target deoxyribonucleic acid (DNA) in a single reaction, saving time and reagents[22-24]. This system, which amplifies two or three different targets, can differentiate *M. tuberculosis* from the NTMs[25,26]. This molecular approach is used in identifying and differentiating microbes because it is able to supply a simple fingerprint of certain bacterial groups when compared to the standard profiles of referent strains. The correct choice of target sequences in the genome is one of the key criteria for detecting and identifying mycobacteria by PCR[27].

Faced with the need to differentiate tuberculosis from other mycobacteria, the aim of this study was to evaluate a system based on multiplex PCR, already optimized by Poroca et al.[26], for identifying mycobacterial species isolated from different clinical samples from patients diagnosed with pulmonary and extrapulmonary tuberculosis. This test, in addition to contributing to a rapid and accurate differential diagnosis between tuberculosis and other mycobacteria, will certainly assist in the early and appropriate therapeutic management of the patient.

## METHODS

### Study population

Thirty-seven male and female patients over the age of 12 years with pulmonary and extrapulmonary tuberculosis diagnoses, who were referred to public clinics in the metropolitan region of Recife of northeastern Brazil between March 2008 and December 2009, were chosen for this study. The types of biological samples collected were chosen based on the different clinical forms of the disease. The diagnosis of tuberculosis was made by the attending physician in accordance with the standards of the American Thoracic Society (ATS), in 2007, based on the isolation of *Mycobacterium* in culture from different samples and visualization of acid fast bacilli (AFB) in the smear microscopy[28].

### Biological specimens

Forty pulmonary and extrapulmonary samples were analyzed, including 26 (65%) samples of pulmonary origin and 14 (35%) samples of extrapulmonary origin, according to the following distribution: 23 (57.5%) from sputum, 3 (7.5%) from a bronchoalveolar lavage (BAL), 8 (20%) from urine, 1 (2.5%) from skin lesion aspiration, 3 (7.5%) from pleural fluid (PL), 1 (2.5%) from a node biopsy and 1 (2.5%) from a bone biopsy. Clinical specimens were analyzed by bacilloscopy, culture, multiplex PCR and polymerase chain reaction restriction enzyme analysis (PRA-*hsp65*) to identify and differentiate mycobacterial species as described below.

### Smear microscopy

The presence of acid fast bacilli in different clinical specimens was determined by Ziehl-Neelsen staining (Stewart, 1953) in the Public Health Laboratory of Recife (Unified Health System - SUS), in accordance with the guidelines of the Brazilian Ministry of Health[29].

### Specimen processing

In total, of 1-5 ml of various clinical specimens (except for the sterile samples collected) was processed using the modified Petroff method in 4% NaOH[29,30].

### Culture

Cultures were performed in a Lowenstein-Jensen medium[29,31] and mycobacterium species were identified using the following biochemical tests: selective inhibition by para-nitrobenzoic (PNB) acid and thiophene-2-carboxylic hydrazide (TCH) acid, niacin accumulation and heat-stable catalase at 68°C[29]. The cultures analyzed using the biochemical tests in this study were considered as references for comparison with the results obtained by multiplex PCR and PRA-*hsp65*.

### DNA extraction

DNA was extracted and purified from reference strains of *Mycobacterium tuberculosis* (H37Rv). In all, 40 species of mycobacteria were isolated on a Lowenstein-Jensen medium from clinical samples obtained from 37 patients diagnosed with pulmonary and extrapulmonary tuberculosis using the conventional method described by Sambrook et al. using a mixture of solvents and phenol chloroform[32].

### Polymerase chain reaction and restriction enzyme analysis

The polymerase chain reaction and restriction enzyme analysis (PRA-*hsp65*) were performed using the techniques described in Telenti et al.[13] The identification was determined by comparing the sizes of the fragments with the algorithm described in the PRASITE site (http://app.chuv.ch/prasite/index.html)[17].

### Multiplex polymerase chain reaction

In multiplex PCR, there are 3 pairs of primers, 1 pair for each target. The gene encoding the antigen of *Mycobacterium tuberculosis* 65KDa, the dnaJ gene and the insertion element IS6110 were used in a single reaction with a mixture containing 10mM Tris-HCl (pH 8.3); 50mM KCl; 2.5mM $MgCl_2$; 2mM dNTP; 2.5U of Taq DNA Polymerase (Invitrogen); and 20pmol, 50pmol and 10pmol of each oligonucleotide pair, respectively. A total of 2µL of DNA at a concentration of 20ng/µL for each strain of mycobacteria was added to the reaction mixture for

a final volume of 50µL. These reaction parameters are in accordance with the conditions described by Poroca et al.[26].

### Statistical analysis

The value of Kappa was determined using SPSS statistical software for Windows (version 18; SPSS, Chicago, IL).

### Ethical considerations

The research was approved by the Ethics Committee of the Aggeu Magalhães Research Center (CPqAM) and the respective ethics committees of the participating hospitals under protocol No. 39/2009.

## RESULTS

### Clinical, epidemiological and laboratory findings

Of the 37 patients diagnosed with tuberculosis, 48.7% (n=18) of the cases originated from the ward, and 59.5% (n=22) were males aged 12 to 64 years (median 39 years). The clinical results showed that 70.3% (n=26) of the patients had pulmonary tuberculosis. The main clinical symptoms found were weight loss (90%), cough (80%) and fever (62.5%). Among the patients' pre-existing diseases, 24.3% (n=9) were infected with the human immunodeficiency virus (HIV) virus and 13.5% (n=5) previously had tuberculosis. In laboratory tests, 82% had chest X-rays and 21.7% had the Mantoux tuberculin skin test. A smear of lung samples was taken from 73% (n =19) of the patients; 17 were sputum samples and 2 were bronchoalveolar lavages, and 94.4% (n=18) of the specimens lung specimens were positive for The presence of acid fast bacilli in different clinical specimens was determined by Ziehl-Neelsen staining (Stewart, 1953) in the Public Health Laboratory of Recife (Unified Health System - SUS), in accordance with the guidelines of the Brazilian Ministry of Health. Mycobacterial strains were isolated in Lowenstein-Jensen culture medium for the 40 biological specimens analyzed **(Table 1)**.

TABLE 1 - Clinical, epidemiological and laboratory characteristics of the 37 patients studied.

| | Pulmonary TB | | Extrapulmonary TB | |
|---|---|---|---|---|
| Characteristic | n | % | n | % |
| Out-patients | 10 | 27.0 | 9 | 24.4 |
| Ward | 16 | 43.2 | 2 | 5.5 |
| Sex | | | | |
| male | 17 | 45.9 | 5 | 13.5 |
| female | 9 | 24.3 | 6 | 16.2 |
| Pre-existing disease | | | | |
| HIV | 1 | 2.7 | 5 | 13.5 |
| TB re-treatment | 4 | 10.8 | 1 | 2.7 |
| Laboratory exams | | | | |
| smear | 19 | 73.0 | 0 | 0.0 |
| culture | 26 | 65.0 | 14 | 35.0 |

HIV: human immunodeficiency virus; TB: tuberculosis.

### Identification of the mycobacterial species by culture and biochemical testing (reference test)

With respect to the reference test, of the 40 mycobacterial strains isolated by culture from the specimens obtained from the 37 patients diagnosed with tuberculosis, 57.5% (n=23) of the strains were identified as belonging to the *M. tuberculosis* complex, 20% (n=8) of the strains were identified as non-tuberculous mycobacteria (NTM) and 22.5% (n=9) of the results were inconclusive.

### Evaluation of multiplex PCR using mycobacterial species

Of the 40 mycobacterial isolates, 27 (67.5%) showed the molecular pattern for *M. tuberculosis* with the simultaneous amplification of the three targets IS6110 (541bp), *dnaJ* (365bp) and *hsp*65 (165bp). In 13 of the samples (32.5%), amplification of a fragment sized 20 to 40 base pairs less than 165bp was observed, which characterized these samples as NTM, according to Poroca et al.[26] **(Figure 1)**.

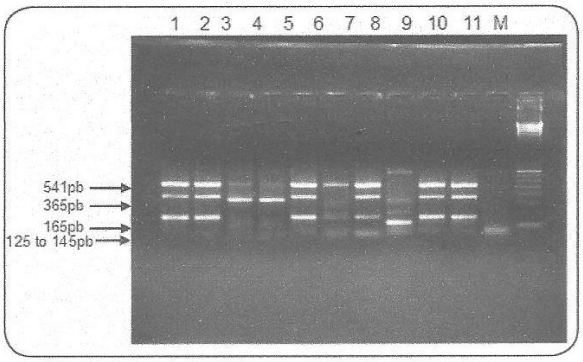


FIGURE 1 - Multiplex PCR of mycobacterial isolates from patients diagnosed with tuberculosis. Bands 1, 2, 5 and 7 represent the mycobacterial species displaying the molecular pattern of *M. tuberculosis*; Bands 3, 4, 6 and 8 represent the mycobacterial species displaying the molecular pattern of NTM; Bands 9 and 10 represent genomic DNA for *M. tuberculosis* (H37Rv); Band 11 represents a negative control for the reaction; Band M represents a 50bp DNA ladder (New England BioLabs, Hitchin, Hertfordshire, UK).

### Species identification using restriction enzyme analysis

When the mycobacterial species were identified by analyzing the banding pattern obtained by the PRA-*hsp65* technique, 29 (72.5%) of the species were identified as the *Mycobacterium tuberculosis* complex; 4 (10%) of the species as *Mycobacterium fortuitum* 2; one (2.5%) of the species as *Mycobacterium abscessus 2, Mycobacterium bolletii/Mycobacterium massiliense;* one (2.5%) of the species as *Mycobacterium gastritis 2/M. kansasii* 6 and one (2.5%) of the species as *Mycobacterium parmense* type 1, whereas 4 (10%) of the results were inconclusive. None of the results indicated the presence of the *M. avium* complex or

*M. intracellulare* among the strains isolated via culture from the pulmonary and extrapulmonary tuberculosis samples.

### Results of the multiplex PCR and PRA-*hsp65* with strains whose results were inconclusive by the reference test (culture and biochemical testing)

In this study, 9 (22.5%) of the mycobacteria samples isolated in culture from the clinical specimens of patients diagnosed with tuberculosis could not be identified by culture and biochemical tests; however, 5 (55.5%) of these strains were identified as *M. tuberculosis* by multiplex PCR, and 4 (44.4%) were identified as NTM. When using the PRA-*hsp65*, 6 (66.6%) strains were identified as an *M. tuberculosis* complex, and 3 (33.3%) could not be identified by PRA-*hsp65* .The diagnosis of tuberculosis in these patients was performed by the medical health service based on smear and/or clinical symptoms.

### Comparison of the reference test with multiplex PCR and PRA-*hsp65*

The results of the multiplex PCR and PRA-*hsp65* were compared to the results of the reference test (culture and biochemical assays) and are summarized in **Table 2**. The correlation calculation between the two tests was conducted using the results from 30 strains identified by culture and biochemical tests, as these results were not considered inconclusive in the reference tests and PRA-*hsp65*.

TABLE 2 - Concordance between the results of multiplex PCR and PRA-*hsp65* with the biochemical and phenotypic tests considered for 30 different isolates of mycobacteria differentiated by phenotypic and biochemical tests.

| Biochemical and phenotypic tests | Multiplex PCR | PRA-hsp65 |
|---|---|---|
| MTBC | 23 | 23 |
| NTM | 7 | 7 |
| Total | 30 | 30 |
| Kappa | 1 | 1 |

MTBC: *Mycobacterium tuberculosis* complex; NTM: non-tuberculous mycobacteria. PCR: polymerase chain reaction; PRA-*hsp65*: polymerase chain reaction and restriction enzyme analysis.

### Data from subjects who had mycobacteria identified as NTM by multiplex PCR and PRA-*hsp65*

We found that 7 (17.5%) strains of mycobacteria were identified as NTM using multiplex PCR and PRA-*hsp65* in 6 individuals who were diagnosed with pulmonary or extrapulmonary tuberculosis. Among those individuals, only one subject had two different isolates derived from clinical urine (non-sterile) and pleural fluid (sterile) specimens. The other 5 patients had only one species of NTM isolated in culture from the clinical urine, sputum or BAL specimens. None of these cases were diagnosed as an NTM infection.

## DISCUSSION

Advances in molecular biology techniques and the information provided by sequencing the complete genome of *M. tuberculosis* prompted the development of new tools for the rapid diagnosis of tuberculosis, including the differentiation of *M. tuberculosis* from other mycobacteria[33,34]. Multiplex PCR has the ability to amplify different targets simultaneously and has been used to detect and identify mycobacteria from the *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria[26,27,35,36].

In this study, when the multiplex PCR was compared with the reference tests (cultures and phenotypic and biochemical tests) to identify 40 mycobacterial isolates, a significant agreement was observed: 22 (100%) of the 37 cases with a final diagnoses of pulmonary or extrapulmonary tuberculosis presented species of the *Mycobacterium tuberculosis* complex (MTBC) using the reference tests. Multiplex PCR identified the presence of the *M. tuberculosis* complex in 27 (67.5%) of the cases. In 13 (32.5%) of the cases, multiplex PCR showed a molecular pattern consistent with an NTM, thus disagreeing with the final diagnosis made by the attending physician. It is important to be cautious when making a differential diagnosis between tuberculosis and other mycobacteria because the clinical symptoms and X-ray images may be similar. In Pernambuco in 2010, approximately 26.7% of the patients were treated without bacteriological confirmation of pulmonary tuberculosis; the diagnosis was based only on clinical and X-ray findings, which are often inconclusive and provide inferior data relative to those obtained in this study[37].

The results of the PRA-*hsp65* technique, when compared with the reference tests, showed the same degree of agreement obtained with the multiplex PCR for the *Mycobacterium tuberculosis* complex and NTM. It is notable that the multiplex PCR, because of its ability to amplify three different, specific targets in the same reaction, has the ability to identify the *Mycobacterium tuberculosis* complex and differentiate it from the species *M. tuberculosis*, *M. bovis* and *M. avium*[26,27]. In contrast, although PRA-*hsp65* does not differentiate between the species of the *Mycobacterium tuberculosis* complex, it is able to identify most of the NTM species[5,38]. This tool is recommended when the culture and phenotypic and biochemical tests suggest the presence of NTM species, but it is costly and time-intensive[5].

During this study, 3 patients diagnosed with pulmonary tuberculosis were beginning their second dose of anti-TB treatment when the species of mycobacteria was identified as NTM by multiplex PCR. In 2 cases, only 1 isolation from a lung sample was obtained, and in one of the cases, the species was identified as *M. gastritis 2/M. kansassi 6* using PRA-*hsp65*. The presence of NTM in a single sample from a non-sterile source requires careful investigation, including requests for new samples to eliminate the possibility of contamination or transitory colonization[11,39]. In this study, no patients were diagnosed by the health service professionals as infected with NTM; therefore, there is a need for patient follow-up using a

new collection of biological specimens, as the diagnosis may be suspect. The present study showed that, despite the high prevalence of tuberculosis in Brazil, the presence of NTM should strongly be considered prior to growing mycobacterial cultures in sterile clinical samples as part of the investigation when patients have symptoms such as an undefined fever.

The findings in this study suggest the need to investigate the presence of NTM in diagnosed lung diseases, following from the recommendations of other studies, which showed that NTM species can be detected in cases where TB is being re-treated[7,8]. The most frequently isolated species belong to the *M. avium* (MAC) complex and *M. kansassi*[40,41]. Ueki et al.[11] demonstrated that the most prevalent species in the lung compartment was *M. kansassi* and MAC in disseminated diseases[11]. For the treatment of pulmonary NTM infections, laboratory support is needed both to identify species and to determine the in vitro profile of resistance to antimicrobial agents. *M. kansasii* remains the most easily treatable form of pulmonary infections; there is a strong correlation between the *in vitro* susceptibility and the *in vivo* response to rifampicin, macrolides and fluoroquinolones. The greatest risk is the emergence of drug-resistant strains, similar to what is observed with *M. tuberculosis*[42], when patients fail to follow the correct treatment regimen.

Among the cases diagnosed as extrapulmonary tuberculosis, we isolated the same species of mycobacteria (*M. fortuitum 2*) in Lowenstein Jensen (LJ) culture medium from samples of urine and pleural fluid, where the bacteriological diagnosis for NTM can be defined according to ATS criteria because the pleural fluid is considered a sterile sample[28]. The *M. fortuitum 2* species were identified by phenotypic and biochemical tests, multiplex PCR and PRA-*hsp65*. *M. fortuitum* is a rapidly growing mycobacterium. Although its pathogenic potential is very low, it is often acquired in hospitals and can infect immunocompromised patients. One patient was HIV-positive and died as soon as he began treatment for tuberculosis, which indicates the increasing clinical importance of identifying mycobacterial species[43,44]. Several studies have demonstrate the importance of *M. fortuitum* infections in immunocompromised patients and found that the most prevalent species in these cases include the *Mycobacterium avium* complex, *Mycobacterium kansasii and Mycobacterium fortuitum*[10,11,45].

It can therefore be concluded that multiplex PCR has the ability to identify and differentiate species of *M. tuberculosis* and NTMs and thus can be used as an auxiliary tool in the differential diagnosis of tuberculosis and diseases caused by NTMs. However, multiplex PCR should not be used as a sole laboratory method; it is important that the differential diagnosis be based on the joint analysis of various parameters using PRA-*hsp65* tests and the sequencing of specific genes that may identify the NTM species.

The speed and accuracy of multiplex PCR to differentiate between the *M. tuberculosis* complex and NTMs enables it to be used as an alternative to phenotypic and biochemical tests when identifying species of the *M. tuberculosis* complex and when screening to determine the NTM species present by sequencing specific genes.

Thus, molecular methods (multiplex PCR, PRA-*hsp65* and sequencing of specific genes) can support diagnoses based on clinical determinations regarding mycobacterial infections, and the implementation of these methods, particularly in reference to services for those patients with tuberculosis (TB), Multidrug-resistant tuberculosis (MDR- TB) and Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS), reduces the possibility of an inadequate diagnosis and treatment.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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## 7.2 Artigo 2- First case report of infection by *Mycobacterium wolinskyi* after mammoplasty in Brazil.

Verificamos durante o desenvolvimento do trabalho da Tese um total de 4 casos de infecções extrapulmonares identificadas após procedimentos invasivos em serviços de saúde do estado de Pernambuco (3 (75%) mamoplastias e 1 (25%) abdominoplastia). As espécies isoladas a partir de amostras clínicas destes casos foram: *M. fortuitum 2 ( 50%),* 1 (25%) *M. wolinskyi* e 1 (25%) *M. novocastrense.*

Para respondermos o primeiro, segundo, terceiro e quarto objetivos especificamente para as infecções extrapulmonares no estado de Pernambuco, trazemos neste segundo artigo a primeira descrição no Brasil de infecção extrapulmonar por *M. wolinskyi* diagnosticada após procedimento cirúrgico invasivo em serviço privado do estado.

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# First case report of infection by *Mycobacterium wolinskyi* after mammoplasty in Brazil

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## Abstract

*Mycobacterium wolinskyi* is a rapidly growing mycobacterium, first described in 1999 as a member of the group *Mycobacterium smegmatis* (*Mycobacterium smegmatis*, *Mycobacterium wolinskyi* and *Mycobacterium goodii*). Only 19 case reports all over the world have been described on literature, none of them in Brazil. On this report, it is described one case of infection after a mammoplasty procedure performed in a private health service in the county of Recife, Pernambuco, Brazil, in 2009. The mycobacteria specie was identified using biochemical tests and sequencing the specific gene *rpoB*. To treat the infection by *Mycobacterium wolinskyi* it was necessary to combine antibiotics for a long period of time associated with surgical procedures of the breast abscesses.

## Case Report

On September 15th, 2009, a 29-year-old woman, 61 kg, 1.69 m stature, Caucasian, with no comorbidities, post-graduated, Brazilian, from Recife-PE was submitted to an elective bilateral reductive mammoplasty on a private hospital of Recife-PE to remove 200 mL of each breast using the *L technique* for resections of excess of skin and breast tissue. Then an ampoule of adrenaline was infiltrated into her breasts, the bandage was realized using saline and Polivinilpirrolidone-iodine. The surgical procedure was concluded in 3 hours. The patient made her bandages at home using water, soap and an antiseptic solution of chlorhexidine gluconate. Healing occurred normally, with no trauma and no presence of inflammatory signs. One year after surgical procedure, on October 17th, 2010, the patient referred edema, heat and pain on her left breast. Although left breast presented no blush and normal aspect of scar.

It was requested a breast ultrasonography (USG) and it was prescribed a non-hormonal anti-inflammatory, Nimesulide 100 mg, one pill a day for 5 days, with no improvement of the signs and symptoms. USG revealed an image of a fluid collection filled by thin echoes, extending from 9 o'clock to 3 o'clock, with an antero-posterior diameter with approximately 2.3 cm, far around 2 cm of the skin with an increase of the echogenicity of the subcutaneous tissue on the region (Figure 1). It was then prescribed treatment with cephalexin, 500 mg every six hours and Nimesulide, 100 mg, one pill a day for 5 days.

As there was no improvement of the clinical conditions, an aspiration of the fluid collection was performed in November 23rd, 2010 on patient's left breast with an entry on the intern superior upper quadrant, obtaining a greenish secretion which was sent for automatized culture and antibiogram, both negative for bacterial growth. After the procedure, it was prescribed ciprofloxacin, 500 mg, 2 pills every twelve hours for 2 days and one pill every twelve hours totaling 10 days, without improvement of the condition. On November 30th, 2010, the patient was submitted to a surgery to drain the breast's abscess, maintaining ciprofloxacin 500 mg, one pill every twelve hours, Diclofenac sodium, 100 mg one pill a day and Dipyrone one pill every six hours for 7 days.

The sample collected in this procedure was sent for automatized culture with antibiogram and for smear tests on acid fast bacilli (AFB), both showing negative results.

After the end of the treatment with antibiotics, on December 13th, 2010 an USG showed an increase of the echogenicity on the cellular subcutaneous tissue and on the breast's fat, associated with 2 collections which presented debris in suspension and irregular and inaccurate contours, measuring: 8.8×3.1×1.7 cm (vol =24.2 $cm^3$), located on the superior upper quadrants of the left breast and another with 2.2×1.2×0.8 cm (vol=1.1 $cm^3$); deeper than the previous one, which was located on the transition of the left lower quadrants, presenting 2 reactive lymph nodes on the left axilla, measuring 1.8 cm and the 1.4 cm, respectively.

After confirming the presence of the collections, another aspiration was performed using USG, on the same breast in December 14th, 2010, and it was also requested in a private laboratory of the city. Another culture and cytological exam of the collected sample, showed one inflammatory cyst and growth of AFB on a specific culture medium, but the mycobacterium specie was not identified due to fungus contamination on the sample.

A Chest X-ray was requested, and it didn't show abnormalities, another USG was performed on January 24th, 2011, which revealed a new fluid collection, homogeneous, measuring: 2.0×0.7×1.5 cm located on the internal superior upper quadrant of the left breast. It was then prescribed vibramycin, 100 mg, one pill every twelve hours and trimethropim sulfamethoxazole, 400/160, one pill every twelve hours for 6 months, and it was also requested a new drainage. The drained material was sent to the Public Health's Central Laboratory – Dr. Milton Bezerra Sobral (LACEN-PE), being isolated non-tuberculous mycobacteria in the culture medium. *Mycobacterium wolinskyi* was identified by sequencing specific genes; this technique was performed at Aggeu Magalhães Research Center, FIOCRUZ-PE. As the patient presented an evident improvement of the clinical conditions, the treatment scheme proceeded for more 6 months, independent on the antibiogram's result (Table 1) remaining

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Key words: *Mycobacterium wolinskyi*, mammoplasty, post-surgical infection.

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asymptomatic for almost 11 months.

After this period, on January 9th, 2012, the inflammatory signs and symptoms reappeared on the left breast, an USG showed 4 cystic images, the biggest at 12 o'clock measuring: 0.7×0.6 cm; the second had slightly thick walls associated with hyperechogenicity of the cellular subjacent subcutaneous tissue at 1 o'clock, measuring: 0.7×0.6×0.5 cm, far 1 cm of the skin and about 4 cm of the nipple; the third cyst presented an heterogeneous content with two adjacent cysts, located at 10 o'clock and measuring: 1.4×1.3×1 cm and 1.3×1.1×0.8 cm, far 3 cm of the nipple and 2 cm of the skin; and the fourth image was located at 5 o'clock measuring: 2.9×1.9×1 cm, far 1 cm of the skin and 4 cm of the nipple. The patient was then submitted to a new surgical procedure to drain the collection and to withdraw the necrotic tissue. This tissue culture revealed one more time the presence of *Mycobacterium wolinsky*, identified by sequencing specific genes. The prescribed therapy was an association of antibiotics, initially under hospital regimen, amikacin 1 g injectable per day with ciprofloxacin 500 mg every twelve hours and doxycycline 100 mg. Amikacin was maintained for 10 weeks under domiciliary regimen, 1 g intramuscular 3 times a week. The other 2 classes of antibiotics were also maintained for 6 months. After this period the patient was released from the therapeutic scheme with complete regression of the clinical symptoms.

## Methods

### *Bacteriology*

The bacilloscopy performed with the samples was negative for AFB. The culture on Löwenstein-Jensen medium revealed AFB growth on less than 7 days, suggesting RGM. The colonies did not show any coloring, they were resistant to the para-nitrobenzoic acid (PNB) and to the Hydrazide of the 2-carboxilic acid (TCH); they did not show rope spoilage and the test for the presence of niacin was negative.[1]

### *Partial sequencing of the rpoB gene*

A 764-bp fragment was amplified and sequenced with primers MycoF (5_-GCAAGGTCACCCCGAAGGG-3_) and MycoR (5_-AGCGGCTGCTGGGTGATCATC-3_).[2] A total of 5 µL of each DNA solution (50 µg/mL) was added to 45 µL of a PCR mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl2, 200 µM each dNTP, 1 µM primers, and 1.0 U of *Taq* DNA polymerase (Promega). PCR mixtures were heated at 95°C for 1 min and then subjected to 35 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 90 s, with a final step of 72°C for 5 min. Amplicons were purified with GFX PCR DNA and a Gel Band purification kit (G&E) and sequenced in an ABI PRISM 3100 sequencer with a BigDye Terminator cycle sequencing kit (Applied Biosystems). The sequences found were edited and aligned by analyzing the sequencing electropherograms using the program BioEdit v7.0.9. The sequences obtained were compared with those deposited in the GenBank database by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The isolate had partial sequence of the rpoB gene with 99% (683/689) similar to GenBank accession number AY262743, which corresponds to Mycobacterium wolinskyi type strain ATCC 700010. The *in vitro* susceptibility test to antibiotics was performed using the microdilution broth assay (MIC) (Table 1).[3]

**Table 1. Antimicrobial susceptibility for *Mycobacterium wolinskyi* isolated on January 24th, 2011.**

| Drugs | MIC (µg/mL) | Interpretation |
|---|---|---|
| Amikacin | 4 | S |
| Cefoxitin | 64 | I |
| Ciprofloxacin | 1 | S |
| Clarithromycin | 16 | R |
| Doxycyclin | 4 | I |
| Moxifloxacin | ≤2 | I |
| Tobramycin | 32 | R |
| Sulfamethoxazole | ≥64 | R |

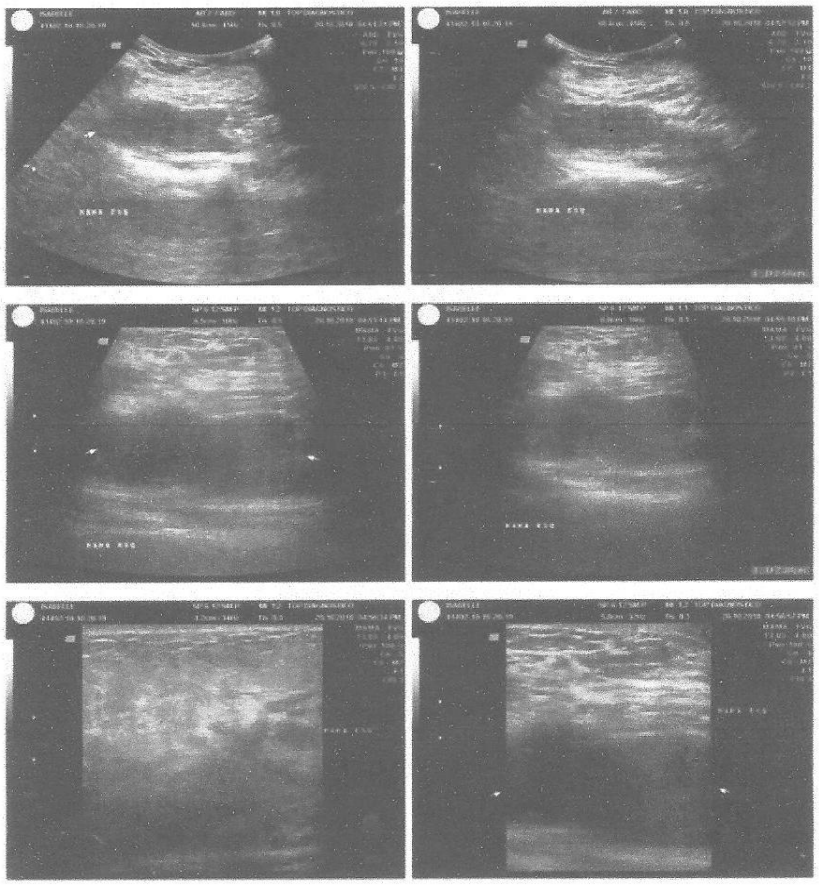

**Figure 1. Left breast's ultrasonography showing fluid collection.**

## Discussion and Conclusions

Among the rapidly growing mycobacteria (RGM), *M. wolinskyi* belongs to *M. smegmatis* group; it was identified for the first time by Brown *et al.* (1999)[4] sequencing 16S rRNA region. The smegmatis group is composed by *M. smegmatis*, *M. wolinskyi* and *M. goodii*. RGM are broadly distributed on the environment, particularly on soil and water, including potable water, biofilms on water distribution piping, swimming pools, sewage and surfaces.[5]

Since its taxonomic description, 19 cases of human infections all over the world were described until now. The majority of infections are post-traumatic and post-surgical, there are no reports of infection after breast aesthetics surgery.[6-8]

Over the last few decades, the majority of the notified cases after infection post mammoplasty are associated with *Mycobacterium fortuitum* (57%) and with *Mycobacterium abscessus* (15.2%). *Mycobacterium wolinskyi* was isolated in only 2 notified cases, representing 2% of all breast infections.[9]

The infection caused by these microorganisms can appear weeks or months after the surgery,[6] with no standard scheme to treat the infection by RGM due to *in vitro* variability and susceptibility of bacteria species. Therefore, it is necessary to identify properly every sample and to determine its sensitivity to antimicrobial agents.[10,11] The choice of the most suited treatment depends on the mycobacteria species involved in the infection, on the clinical presentation and on the patient's immunological condition. These bacteria are capable of producing biofilms, which makes their resistance to antibiotics easier.[12] In general, procedures of drainage, debridement contribute to the resolution of the cases when associated with antibiotic therapy.[6,8]

The typical profile of the *in vitro* susceptibility of *Mycobacterium wolinskyi* is: susceptibility to amikacin, imipenem and trimethropim sulfamethoxazole; resistance to tobramycin; intermediate susceptibility to doxycyclin and ciprofloxacin; and susceptibility to cefoxitin and clarithromycin.[13] Its resistance to tobramycin is a feature that distinguishes *Mycobacterium wolinskyi* from the other members of *Mycobacterium smegmatis* group.[4]

The present case is the first report of infection by *Mycobacterium wolinskyi* after mammoplasty in Brazil. There was no notification of outbreaks during this period at the hospital where the surgical procedures were performed. It is more likely that the bacteria infected the breast at the moment of the mammoplasty, since the presence of inflammatory signs (edema and pain) and abscesses were detected one year after the surgery, with no breast trauma or piercing in the region in this period. It was also observed the absence of comorbidities, presenting similar evolution with the cases which are associated with this type of mycobacterium described on literature.[6,8] To achieve cure, it was necessary to perform several drainage procedures of the abscesses combined with long term therapy using antibiotics, anti-inflammatories and analgesics. This case reassures the occurrence of postsurgical infections by non-tuberculous mycobacteria which must be considered, by health professionals, an important cause of morbidity for human beings.

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## 7.3 Artigo 3- Cases of pulmonary infection by nontuberculous mycobacteria in the state of Pernambuco, endemic region for tuberculosis in northeastern Brazil.

Os objetivos um, dois, três e quatro desta Tese foram atendidos e seus resultados apresentados neste terceiro artigo, especificamente para as infecções pulmonares por MNT.

Neste artigo foi descrito pela primeira vez o perfil clínico, epidemiológico e laboratorial dos casos de micobacterioses pulmonar por MNT, os fatores que estão associados à doença pulmonar. Além da frequência, diversidade e perfil de resistência a antimicrobianos das espécies de MNT no estado de Pernambuco que é sabidamente uma região endêmica para tuberculose.

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# Cases of pulmonary infection by nontuberculous mycobacteria in the state of Pernambuco, endemic region for tuberculosis in northeastern Brazil.

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## ABSTRACT

Currently, the gender *Mycobacterium* is composed by 165 species and 13 subspecies. Composed by the *Mycobacterium tuberculosis* Complex (MTBC) *(M. bovis, M. bovis-BCG, M. africanum, M. microti, M. canettii, M. caprae, M. pinnipedii), M. leprae* and other species so-called nontuberculous mycobacteria (NTM) or atypical mycobacteria, these with different phenotypic, genetic and pathogenic features. There are variations on the geographic distribution of the NTM species, which are associated to diseases in different continents. In endemic countries for tuberculosis (TB) there is a lack of regional studies to evaluate the real prevalence of infections by NTM. The state of Pernambuco, located in northeastern Brazil, is an endemic region for tuberculosis and reports about diseases which are caused by NTM are scarce. In this study, clinical and epidemiological profiles of pulmonary cases caused by NTM were evaluated, and also the diversity, frequency and the resistant profile to antimicrobial by NTM species on residents of the state of Pernambuco, between July 2010 and June 2013. A total of 21 cases of pulmonary NTM were identified and compared to 55 cases with clinical and/or radiological evidence of pulmonary tuberculosis. In bivariate analysis, it was verified an association with the previous occurrence of tuberculosis (93.3%) and with the gender, the majority of cases in males (67.7%). It was also observed that the average age of NTM cases, (52 ± 14.7), was significantly higher than the cases of pulmonary tuberculosis (41± 14.8). Regarding the species, it was seen that the majority of patients with pulmonary disease by NTM in Pernambuco were infected with a diversity of mycobacteria, similar to studies performed in another Brazilian regions; except for *M. fortuitum* and *M. asiaticum*. Therefore, the present study contributes in an unprecedented way to elucidate the frequency and diversity of NTM species which are associated with pulmonary disease, as well allowing a greater scientific understanding of this pathology in the state of Pernambuco, northeastern Brazil.


**Keywords:** Nontuberculous Mycobacteria, Differential diagnosis, Risk Factors.

## INTRODUCTION

Currently, the gender *Mycobacterium* is composed by 165 species and 13 subspecies (Tortoli, 2006; Euzéby, 2013). Composed by the *Mycobacterium tuberculosis* Complex (MTBC) *(M. bovis, M. bovis-BCG, M. africanum, M. microti, M. caprae, M. pinnipedii), M. leprae* and other species so-called nontuberculous mycobacteria (NTM) or atypical mycobacteria; these with different phenotypic, genetic and pathogenic features ( Brasil, 2008; Tortoli, 2003; Zamarioli et al, 2008). NTM are also divided in rapidly growing mycobacteria

(RGM) and slowly growing mycobacteria (SGM), in other words, those which form colonies in culture medium within seven days and those which need more time of incubation, respectively (Runyon, 1959).

NTM are widely spread in the environment, being isolated from the water, including piped water, soil, animals, surgical equipment and also in disinfectant solutions. The infection occurs by inhalation, inoculation or ingestion of contaminated material by mycobacteria, which may cause pulmonary diseases and infections of surgical wounds in different tissues; however, the person-to-person transmission seems not to occur (Cowman et al, 2012; Gómez, 2009).

Several forms of the disease caused by NTM have been described in humans, affecting lungs, ganglions, skin, as well as in the disseminated form. Pulmonary cases caused by NTM generally affects patients with pulmonary diseases, such as pneumoconiosis, chronic obstructive pulmonary disease, preexisting tuberculosis, chronic bronchitis, bronchiectasis and esophageal disease, associated with the chronic aspiration of food through the respiratory tract (Griffith et al, 2007; Jeong et al, 2004; Jarzembowski, 2008; Bodle et al, 2008; Glassroth, 2008; Sexton & Harrison, 2008; Griffith, 2010).

There is a variable geographic distribution of NTM species, associated with diseases in different continents, *M. kansasii* is frequently found on pulmonary infections in the USA, Europe and South Africa, besides, mycobacteria from the *Mycobacterium avium* Complex (MAC) which include *M. avium*, *M. intracellulare*, *M. colombiense* and *M. chimaera* (Tortoli, 2003; Tortoli et al, 2004) have a great worldwide clinical importance (Griffith et al 2007). In different Brazilian regions, it is also observed a diversity of NTM species which are isolated from pulmonary cases, *M avium*, *M. intracellulare*, *M. kansasii*, *M. fortuitum*, *M. chelonae*, *M. abscessus* and *M. massiliense* were the most frequent mycobacteria on pulmonary diseases (Barreto & Campos, 2000; Matos et al, 2004; Costas et al, 2012).

The clinical evaluation of the pulmonary disease is frequently complicated due to its similarity of symptoms with the preexisting pulmonary diseases. Pulmonary diseases' signs and symptoms caused by NTM are variable and unspecific and most of the times, the clinical profile resembles the chronic evolution of tuberculosis (Griffith et al 2007; Hadad et al, 2005).

The differential diagnosis between tuberculosis (TB) and other mycobacteriosis in endemic regions for TB is essential since the diseases present peculiarities related to their epidemiology, prognosis and treatment. Because of their clinic similarities, only the bacteriological diagnosis is considered confirmatory. Thereunto, the participation of a

specialized laboratory with an adequate infrastructure for isolating and identifying species is highly necessary (São Paulo, 2005). It is required to be cautious when performing the diagnosis of mycobacteriosis; isolating NTM from non-sterilized clinic specimens may cause a transitory colonization or contamination. The *American Thoracic Society* recommends the mycobacteriosis' diagnosis to be carried out with basis on a series of bacteriological, clinical and radiological criteria (Griffith et al 2007; Centro de Referências Professor Hélio Fraga, 2005; Brunello et al, 2001).

The infections caused by NTM are increasing worldwide; however, the magnitude and regional distribution of these cases in endemic countries for tuberculosis are still not well known (Gopinath & Singh, 2010). In Brazil, the most cases of NTM infections occur in the southeast of the country ( Zamarioli et al, 2008; Ueki, 2005; Campos, 2000; Pedro et al, 2008). Costa et al (2012) showed in a study held in the country's north that a great variety of NTM species may be involved in the pulmonary disease and that the differential diagnosis between TB and NTM is essential for the proper treatment of the cases. In the northeastern part of the country, there are no studies which bring epidemiological information and variability of the involved species in the pulmonary disease caused by NTM (Costa et al, 2012).

Therefore, the present study intends to seek clinical and epidemiological profiles of pulmonary cases caused by NTM comparing them with cases of pulmonary tuberculosis, besides the diversity, frequency and resistance profile to antimicrobial by the NTM species from residents of the state of Pernambuco.

## METHODOLOGY

**Type of study:** Cross-sectional study with two comparative groups.

**Case selection of NTM and clinical samples**

The patients who were included in this study presented besides the suggested symptoms of the mycobacterial disease, independent from the bacilloscopy result, the isolation in specific medium of nontuberculous mycobacteria in at least two clinical pulmonary samples (sputum and bronchoalveolar washing), with the identification of the NTM species through phenotypic, biochemical and molecular techniques (Griffith et al 2007). The confirmatory clinical diagnosis was performed by the assistant physician of the health service, blindly applied. The cases of pulmonary mycobacteriosis were forwarded by the bacteriology service of the Central Laboratory of Public Health - Dr. Milton Bezerra Sobral (LACEN – PE), where the culture and the differentiation between bacteria from MTBC and NTM were performed.

Clinical, epidemiological and laboratorial data from the NTM cases were extracted from the existing database of the State Health Department (Ambulatory and Laboratorial Manager – ALM) for registering users of the Unified Health System, as well as the present information from the patients' medical records.

**Case selection of pulmonary tuberculosis**

55 individuals were included with clinical and/or radiological evidence of tuberculosis and isolation of *M. tuberculosis* in pulmonary clinical samples (sputum and/or brochoalveolar washing), through the direct exam and/or culture or evident clinical improvement after treatment.

**Bacilloscopy and culture with biochemical tests**

Bacilloscopy was performed using Ziehl-Neelsen method to identify and quantify the Acid-Alcohol Resistant Bacilli (AARB) in accordance with the technical norms from the National Manual of Tuberculosis and another Mycobacteria Surveillance (Brasil, 2008). The biological specimens were initially decontaminated by Petroff's method (NaOH 4%), (Brasil, 1994). The samples were posteriorly inoculated in Lowestein-Jansen medium (Difco, Sparks, EUA) and incubated at 35°-37°C, in the absence of light for at least eight weeks or until the colonies have appeared. Isolates from the *M. tuberculosis* Complex were distinguished from NTM by the colonies' morphology, cord-like factor production, susceptibility to the para-nitrobenzoic acid (PNB), niacin production and absence of thermo stable catalase at 68ºC by *Mycobacterium tuberculosis* (Brasil, 2008).

**Molecular identification**

All the isolates in this study were identified through sequencing specific genes; *hsp65* for slowly growing mycobacteria, and *rpoB* for rapidly growing mycobacteria (Adékambl et al, 2003; Kim et al, 2005; Shin et al, 2006).

**Susceptibility test to antimicrobial agents**

Rapidly and slowly growing mycobacteria were tested against the main antimicrobial compounds using the broth microdilution assay (BMA), following the protocol from the manual of Clinical and Susceptibility Laboratory Standards Institute CLSI (2011) for mycobacteria, Nocardia and another aerobic actinomycetes (CLSI, 2011). The susceptibility profile to the drugs by slowly growing mycobacteria was built using the respective antimicrobial compounds: amikacin, ciprofloxacin, clarithromycin, moxifloxacin, sulfamethoxazole, rifampicin, ethambutol, isoniazid and streptomycin. For the rapidly

growing mycobacteria were tested: amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxicyclyn, moxifloxacin, sulfamethoxazole and trobramycin.

**Ethical considerations**

The present work was approved by the Ethics Committee of Aggeu Magalhães/ Fiocruz (N°06/2013).

**Statistical analysis**

Initially, a descriptive analysis was held to characterize the sample as its clinical, epidemiological and laboratorial profiles, besides life habits and type of health service. Thereafter, a bivariate analysis was performed, aiming the identification of the association between the occurrence of NTM and the investigated factors. To do so, the tests of chi-square or Fisher exact test were performed when necessary, besides the t-Student test. All conclusions were taken at the significance level of 5%. For the data analysis SPSS v 8.0 was used.

## RESULTS

**Patients' characteristics**

Between July 2010 and June 2013, the Central Laboratory of Public Health - Dr. Milton Bezerra Sobral (LACEN – PE), reference on tuberculosis' diagnosis, received 4,808 pulmonary samples of patients with suspicion of mycobacterial infections, from these, 1,506 (95.4%) were infected with MTBC and 72 (4.6%) samples had isolation of NTM (33 individuals). Among the 33 individuals with positive NTM, 21 (63.6%) presented at least two positive cultures from sputum samples or one sample of bronchoalveolar washing (BAW) with the same NTM specie, besides the clinical and respiratory profiles, which were compatible with mycobacterial disease (Griffth et al, 2007). The remaining 12 (36.3%) could not be confirmed as pulmonary NTM cases due to: a) only one pulmonary sample was collected and delivered to the laboratory, b) only in one sample NTM was isolated, being the rest negative, c) clinical data with conduct and therapeutic development were not included on the Ambulatory and Laboratorial Manager (ALM) database, neither did the medical records at the time of the research.

All 21 patients were forwarded from the public health services and considered suspect cases of pulmonary tuberculosis by the responsible physicians, based on the results from the bacilloscopy and search of AARB, besides the clinical symptoms and radiological signs of alteration. Among the 21 cases of pulmonary mycobacteriosis, according to the criteria of ATS (2007), only 7 (33.3 %) were treated with antimicrobial drugs for NTM, but the majority, 14 (66.7%), had the TB diagnosis made by the physicians from the health services

who have started the treatment with a specific schema. The epidemiological and laboratorial characteristics of the 21 patients with pulmonary diseases caused by NTM, from the ATS (2007) criteria, are described at table 1.

**Table 1 - Cases of Pulmonary NTM by criteria by ATS criteria in the State of Pernambuco**

| Nº | Gender | Age | Occupation | Area | AFB smear | Nº positive cultures | Clinical specime | Other associated conditions | Species |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 56 | retiree | urban | + | 3 | sputum | cancer | *M. kansasii* |
| 2 | F | 34 | case-worker | urban | + | 3 | sputum | - | *M. kansasii* |
| 3 | F | 49 | dentist | urban | + | 2 | sputum | - | *M. abscessus subsp bolletti* |
| 4 | F | 61 | retiree | urban | + | 3 | sputum | previous tuberculosis | *M. fortuitum* |
| 5 | M | 75 | retiree | urban | - | 3 | sputum | previous tuberculosis | *M. abscessus subsp abscessus* |
| 6 | M | 54 | driver | urban | + | 1 | BAL | previous tuberculosis | *M. kansassi* |
| 7 | F | 44 | housewife | urban | + | 2 | sputum | previous tuberculosis | *M.asiaticum* |
| 8 | M | 32 | unemployed | urban | + | 4 | sputum | previous tuberculosis | *M. kansasii* |
| 9 | M | 28 | general services | urban | - | 8 | sputum | previous tuberculosis | *M. kansasii* |
| 10 | M | 55 | retiree | urban | - | 2 | sputum | previous tuberculosis | *M. kansasii* |
| 11 | M | 63 | merchant | urban | - | 2 | sputum | - | *M. kansasii* |
| 12 | F | 43 | retiree | urban | + | 4 | sputum | - | *M. kansasii* |
| 13 | M | 34 | unemployed | urban | - | 2 | sputum | previous tuberculosis | *M. kansasii* |
| 14 | M | 59 | retiree | urban | + | 2 | sputum | - | *M. abscessus subsp abscessus* |
| 15 | F | 58 | retiree | urban | + | 2 | sputum | previous tuberculosis | *M. kansasii* |
| 16 | M | 47 | building inspector | urban | + | 2 | sputum | previous tuberculosis | *M. kansasii* |
| 17 | M | 69 | retiree | urban | + | 3 | sputum | COPD and previous tuberculosis | *M.intracellulare* |
| 18 | M | 57 | attendant | urban | - | 3 | sputum | - | *M. fortuitum* |
| 19 | M | 69 | driver | urban | + | 3 | sputum | previous tuberculosis | *M. kansasii* |
| 20 | F | 74 | retiree | urban | + | 3 | sputum | previous tuberculosis | *M. abscessus subsp bolletti* |
| 21 | M | 28 | merchant | urban | + | 2 | sputum | previous tuberculosis | *M.intracellulare* |

Patients with pulmonary NTM had the average age higher than the group with pulmonary TB (p=0.006). The average age on the group with NTM was of 52.0 ± 14.7 years old, and 57% (12/21) of the patients had more than 57 years old. From the group with pulmonary tuberculosis the average age was of 41.0 ± 14.8 and 14 (67.7%) were male. Concerning the average time between the appearance of the symptoms and the final diagnosis for pulmonary NTM or tuberculosis, 11 (11/21, 52.4%) had the diagnosis done after 6 months, and 9 (9/21, 42.9%) in a year or more after the beginning of the symptoms. A total of 12 (12/21 57%) patients were smokers and 8 (38%) considered themselves as alcoholics, while patients with TB, 13 (13/55, 23.5%, p 0,007), were smokers.

As to the presence of comorbidities, at least one was found in 15 (71.4%) patients. The report of previous tuberculosis was made by most of the cases of pulmonary NTM (14/15, 93.3%), while in the group with pulmonary tuberculosis only 6 were made (6/32, 18.8%, p <0.001). None of the NTM cases were carriers of the HIV virus, while 17 (17/32, 53%, p 0,002) patients with pulmonary tuberculosis were seropositive. Cancer and Chronic Obstructive Pulmonary Disease (COPD) were found in only one of each case (1/15, 6.7%).

In the group with pulmonary NTM, all cases presented cough; productive cough was reported in 66.7% (14/21) of the patients and hemoptysis in only one case (1/21, 4.8%). Loss of weight was observed in 66.7% (14/21) of the individuals. Fever was not present in 57.1%

(12/21) of pulmonary NTM cases; however, it was a present symptom in 74.1% (40/54, p 0,015) of the individuals with pulmonary TB. In most cases (20/21, 92.5%) of the individuals with NTM, the presence of inflammatory signs was not observed (pain, redness and swelling).

Radiological alterations were observed in all 21 patients with NTM, but only 9 (42.9%) were found in the database (ALM) or in the medical records. Among the radiological alterations, bronchiectasis was observed in 55.5% (5/9) of the patients, followed by fibrosis in 33.3% (3/9), cavitation in 33.3% (3/9), pulmonary infiltrate in 22.2% (2/9), pleural thickening in 22.2% (2/9), and pulmonary nodules in 11.1% (1/9). Emphasizing that 44.4% (4/9) showed more than two different types of alterations identified by X-Ray (Table 2).

**Table 2 - Clinical symptoms and changes in X-ray nine patients with NTM lung**

| Nº | clinical symptoms | Chest X-ray |
|---|---|---|
| 2 | dry cough, weight loss, no fever or signs of inflammation | pleural thickening e infiltrate |
| 3 | productive cough, weight loss, no fever or signs of inflammation | bronchiectasis |
| 8 | productive cough, weight loss, fever and no signs of inflammation | cavity |
| 11 | productive cough, weight loss, fever and no signs of inflammation | bronchiectasis, fibrosis e pulmonary nodules |
| 13 | dry cough, weight loss, fever and no signs of inflammation | bronchiectasis |
| 14 | productive cough, weight loss, fever and no signs of inflammation | pleural thickeninge fibrosis |
| 17 | productive cough, weight loss, no fever or signs of inflammation | bronchiectasis, fibrosis, cavity e infiltrate |
| 20 | productive cough, weight loss, fever and no signs of inflammation | bronchiectasis |
| 21 | productive cough, weight loss, fever and presence of lymph nodes in the neck | cavity |

**NTM isolates**

Six different species of NTM were identified from the isolation in culture of clinical samples from patients with pulmonary mycobacteriosis according to the criteria from the ATS (2007), including: 12 (57.1%) *M. kansasii*, 2 (9.5%) *M. intracellulare*, 2 (9.5%) *M. abscessus subsp abscessus*, 2 (9.5%) *M. abscessus subsp bolleti,* 2 (9.5%) *M. fortuitum* and 1 (4.8%) *M. asiaticum*. The pulmonary infection by *M. abscessus subsp bolleti* has occurred only in women with average age of 55.5 years old. In men, the specie which was associated with pulmonary disease was *M. kansassi* 10 (83.3%), with average age of 48.1 years old. From the 12 individuals who did not fit in the diagnostic standards of the ATS (2007) for NTM disease, the identified species were *M. fortuitum*, *M. kansasii*, *M. abscessus subsp bolleti*, *M. abscessus subsp abscessus* and *M. szulgai*.

**Susceptibility tests to antibiotics**

Fifteen (15/21, 71.4%) isolates of nontuberculous mycobacteria were evaluated regarding the sensitivity or resistance to antimicrobial drugs used in the clinical practice for

SGM and RGM. From the SGM group, 10 isolates of *M. kansassi* and 1 of *M. intracelullare* were tested, and from the RGM group, 2 isolates of *M. fortuitum*, 1 of *M. abscessus subsp bolletti* and 1 of *M. abscessus subsp abscessus* were tested. The table below summarizes the pattern of the antimicrobial resistance of SGM and RGM which were isolated from clinical samples of 15 cases of pulmonary mycobacteriosis in the state of Pernambuco (Table 3). All SGM and RGM which were tested have shown sensitivity *in vitro* for amikacin.

**Table 3 - The profile of antimicrobial resistance of MNT which were isolated from clinical samples of 15 cases of pulmonary mycobacteriosis**

| Resistance to antimicrobials | *M. abscessus subsp abscessus* | | *M. fortuitum* | | *M. abscessus subsp bolletti* | | *M. kansasii* | | *M. intracellulare* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **N** | **%** | **N** | **%** | **N** | **%** | **N** | **%** | **N** | **%** |
| Amikacin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cefoxitin | 1 | 100 | 0 | 0 | 0 | 0 | . | . | . | |
| Ciprofloxacin | 1 | 100 | 0 | 0 | 1 | 100 | 3 | 30 | 0 | 0 |
| Clarithromycin | 1 | 100 | 1 | 50 | 1 | 100 | 1 | 10 | 0 | 0 |
| Doxycycline | 1 | 100 | 0 | 0 | 1 | 100 | . | | . | |
| Moxifloxacin | 0 | 0 | 0 | 0 | 1 | 100 | 0 | 0 | 0 | 0 |
| Tobramycin | 1 | 100 | 2 | 100 | 1 | 100 | . | . | . | |
| Sulfamethoxazole | 1 | 100 | 2 | 100 | 1 | 100 | 4 | 40 | 1 | 100 |
| Rifampicin | . | | . | | . | | 1 | 10 | 1 | 100 |
| Ethambutol | . | | . | | . | | 5 | 50 | 1 | 100 |
| Isoniazid | . | | . | | . | | 0 | 0 | 1 | 100 |
| Linezolid | . | | . | | . | | . | | 1 | 100 |
| Streptomycin | . | | . | | . | | 4 | 40 | 1 | 100 |
| **Total** | **1** | | **2** | | **1** | | **10** | | **1** | |

## DISCUSSION

In this study, 21 individuals were characterized as cases of pulmonary NTM according to the criteria from the ATS (2007) between July 2010 and June 2013 in the state of Pernambuco, representing 63.6% of all the samples which had positive culture to NTM. A total of (12/33, 36.4%) patients presented positive culture to NTM although they have not filled the diagnostic criteria for pulmonary infections caused by nontuberculous mycobacteria. It does not necessarily mean that the presence of NTM would not be the disease's cause, due unfortunately to the lack of accompaniment of the patients in whom the final diagnostic confirmation was not possible. According to Griffith et al (2007), monitoring these cases is highly necessary; with the lack of knowledge about NTM's physiopathology, it is required to have the identification between colonization and infection of slow evolution.

Concerning the risk factors which are associated to the diseases caused by NTM, the existence of a previous case of tuberculosis was the most present factor in this study. Some specific risk factors of the pulmonary diseases caused by NTM were identified in different studies, such as chronic pulmonary disease, advanced age, gender (male or female), HIV

infection and previous tuberculosis. Being the last factor the most important historically (Griffith et al, 2007; Marras & Daley, 2002). Different factors prove that the existence of previous tuberculosis may be a risk factor and also a facilitator to the pulmonary infection by NTM, such as the pulmonary alterations caused by *M. tuberculosis* and malnutrition, which generally is associated with this disease (Marras & Daley, 2002; Gupta et al, 2009). Cancer and COPD were not frequent (one case of each), agreeing with what was found by Costa et al (2012) in a study which was accomplished in the state of Pará, differently from what was found on the cases of NTM in the United States (Prevolts et al, 2010; Winthrop et al, 2010). It was noticed that males were more infected by NTM, agreeing with the published results of Marras and Daley (2002) and being contrary to the researches which relate that females are more affected by pulmonary diseases caused by NTM (Prevolts et al, 2010; Winthrop et al, 2010; Freeman at al, 2007; Cassidy et al, 2009). However, when we stratified by NTM species, we found that *M. abscessus subsp bolleti* has only caused disease in women. Griffith et al (2003) have found a predominance of females (65%) among 154 cases of pulmonary diseases caused by RGM. The cases of pulmonary NTM had an average age of 51 years old, and 57 % were above 57 years old, similar to what was found in other studies in Brazil and in the world (Winthrop et al, 2010; Cook, 2010; Billinger et al, 2009; Costa et al, 2013). New studies are necessary to elucidate what are the reasons of the possible association of pulmonary diseases caused by NTM related to the gender.

Radiological alterations on the pulmonary diseases caused by NTM have been classified basically as founds of cavitation (“classical form”) or nodules, associated with bronchiectasis (“non-classical”) (Martinez et al, 2007; Miller, 1994, Ellis & Hansell, 2002). However, some cases of NTM may not fit in this classification because they present the two forms combined (Costa et al, 2013). 44% of the evaluated cases in this study have presented more than two different types of X-Ray alterations, not fitting in the suggested classification by these authors.

Among the individuals who were classified as having pulmonary cases of NTM, 66.7% (14/21) were treated with the therapeutic scheme for tuberculosis and only 33.3% (7/2) were submitted to specific antimicrobial drugs for NTM. Besides, the diagnostic definition between tuberculosis and pulmonary NTM was performed in 52.4% (11/21) of the cases six months after the appearance of the symptoms and in 42.9% (9/21), in a year or more after the beginning of the clinical profile. These data point to the need for the differential diagnosis between tuberculosis and pulmonary mycobacteriosis, including the identification of the mycobacteria specie. Yet, in endemic countries for TB there is a delay on diagnosing and

identifying NTM species due to the lack of infrastructure of the laboratories and the directed attention of professionals and programs to TB. For this reason, most of the individuals have their diagnosis based only on the clinical profile and on the bacilloscopy's result, receiving an inadequate treatment, which can lead to a bacterial resistance (Gopinath & Singh, 2010).

For not being transmissible, the diseases which are caused by NTM are not required to be notified, except in cases of infection after surgical procedures in health services, leading to a lack of official records that can estimate its prevalence in the country (Griffith et al, 2007; Ueki, 2005). Likewise, there is a shortage of information about the diversity and frequency of NTM which are associated with pulmonary diseases, especially in endemic countries for TB where there is a deficiency of regional studies to evaluate the real prevalence of the infections by NTM (Gopinath & Singh, 2010).

The knowledge about the diversity and epidemiology of NTM species associated with pulmonary diseases is important because: (i) it allows the proper choice of laboratorial methods of diagnosis (ii) enables the recognition of the species which are associated to the disease and (iii) provides the information that will serve to improve the organization of the health services' network to attend these patients (Costa et al, 2012). In this study the sequencing of the specific genes *hsp65* and *rpoB* was performed to identify NTM species which were isolated from pulmonary samples, and it was observed that the most frequent isolated species were *M. kansasii* followed by *M. intracellulare*, *M. abscessus subsp abscessus*, *M. abscessus subsp bolleti*, *M. fortuitum* and *M. asiaticum*, respectively. When comparing it with other studies about pulmonary infections caused by NTM in Brazilian regions, it was found that in Pernambuco, the species which are associated with pulmonary disease are similar to the species found on the country's southeast and in the state of Bahia, where species from *M avium, M. kansasii, M fortuitum* and *M. abscessus* Complexes were associated with cases of pulmonary disease by NTM (Barreto & Campos, 2000; Matos et al, 2004; Ueki, 2005). However, it was different from what was observed by Costa et al (2012) in a study performed in the state of Pará, where the species *M. massiliense, M. simiae* followed by *M. intracellulare* and *M. avium* were the most frequent, probably due to characteristic environmental factors of this particular Brazilian region. Yet, the present study has identified a case where *M. asiaticum* was associated with pulmonary disease. This NTM was firstly described as a cause of disease in humans in 1980, and since then, only a few cases have been reported on literature. The major series of cases was in 24 patients from Australia and most of them were treated with drugs against tuberculosis (Grech et al, 2010). The pattern of antimicrobial susceptibility is not clearly established due to the lack of experience with this

NTM, but some reports suggest that *M. asiaticum* is susceptible to ethambutol, ethionamide, rifampicin, isoniazid and streptomycin, while other authors found resistance to isoniazid and rifampicin (Taylor et al, 1990; Dawson et al, 1995; Wayne & Sramek, 1992). Different studies show the success of the treatment to this NTM with anti-TB drugs (Dawson et al, 1995; Wayne & Sramek, 1992; Blacklock et al, 1983). The case of pulmonary disease caused by *M. asiaticum,* in this study, was treated with the therapeutic schema for tuberculosis for six months and it had a good response to the therapy.

The identification of NTM species plays a fundamental role on a proper therapeutic schema's choice to the patient, because it provides the first indication of antimicrobial susceptibility (Brown-Elliott et al, 2003; Wilson et al, 2001). The treatment of these infectious agents should be individualized and taken into account the drug sensitivity studies of the strains which cause disease (Alcaide & Esteban, 2010). However, there are several obstacles in the specific treatment for the diseases caused by NTM; perhaps the biggest one is that *in vitro* susceptibility testing may not be an efficient guide for the *in vivo* response to antibiotics (Griffith & Aksamit, 2011). Some species share this characteristic, such as *M. avium*, *M. abscessus*, *M. chelonae* , *M. immunogenum* , *M. scrofulaceum* , *M. xenopi* , *M. szulgai* , *M. simiae* and *M. malmoense* Complexes (Griffith & Aksamit, 2011). It was observed in this study, through antimicrobial sensitivity tests made with RGM, that the species *M. abscessus subsp abscessus* and *M. abscessus subsp bolleti* have shown resistance to most of the utilized drugs, being amikacin the only drug in which an *in vitro* activity was observed against both species. We agree with reports from other studies which demonstrate the resistance of *M. abscessus* to several drugs, leading to a difficulty of cure and for being often necessary to make surgical interventions in some cases of limited disease (Cowman et al, 2012; Jurand et al, 2011; Emmet & Paul, 2010). At the present time, there is not a safe antibiotic schema, even having basis on the *in vitro* susceptibility to achieve cure in the pulmonary disease caused by *M. abscessus* (Griffith et al, 2007). In this study it was found a case of one patient in whom *M. abscessus subsp bolleti* was isolated and despite of having the antimicrobial tests done, there was no success with the treatment, even with the addition of amikacin on the therapeutic scheme, extending it for more than a year.

The pulmonary disease caused by *M. fortuitum* is clinically similar to the disease caused by *M. abscessus* (Griffith et al, 1993). However, this NTM specie rarely affects the lungs, being more common in infections of the skin, bone and soft tissues (Griffith et al, 2007). It is important to have the therapy based on susceptibility, using at least two agents which have shown *in vitro* activity against this pathogen, and monitoring at least for 12

months, with negative sputum cultures (Griffith et al, 2010). The two individuals from this study who had pulmonary disease associated with *M. fortuitum* were submitted to a treatment for over than a year with the association of more than two drugs and they have obtained an evident clinical improvement. *M. kansasii* generally provides a better therapeutic response among the NTM which cause pulmonary disease, and it has a good clinical correlation between *in vitro* results and the drugs. It normally presents susceptibility to rifampicin, macrolides and fluoroquinolones besides a good *in vivo* response to these antimicrobial agents. The biggest impediment seems to be the patient's access to the therapy, leading to the risk of acquired resistance to these drugs, similarly observed with M. tuberculosis. (Jurand et al, 2011). In this study, among the cases with the isolation of *M. kansasii* and in which the antibiogram was performed, only one isolate presented resistance to rifampicin, and after the unsuccessful therapeutic scheme for TB, the treatment with amikacin, clarithromycin and ciprofloxacin had begun with an evident clinical improvement. Alcaide & Esteban (2010) showed that other second choice agents presented activity against *M. kansasii*, such as the fluoroquinolones and sulfamethoxazole; however, pyrazinamide is contraindicated due to the *in vitro* resistance of *M. kansasii* to this drug.

Finally, we highlight the relevance of the study, which aims to contribute on elucidating in an unprecedented way in the state of Pernambuco, the frequency and diversity of the NTM species which are associated with pulmonary diseases, besides seeking the real magnitude of this pathology in a region that is known for being endemic for tuberculosis.

Our data show that there is a variety of NTM species which are involved in cases of pulmonary bacteriosis in the state of Pernambuco. In this context, it is necessary to perform the differential diagnosis between tuberculosis and pulmonary disease caused by NTM, especially in individuals with reports of previous tuberculosis or who failed TB's treatment. It was also verified that identifying the specie is one factor that should be considered when performing the final diagnosis and that the absence of a regional reference center may be reflecting in mistakes on the diagnosis and on inadequate treatments.

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## 8 CONCLUSÕES

a) Em regiões endêmicas para tuberculose podemos considerar que uma das principais razões para subdiagnóstico das infecções causadas por MNT sejam os limitados recursos laboratoriais disponíveis. Os testes convencionais utilizados para o diagnóstico das micobacterioses e tuberculose (baciloscopia e cultura e testes bioquímicos) deixam lacunas na capacidade de realizar o diagnóstico diferencial entre a tuberculose e as doenças causadas por micobacterias não tuberculosas. As técnicas de biologia molecular (PCR multiplex, PRA-hsp65 e sequenciamento de genes específicos) podem ser ferramentas valiosas para identificação das espécies de micobactérias reduzindo o tempo necessário para diagnóstico correto e início do tratamento adequado. Porém para a implantação de técnicas moleculares nos laboratórios de referências é necessário além da disponibilidade de recursos financeiros, pessoal treinado para a realização dos testes;

b) Os casos de micobacteriose extrapulmonar, no estado de Pernambuco, foram identificados em mulheres submetidas a procedimentos cirúrgicos invasivos (mamoplastia e abdominoplastia) e as espécies associadas a estes casos foram as micobactérias de crescimento rápido (MCR) : *M. fortuitum 2 ( 50%),* 1 (25%) *M. wolinskyi* e 1(25%) *M. novocastrense.* Durante o período do estudo não foram notificados surtos de infecções por MCR em serviços públicos ou privados do estado. No entanto, diante do difícil tratamento destas infecções e os sérios danos causados à saúde humana dos indivíduos infectados, faz-se necessário o controle sistemático dos procedimentos de esterilização dos instrumentos cirúrgicos nos serviços de saúde para a prevenção de possíveis novos casos;

c) Os casos de infecção pulmonar foram a maioria (84%) entre as infecções por MNT no estado de Pernambuco. As principais características encontradas nestes casos foram: Sexo masculino, idade superior a 50 anos e estória de infecção prévia por tuberculose;

d) O tratamento para as MNT deve ser individualizado e levar em consideração os estudos de sensibilidade a drogas das cepas causadoras da doença. Neste estudo observou-se que as espécies *M. abscessus subsp abscessus* e *M. abscessus subsp bolleti* isoladas de casos pulmonares demonstraram resistência à maioria das drogas utilizadas, sendo a amicacina a única droga em que foi observada atividade *in vitro* contra as duas espécies refletindo em grande dificuldade no tratamento destes indivíduos;

e) Nossos dados demostram que existe uma variedade de espécies de MNT envolvidas em casos de micobacteriose pulmonar no estado de Pernambuco. Neste contexto, faz-se necessário o diagnóstico diferencial entre tuberculose e doença pulmonar por MNT, sobretudo em indivíduos com relato de tuberculose anterior ou que falharam o tratamento para TB.

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**APÊNDICE A – Protocolo para coleta de dados clínicos, epidemiológicos e laboratoriais.**

**PROTOCOLO DE COLETA DE DADOS**

**PESQUISA DE MICOBACTERIAS ( caso –controle)**

**IDENTIFICAÇÃO**

| | |
|---|---|
| **1. Número da ficha na pesquisa** | **2. Data da entrevista** _____/_____/_____ |
| **3. Procedência:**<br>1. Ambulatório<br>2. Enfermaria | **4. Número do Prontuário do Hospital** |
| **5. Hospital de origem:**<br>1. Hospital das Clínicas<br>2. Hospital Otávio de Freitas<br>3. IMIP<br>4. Hospital Barão de Lucena<br>5. Outro: _____ | **6. Número do SAME** |

**DADOS DO PACIENTE**

**7. Nome Completo do Paciente**

_____

**8. Nome da Mãe ou Responsável**

_____

| | | |
|---|---|---|
| **9. Data de nascimento** _____/_____/_____ | **10. Idade do paciente** ____ anos ____ meses | **11. Sexo**<br>1. Feminino<br>2. Masculino |

**12. Endereço**

_____

_____

**Ponto de referência**

_____

| | | |
|---|---|---|
| **13. Bairro** _____ | **14. Cidade** _____ | **15. UF** |
| **16. Telefone Res. e Celular**<br>(____)_____ - _____<br>(____)_____ - _____ | **17. CEP** _____ | **18. Zona de localização da moradia**<br>1. Urbana<br>2. Rural |
| **19. Grau de instrução**<br>1. Analfabeto<br>2. Iniciou alfabetização<br>3. 1° grau<br>4. 2° grau<br>5. 3° grau<br>6. outro | **20. Qual é a sua ocupação?** _____ | |
| | | |
| | | |

**ANTECEDENTES DO PACIENTE**

| | |
|---|---|
| **21. Tem alguma doença de base?**<br>1. Sim<br>2. Não<br>8. Não sabe informar ☐ | **22. Qual doença?**<br>1. Asma 6. Fibrose Cística<br>2. Pneumonia 7. HIV/AIDS<br>3. Bronquite 8. Câncer<br>4. Tuberculose 9. Renal<br>5. Diabetes 10. Outro: _____ ☐ |
| **23. Uso de imunossupressor ?**<br>1. Sim<br>2. Não ☐<br>Qual: _____ | **24. Já fez TTO para tuberculose?**<br>1. Sim<br>2. Não ☐<br>Qual: _____<br>**Caso sim, quanto tempo?** _____<br>**Caso sim, número de TTO anteriores:** _____ |
| **25. Já foi submetido a algum processo cirúrgico?**<br>1. sim ☐<br>2. não | **26. Qual o procedimento cirúrgico?**<br>1. Invasivo ☐<br>2. Não invasivo ☐ |
| **27. A quanto tempo realizou o procedimento cirúrgico**<br>**1. < de 1 ano** ☐<br>**2. 1 a 3 anos** ☐<br>**3. > 3 anos** ☐ | |

| **Hábitos de vida** | |
|---|---|
| **27. O senhor(a) fuma?**<br>**1. Sim** ☐<br>**2. Não** ☐ | 28. **Com qual que frequência você toma cerveja, vinho, pinga ou qualquer outro tipo de bebida alcoólica?**<br>1 - Todos os dias 5 - 2 a 3 dias por mês<br>2 - Quase todos os dias 6 - Uma vez por mês<br>3 - 3 a 4 dias por semana 7- Menos de uma vez por mês<br>4 - 1 a 2 dias por semana ☐ |
| **29. O senhor(a) usa drogas**<br>**1. sim** ☐<br>**2. não** ☐<br>**Se sim qual?**_____ | 1. |
| **Características clínicas** | |
| **30. Há quanto tempo o paciente está doente?**<br>**_____meses_____dias** | 31.Tem febre?<br>**1.Sim** ☐<br>**2.Não** ☐ |
| 32. Tem tosse?<br>**1.Sim** ☐<br>**2.Não** ☐ | **33. Tipo de tosse?**<br>**1. seca**<br>**2. produtiva**<br>**3. hemoptise**<br>**9.inaplocável** |
| 34. Tem dispnéia ou falta de ar?<br>**1.Sim** ☐<br>**2.Não** ☐ | **35. Perda de peso?**<br>**1.Sim** ☐<br>**2.Não** ☐ |
| 36. **Notou linfonodo ( íngua) aumentado?**<br>1. Sim<br>2. Não ☐<br>9. Inaplicável | **37. Local do linfonodo**<br>**1. Pescoço**<br>**2. Axila**<br>**3. Região inguinal (virilha)**<br>**Outros**_____ |
| 38. Presença de sinais inflamatórios (calor, dor, vermelhidão):<br>1. Sim ☐<br>2. Não,<br>8. Não investigado | |

| | Exames laboratoriais | |
|---|---|---|
| **39. Realizou Cultura**<br>1. Sim<br>2. Não | 40. Resultado da cultura<br>1.Positiva<br>2. Negativa<br>9. Inaplicável ☐<br>_____Cruzes Resultado da Cultura Data:___/____/_____<br>10. | |
| **41.Material da cultura**<br>1. Escarro<br>2. Lavado gástrico<br>3. Líquor ☐<br>4. Biópsia<br>5. Outro material<br>9. Inaplicável<br>Outro _____ | 42. Tempo de crescimento bacteriano em cultura<br>1. 1ª semana<br>2. 2ª semana<br>3. 3ª semana ☐<br>4. 4ª semana<br>5. 5ª semana ou mais<br>9. inaplicável | |
| **43. Realizou Baciloscopia?**<br>1. Sim<br>2. Não ☐<br>Data:___/____/_____ | **44. Resultado da bacilos copia:**<br>2. Positiva<br>3. Negativa<br>9. Inaplicável ☐<br>_____Cruzes | **45. Material da baciloscopia:**<br>1. Escarro<br>2. Lavado gástrico<br>3. Líquor ☐<br>4. Biópsia<br>5. Outro material<br>9. Inaplicável<br>Outro _____ |
| **46. Realizou tomografia ou ultrassonografia?**<br>**1. Sim**<br>**2. Não** ☐ | **47. Resultado Tomografia:**<br>**1. Normal**<br>**2. Alterado** ☐<br>**Alterações:** | **48. Resultado da ultracenografia:**<br>**1. Normal**<br>**2. Alterado**<br>**Alterações:** |
| **49. realizou biópsia?**<br>**1. Sim**<br>**2. Não** ☐ | **50. Resultado da biopsia:**<br>**1. Normal**<br>**2. Alterado**<br>**Alterações:** | |
| **51. Realizou RX tórax?**<br>**1. sim**<br>**2. não** ☐ | **52. Resultados RX tórax:**<br>**1. Normal**<br>**2. Alterado**<br>**Alterações:** | |

## Diagnóstico final e evolução

53. Diagnóstico final pelo médico do Serviço de saúde
1. Tuberculose pulmonar
2. Tuberculose extrapulmonar ☐
3. Micobacteriose pulmonar
4. Micobacteriose extrapulmonar

**54. Se extrapulmonar qual a forma**
**1. pleural**
**2. ganglionar**
**3. Renal**
**4. óssea** ☐
**5. Miliar**
**6. Nasocomial**
**7. outras**

55. Iniciou tratamento
1. sim
2. não ☐

**56. Qual o esquema terapêutico?**

_____

**57- Resposta ao tratamento específico (feita pelo médico acompanhante)**

1. Melhora clínica evidente após 30 dias de início do tratamento (Resposta ao tratamento)
2. Não houve melhora clínica evidente após 30 dias de início do tratamento (Não houve resposta ao tratamento) ☐
9. Inaplicável

| Entrevistador: | Assinatura: |
|---|---|
| _____ | _____ |

## DIAGNÓSTICO MOLECULAR

| **58. Realizou sequenciamento de gene específico** | **59. Identificação da espécie** | **60. Qual espécie de micobactéria identificada?** |
|---|---|---|
| 1. Sim<br>2. Não ☐<br>Data:___/____/_____ | 1. Sim<br>2. não<br>9. Não se aplica ☐ | _____<br>_____ |
| **61. Resistência a Antibióticos?** | **64. Qual o perfil de resistência?** | |
| **1. Sim**<br>**2. Não** ☐ | _____<br>_____ | |

Responsável pelos exames moleculares:_____

**ANEXO A- Parecer do comitê de ética em pesquisa-CPqAM/Fiocruz**

CEP
Comitê de Ética em Pesquisa

**Título do Projeto:** "Fatores e espécies de Micobactérias não tuberculosas associados aos casos de micobacterioses pulmonar e extrapulmonar no estado de Pernambuco: estudo caso-controle".
**Pesquisador responsável:** Andrea Santos Lima.
**Instituição onde será realizado o projeto:** CPqAM/FIOCRUZ
**Data de apresentação ao CEP:** 15/10/12
**Registro no CEP/CPqAM/FIOCRUZ:** 34/12
**Registro no CAAE:** 07382012.4.0000.5190

**PARECER Nº 06/2013**

O Comitê avaliou as modificações introduzidas e considera que os procedimentos metodológicos do Projeto em questão estão condizentes com a conduta ética que deve nortear pesquisas envolvendo seres humanos, de acordo com o Código de Ética, Resolução CNS 196/96, e complementares.

O projeto está aprovado para ser realizado em sua última formatação apresentada ao CEP e este parecer tem validade até 06 de março de 2016. Em caso de necessidade de renovação do Parecer, encaminhar relatório e atualização do projeto.

Recife, 06 de março de 2013.

Coordenadora do CEP/CPqAM

Observação:
Anexos:
- Orientações ao pesquisador para projetos aprovados;
- Modelo de relatório anual com 1º prazo de entrega para 06/03/2014.

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