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Critically Appraised Topic

Transmission of nontuberculous Mycobacteria (NTM) between patients with cystic fibrosis: is there evidence for person-to-person transmission? Which techniques are available to investigate the NTM transmission?

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Date: 08/03/2016

Clinical bottom line

Nontuberculous Mycobacteria (NTM) are pathogens causing chronically lung infections, especially in cystic fibrosis (CF) patients. The last decades, the prevalence of NTM in CF patients is rising due to several reasons. As the treatment comprises several months and is very intensive for the patient, newly infected patients must be avoided. Until 2012, it was believed the acquisition of NTM in CF patients was only due to environmental exposure, but in 2012 Aitken *et al.* were the first to suspect person-to-person transmission of NTM within the CF population. One year later, Bryant *et al.* also had strong evidence for person-to-person transmission; however, Harris *et al.* could not confirm this. Until now, it is not yet clearly understood whether person-to-person transmission occurs. The main limitation and difficulty of most studies is the fact that each CF center only consists of a few NTM positive CF patients, which makes it difficult to interpret results. Therefore one of the 'to do actions' in the future should include the creation of a database where all the information of NTM isolates from different CF centers is available. This would facilitate making stronger conclusions, as it would be possible to compare much more NTM isolates.

Over the past 2 decades, NTM have emerged as important pathogens in the setting of CF lung disease. Although the incidence of NTM disease in the general population of industrialized countries is approximately 1 in 100,000 there is a 10,000-fold greater prevalence of these organisms in respiratory cultures from patients with CF[1]. In most studies the prevalence of positive NTM cultures and/or NTM infection within CF patients is 6% to 13% and tends to increase[2–4,5]. In reality, the importance and clinical significance of NTM in CF patients seems to vary depending on the mycobacterial species and on between patients. In many cases, an important question following a positive NTM cultures in CF patients will be to determine whether this is due to contamination, a transient colonization or a clinically relevant infection for which a targeted treatment is necessary[1,6,7].

The Infectious Diseases Society of America and the American Thoracic Society (IDSA/ATS statement, published in 2007) suggest NTM screening in adult and adolescent CF patients once a year. On top of this annual screening, CF patients – including younger children – must be evaluated for NTM infection in the presence of clinical deterioration which is unresponsive to a treatment targeting usual bacterial pathogens[8]. Although it is clear that CF patients should be screened, it is very important that an efficient and effective screening method will be determined[4,5,8].

There remain some uncertainties in regard to the infection route of NTM. Initial studies have suggested acquisition of NTM from the environment, but the idea of environmental contamination as the only route of infection may need to be reviewed[4,9]. The concern that *M. abscessus* could spread horizontally between CF patients has recently increased[1,2,5].

As the University Hospital Saint-Luc Brussels acts as one of the 7 CF centers in Belgium, it is important to acquire knowledge about the optimal NTM screening method and furthermore to investigate if NTM person-to-person transmission (direct or indirectly) in CF patients occurs. Therefore, we should examine the best laboratory technique for epidemiological studies by doing a review of the literature.

Question(s)

1. What is the importance and clinical significance of NTM in CF patients?
2. How should NTM screening in CF patients be performed (pre-analytical and analytical considerations)?
3. Is there evidence for person-to-person transmission in CF patients (direct or indirect transmission)?
4. Which techniques are available to investigate the NTM transmission in CF patients?

Search terms

1. MeSH Database (PubMed): MeSH term: "Atypical Mycobacterium", "Mycobacterium avium Complex", "Nontuberculous Mycobacterium"
2. PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters: "NTM transmission", "NTM CF patients", "NTM lung disease treatment", "M. abscessus CF", "M. avium complex CF"
3. Guidelines: "CF Clinical Care Guidelines - Cystic Fibrosis Foundation (CFF)", An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases", "Infection Prevention and Control Guideline for Cystic Fibrosis: 2013 Update - CFF and SHEA", "US Cystic Fibrosis Foundation and European Cystic Fibrosis Society consensus recommendations for the management of non-tuberculous mycobacteria in individuals with cystic fibrosis - CFF and ECFS"

Relevant evidence/References

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1 INTRODUCTION

1.1 CYSTIC FIBROSIS (CF)

Cystic fibrosis (CF) is the most frequent genetic disease in Europe, with an estimated prevalence ranging between 1/8,000 and 1/10,000[10]. CF is caused by autosomal recessive mutations in the CF transmembrane conductance regulator (CFTR) gene on chromosome 7. This leads to a reduction in the transport of sodium and chloride across epithelial surfaces, resulting in viscous respiratory and gastrointestinal secretions which on its turn lead to multi-organ disease. The obstruction of the small and medium-sized airways is the main cause of morbidity and mortality among CF patients, due to bronchiectasis, chronic airway infections, and progression toward respiratory failure. Currently the expected lifespan of CF patients is 37 years[5].

Even patients which are heterozygous for CFTR gene mutations have higher risk to develop bronchiectasis and NTM lung disease. Although these patients do not meet criteria for CF, they have bronchial epithelial ion and water transport abnormalities which may lead to the development of bronchiectasis.

1.2 TAXONOMY AND CLASSIFICATION OF NONTUBERCULOUS MYCOBACTERIA (NTM)

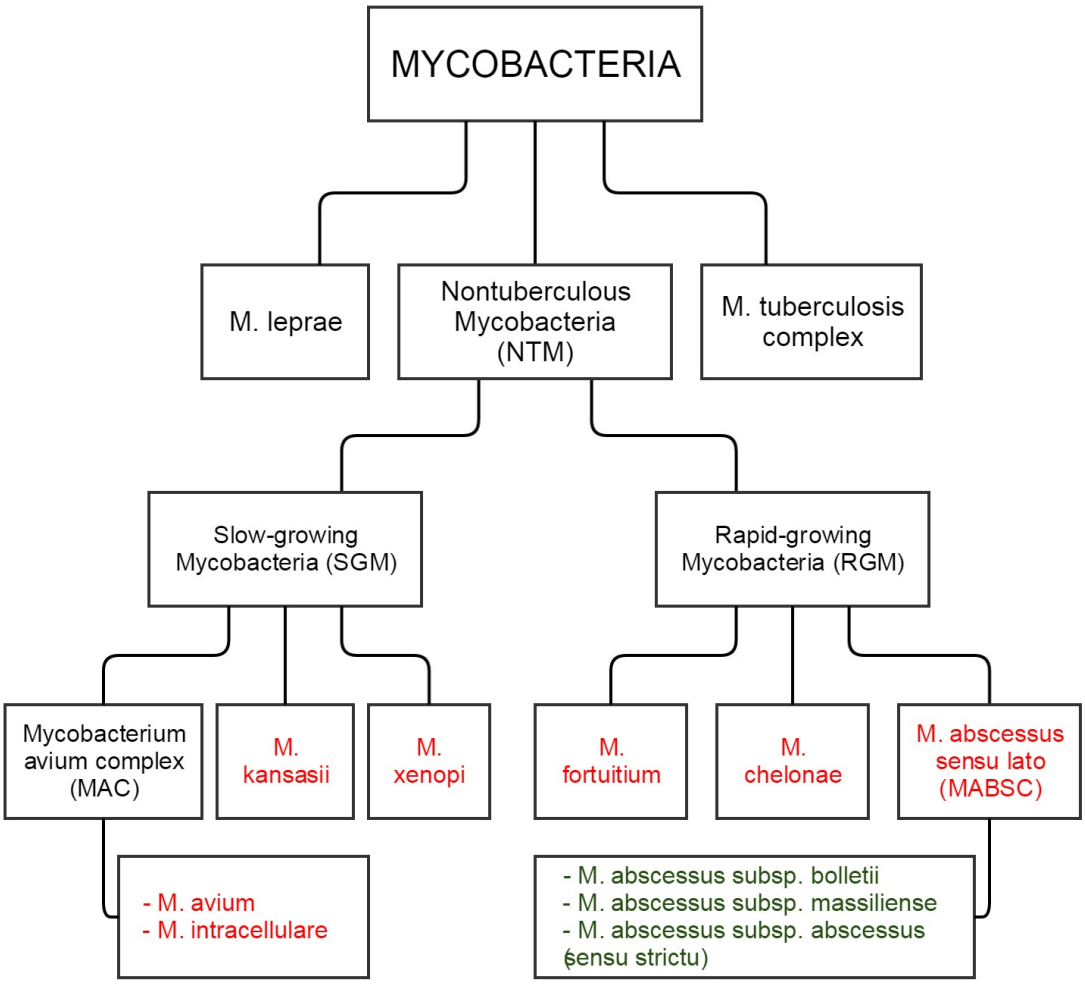
Mycobacteria are aerobic, non-motile organisms that appear positive with acid-fast alcohol stains. They have a lipid rich, hydrophobic cell wall, which is substantially thicker than most other bacteria. The thickness and composition of the cell wall renders Mycobacteria impermeable to hydrophilic nutrients and resistant to heavy metals, disinfectants, and antibiotics.

In the past, NTM or atypical Mycobacteria were considered as benign environmental ubiquitous bacteria, that can be found in water and soil, associated with random colonization and only rarely with genuine infection of the airway. Since the end of the eighties, NTM have gained importance because it was widely accepted that NTM are opportunistic micro-organisms capable of causing diseases in a different range of locations (skin and soft tissues, lymph nodes and lungs) as well as disseminated disease, especially in patients with underlying diseases, such as acquired immune deficiency syndrome (AIDS) or CF.

NTM represent over 150 different species, most of which do not seem to cause human diseases except in individuals with immune deficiency.

NTM can be roughly divided in slow-growing Mycobacteria (SGM) with *Mycobacterium avium complex* (MAC), consisting of the species *M. avium and M. intracellulare,* as the most important entity, and rapidly-growing Mycobacteria (RGM) with *M. abscessus complex* (MABSC) as the most important species (see figure 1). 10 years ago, the latter species was divided up in 3 subspecies based on *rpoB* sequences: *M. abscessus subsp. bolletii, M. abscessus subsp. massiliense and M. abscessus subsp. abscessus* (*M. abscessus sensu stricto*)[11].

Figure 1: Classification of Mycobacteria (red: species, green: subspecies)



1.3 CLINICAL SYNDROMES OF NTM INFECTIONS

Although NTM can affect all organs of the human body, they primarily affect the lungs. Progressive pulmonary disease is usually associated with bronchiectasis or chronic obstructive pulmonary disease (COPD). Symptoms are generally nonspecific, however, virtually all patients have chronic or recurring cough. This typically progresses slowly and may include: blood in sputum (mucus and other matter brought up from the lungs, bronchi, and trachea), cough, fever, nausea, night sweats, weight loss,…

Other clinical syndromes include:

- superficial lymphadenitis: in children (mostly cervical lymphadenitis)
- skin and soft tissue infection
- disseminated disease: in immunocompromised patients

1.4 EPIDEMIOLOGY OF NTM LUNG INFECTIONS IN THE CF POPULATION

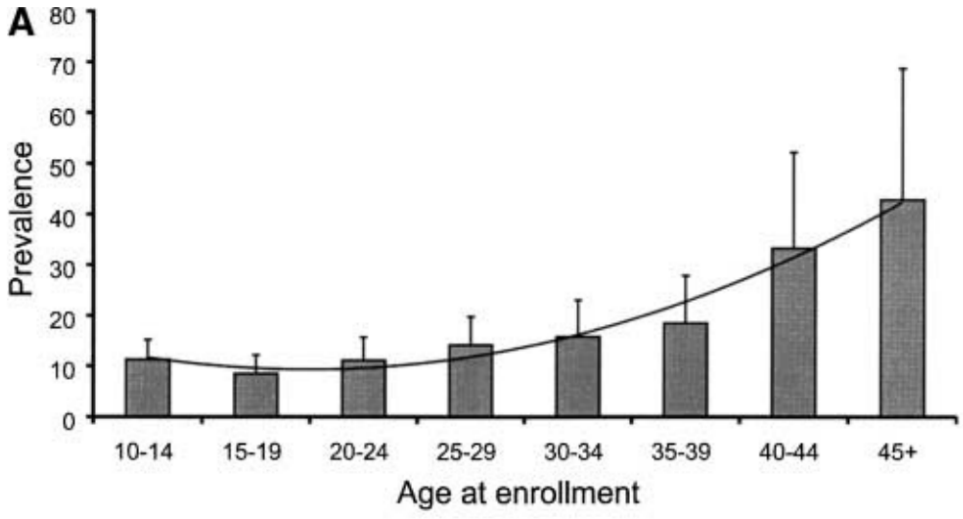
The prevalence of positive NTM cultures in CF patients varies between the region and increases with the age of the patients. One of the reasons that explains a higher prevalence of NTM among CF patients compared to the general population is the underlying structural airway disease and altered mucociliary clearance that acts as predisposing factors[12].

The NTM species most commonly reported among CF patients are MAC and MABSC. Other less commonly isolated species include *M. simiae, M. kansasii, M. gordonae* and *M. fortuitum*.

In Europe the most important NTM in lung disease within CF patients is MABSC, which has a prevalence of 13% in CF patients[4]. In the USA, there have been reported more infections due to MAC compared to MABSC. The prevalence is rising due to several reasons including improved laboratory practices nowadays, improved patient survival and inhaled antibiotic usage[1,6-7]. All these facts are responsible that NTM have emerged as new pathogens in CF in the last 20 years. However, not all positive NTM cultures represent infection.

According to a study performed in the USA, the prevalence of NTM seems to be highly correlated with age, approaching 40% in patients older than 40 years compared with 10% in children and young adults (see graphic 1)[5,9].

Graphic 1: Prevalence of subjects with at least one positive culture for NTM from each age category (Olivier *et al.*, 2003)

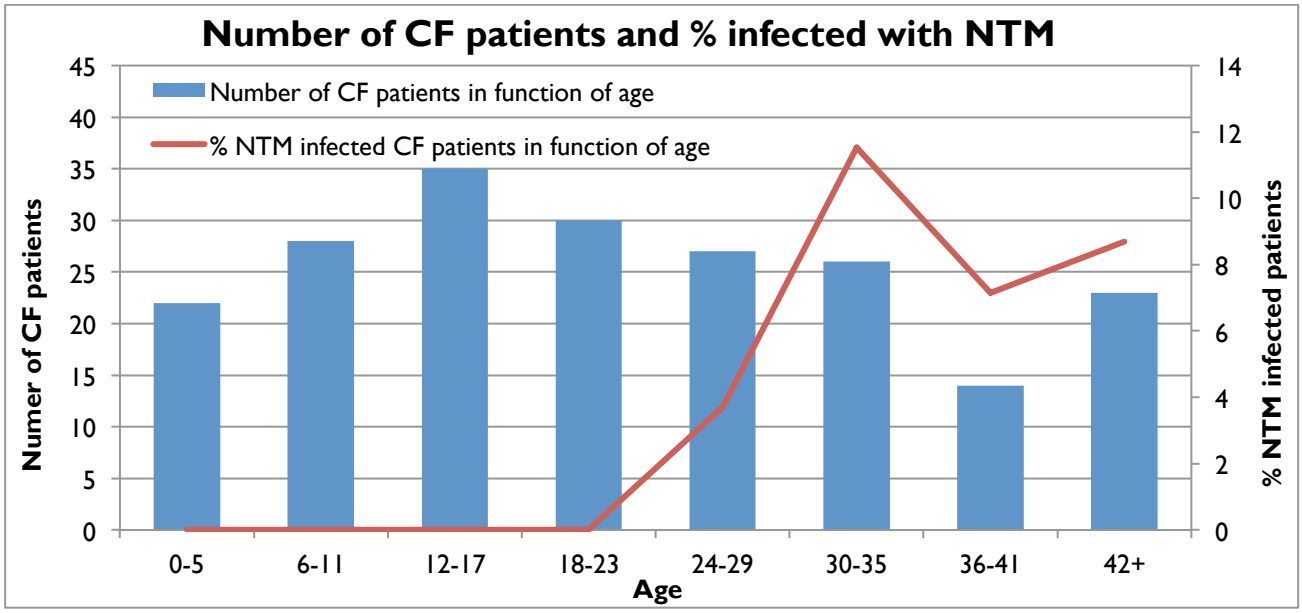


In the CF center of the university Hospital Saint-Luc Brussels 205 CF patients are treated, including 85 children (< 18 years old). 6 patients are chronically infected with NTM: 5 MABSC and 1 MAC

(see graphic 2). Additionally one patient had a positive culture with M. gordonae, a non-pathogenic NTM.

The prevalence is 3.4% in the whole population and 5.8% in the adult (> 18 years old) population, with a peak in the > 30 years old patients (7-12%). The prevalence in our center is slightly lower than the prevalence mentioned in the literature.

Graphic 2: Number of CF patients and proportion of CF patients infected with NTM (data of Saint-Luc Hospital, Brussels)



In table 1 the 7 CF patients which are NTM positive are listed. The data presented illustrate the important variation that exists between patients in regard to NTM infection. Patients 1 and 2 probably presented contamination; patient 5 had 2 positive smears and cultures, which may be associated to a short infection episode. Patients 3, 4, 6 and 7 presented numerous and repeated positive samples, and therefore a more chronic form of NTM lung disease.

Table 1: CF patients with NTM positive stain and culture

Patient	Stain +	Culture +
1	0/22	2/22
2	0/21	1/21
3	22/42	26/42
4	5/11	5/11
5	2/9	2/9
6	10/32	8/32
7	49/87	65/87

1.5 DIAGNOSIS OF NTM LUNG INFECTIONS

CF patients suffer from chronic lung infections due to disruption of exocrine function that does not allow them to clear certain microorganisms (table 2).

In young children, infections are caused by organisms frequently seen in non-CF pediatric patients such as *H. influenzae, S. pneumoniae* and *S. aureus*. Later on, *P. aeruginosa* becomes the most important pathogen involved in chronic lung infection and is capable to form a biofilm (mucous colonies). Finally pathogens such as *S. maltophilia* and *Achromobacter* species may be acquired during a hospital or clinic visit.

As mentioned before, in CF patients, pathogens such as NTM have been isolated with increasing frequency. Therefore Mycobacterial culture should be added to the routine cultures for CF patients.

Table 2: Laboratory Diagnosis of Pulmonary Infections in Cystic Fibrosis (IDSA guideline: A Guide to Utilization of the Microbiology Laboratory for Diagnosis of Infectious Diseases: 2013 Recommendations by the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM))

Etiologic Agents	Diagnostic Procedures	Optimum Specimens	Transport Issues; Optimal Transport Time
Bacteria			
Staphylococcus aureus *Haemophilus influenzae* *Streptococcus pneumoniae* Enteric bacilli *Pseudomonas aeruginosa* *Stenotrophomonas maltophilia* *Achromobacter* spp	Culture	Expectorated sputum; throat swabs[a]; other respiratory samples	Sterile container, RT, 2 h; >2–24 h, 4°C
Burkholderia cepacia complex	Culture using *Burkholderia cepacia* selective agar	Throat swabs[a], expectorated sputum; other respiratory cultures	Sterile container, RT, 2 h; >2–24 h, 4°C
Opportunistic glucose nonfermenting gram-negative rods *Burkholderia gladioli* *Ralstonia* spp *Cupriavidus* spp *Pandorea* spp	Culture	Expectorated sputum; throat swabs[a]; other respiratory samples	Sterile container, RT, 2 h; >2–24 h, 4°C
***Mycobacterium* spp**			
Mycobacterium abscessus *Mycobacterium avium* complex	Mycobacteria culture Mycobacteria culture	Expectorated sputum, bronchoscopically obtained cultures; other respiratory cultures	Sterile container, RT, 2 h; >2–24 h, 4°C
Fungi			
Aspergillus spp *Scedosporium* spp *Trichosporon*	Calcofluor -KOH or other fungal stain Fungal culture	Expectorated sputum, bronchoscopically obtained cultures; other respiratory cultures	Sterile container, RT, 2 h; >2–24 h, 4°C
Viruses			
RSV Influenza Adenovirus Rhinovirus Coronavirus Parainfluenza virus Human metapneumovirus	Rapid antigen detection DFA Viral culture methods NAAT[b]	Nasal aspirates, nasal washes, NP swabs, throat washes, throat swabs; bronchoscopically obtained specimens	Transport in viral transport media, RT or 4°C, 5 d; –70°C, >5 d

Microbiological diagnosis of NTM is challenging, and furthermore – as illustrated above, the interpretation of a positive result has to be done with caution, as distinguishing infection from colonization can be difficult. The term colonization has been used when NTM are recovered more than once without clinical symptoms[13-14]. For this reason, a single positive culture from nonsterile sources including the respiratory or digestive tract does not systematically indicate infection or disease. When interpreting a positive result, the microbiologist and the clinician must keep in consideration that NTM may colonize the airways without causing disease, but at the same time, impaired pulmonary clearance mechanisms can contribute to the evolution from colonization to infection[15].

In 2007 the ATS/IDSA has composed some criteria to facilitate the diagnosis of NTM lung infection[8]. These criteria are valid for the whole population and have not been specifically written for CF patients, but the Cystic Fibrosis Foundation (CFF) and the European Cystic Fibrosis Society (ECFS) recommend using the ATS/IDSA criteria also for the diagnosis of NTM pulmonary disease within CF patients[13]. We should also take into consideration that the risk of NTM infection is much higher in the CF population due to underlying bronchiectasis, so the probability to be actually infected with a positive NTM culture in the CF population is much higher compared to the whole population.

For NTM lung diagnosis, first of all, pulmonary symptoms + radiographic features of NTM lung disease must be present (see section 1.3 Symptoms of NTM infection) and other diagnoses must be excluded. The second criterion includes positive microbiological NTM culture (see table 3).

Table 3: Clinical and microbiological criteria for diagnosing NTM lung disease (An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases, 2007)

Clinical (both required)

1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or a high-resolution computed tomography scan that shows multifocal bronchiectasis with multiple small nodules (A, I)*

and

2. Appropriate exclusion of other diagnoses (A, I)

Microbiologic

1. Positive culture results from at least two separate expectorated sputum samples (A, II). If the results from (1) are nondiagnostic, consider repeat sputum AFB smears and cultures (C, III).

or

2. Positive culture result from at least one bronchial wash or lavage (C, III)

or

3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM (A, II)
4. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination (C, III)
5. Patients who are suspected of having NTM lung disease but do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded (C, III)
6. Making the diagnosis of NTM lung disease does not, *per se*, necessitate the institution of therapy, which is a decision based on potential risks and benefits of therapy for individual patients (C, III)

As mentioned above, a single positive sputum culture (especially with a small number of organisms) is generally regarded as vague for diagnosis of NTM lung disease.

Tsukamura *et al.* showed that patients with MAC isolated from three specimens (3/3) had progressive radiographic abnormalities in 98% of the cases. Patients with MAC isolated from two specimens (2/3) showed 90% radiographic abnormalities. This in contrast to patients with only one positive out of three specimens (1/3) who had radiographic abnormalities in only 2% of the cases[16] (see table 4).

Table 4: Progressive radiographic abnormalities in function of positive NTM (M. avium complex) samples (Tsukamura *et al.*, 1991)

# positive NTM samples (MAC)	progressive radiographic abnormalities
1	2%
2	90%
3	98%

However, these findings should be interpreted with caution, since the patients included in the study of Tsukamura *et al.* not only consisted of CF patients. CF patients usually have pre-existing radiographic abnormalities (bronchiectasis,…).

1.6 TREATMENT AND PITFALLS OF NTM LUNG INFECTIONS

Infections due to NTM are difficult to treat because they are intrinsically resistant to the classical anti-tuberculosis drugs (rifampicin, isoniazid and ethambutol) and furthermore to most of the antibiotics that are currently available. The antibiotics, used in some cases, are listed in table 5[15]. Especially macrolide antibiotics, i.e. clarithromycin and azithromycin, are very important for the treatment of NTM.

Table 5: Antibiotics used in Mycobacterium abscessus pulmonary infections

Antibiotic	Frequency	Route
Amikacin	7 - 10 mg/kg once daily	IV
	250 – 500 mg twice daily	nebulized
Azithromycin	250 – 500 mg once daily	oral
Clarithromycin	500 mg twice daily	oral
Cefoxitin	4 g twice daily	IV
Imipenem	750 – 1000 mg twice daily	IV
Linezolid	300 – 600 mg once daily	oral
Tigecycline	25 – 50 mg daily	IV

Resistance to aminoglycosides, β-lactam antibiotics,… are already well known, but recently a inducible erythromycin ribosomal methylation (*erm(41)*) gene has been recognized in *M.*

abscessus and has been associated with poor clinical response to treatment with macrolides[11,17]. The *erm(41)* gene encodes for enzymes that are able to methylate the 23S ribosomal RNA within the 50S ribosomal subunit, resulting in reduced binding affinity of macrolides. The relatively slow induction of macrolide resistance has led the Clinical and Laboratory Standards Institute (CLSI) to recommend 14 days incubation of rapidly growing Mycobacteria isolates when tested against clarithromycin. In general, the *erm(41)* gene is present, but not expressed in all 3 *M. abscessus* subspecies: *M. abscessus subsp. abscessus* and *M. abscessus subsp. bolletii* have universal expression of an inducible *erm(41)* gene conferring macrolide resistance whereas the *erm(41)* gene in *M. abscessus subsp. massiliense* strains contains a large 397-bp deletion, resulting in a nonfunctional *erm(41)* gene and consequently no macrolide resistance[11].

However, Nash *et al.* described two strains of *M. abscessus subsp. abscessus* which contained a nonfunctional *erm(41)* gene, resulting in sensibility to clarithromycin[17]. This was due to a T-to-C substitution at position 28 of the gene (T28C).

A second mechanism resulting in macrolide resistance in *M. abscessus* includes point mutations in the *rrl* gene, encoding 23S rRNA[18].

2 QUESTION 1: WHAT IS THE IMPORTANCE AND CLINICAL SIGNIFICANCE OF NTM IN CF PATIENTS?

Previously thought as a group of rather benign environmental bacteria associated with random colonization and only rarely with genuine infection of the airway, perceptions have now changed. Pulmonary disease caused by NTM may occur as a component of disseminated infection, but often the disease only affects the lungs.

It has already been mentioned that the prevalence of NTM in CF patients is 6-13%. The proportion of those who are chronically infected is not clear. In a study of Martiniano *et al.*, 96 (26 MABSC and 70 MAC) out of 650 CF patients (prevalence: 14.8%) had positive NTM cultures. 38.5% of CF patients with a first positive NTM culture (37 out of 96 patients) suffered active NTM infection and did meet the ATS/IDSA definition (at least two positive cultures and clinical and radiographic progression, see table 3)[19]. The frequency of active NTM disease was not statistically different in subjects infected with MABSC (46% active NTM disease) compared with the MAC infected patients (36% active NTM disease). In the 3 years after the first positive NTM culture, subjects with active disease had a persistently increased rate of decline in the forced expiratory volume in 1 second (FEV_1).

Winthrop *et al.* showed that approximately half of those with positive NTM respiratory cultures fulfilled clinical criteria for active infection[13].

Unlike *M. tuberculosis*, the isolation of NTM in pulmonary specimens does not systematically equate with disease. Frequently, NTM are first detected in a CF sputum sample in the absence of clinical suspicion, as part of routine screening. In some instances the mycobacterial organisms are pathogenic, but in others they are commensal.

The role of NTM as lung pathogens in CF patients is often underestimated because clinical symptoms and radiographic features are nonspecific and overlap considerably with progression of lung disease due to underlying CF. Recently, a large retrospective study of 1216 CF patients showed that positive NTM cultures were associated with progressive deterioration in lung function[20].

Overall, NTM has emerged as significant pathogens in CF patients, linked to disease progression and poor outcomes. Clinical course is similar to that of tuberculosis (TB), with insidious and slow progression that requires combined treatment for various months. Until recently, many centers considered *M. abscessus* infection to be a contraindication for lung transplantation, but nowadays the presence of MABSC or MAC infection despite optimal therapy is not an absolute contraindication to lung transplant, according to the CFF and ECFS[13].

There are no data of mortality directly associated to mycobacterial infection in CF patients, since these patients usually suffer simultaneous infections by different microorganisms.

Overall we can conclude that NTM (especially MABSC and MAC) are significant pathogens in CF patients. One single positive culture for NTM doesn't mean infection as this could be contamination or colonization, but in 30-50% of those patients (several positive cultures and furthermore clinical and radiological findings of pulmonary disease) NTM can certainly be the cause of serious lung infections with progressive deterioration in lung function.

3 QUESTION 2: HOW SHOULD NTM SCREENING IN CF PATIENTS BE PERFORMED (PRE-ANALYTICAL AND ANALYTICAL CONSIDERATIONS)?

3.1 FREQUENCY OF NTM SCREENING IN CF PATIENTS

Both the "Infection Prevention and Control Guideline for Cystic Fibrosis: 2013 Update" published by the CFF and the Society for Healthcare Epidemiology of America (SHEA) and the "US Cystic Fibrosis Foundation and European Cystic Fibrosis Society consensus recommendations for the management of non-tuberculous mycobacteria in individuals with cystic fibrosis" published by the CFF and the ECFS recommend NTM screening (culture + smear) be performed annually in spontaneously expectorating individuals with a stable clinical course[10].

Patient receiving NTM treatment should have expectorated (or induced sputum) samples sent for NTM culture and smear each 1 or 2 months, throughout the entire course of treatment to assess the microbiological response.

3.2 NTM SCREENING IN CF PATIENTS

3.2.1 Preferred specimen

For the diagnosis of NTM lung disease, collecting early-morning sputum specimens is preferred. The use of bronchoalveolar lavage fluid (BAL) is also justified.
It is advised against the use of oro-pharyngeal swabs for NTM screening, because they absorb too little material and because of their hydrophobic properties, which hinder the release of mycobacteria from the swab[10,21,22].

Preferably respiratory specimens must be transported within 2 hours at room temperature, or can be kept 24 hours in the refrigerator[8].

3.2.2 Decontamination methods

Optimal recovery of Mycobacteria from non-sterile clinical specimens (sputum, bronchial wash, skin, soft tissue, gastric lavage, and urine) requires liquefying the organic debris (digestion) and elimination of the commensal flora including pathogens such as *P. aeruginosa* (decontamination). Since NTM grow relatively slowly, this decontamination step prior culture is very important to avoid overgrowth of other bacteria in respiratory specimen. On the other hand decontaminating procedures are toxic to Mycobacteria. So it is a question of finding a balance between recovery of Mycobacteria and toxicity against Mycobacteria. In

contrast, sterile sites such as CSF, bone marrow, blood, and biopsy sites do not need a decontamination step prior to culture. Several methods for digestion and decontamination prior culture will be discussed below[23]. The CFF and the ECFS recommend respiratory specimen be decontaminated using the standard NALC-NaOH method and if the sample remains contaminated with Gram-negative bacteria after standard NALC-NaOH decontamination, it should be further treated with either 5% oxalic acid or 1% chlorhexidine[13].

3.2.2.1 NALC-NaOH method

The N-Acetyl-L-Cysteine sodium hydroxide (NALC-NaOH) method is the most commonly used procedure. NALC is a very effective mucolytic agent that digests sputa within a few minutes. NaOH acts as a digestant-decontaminant.

Pro: as it is a combination of products, a reduced concentration of the digestant-decontaminant NaOH is used, with reduced toxicity for Mycobacteria as consequence.

Contra: the final working reagent has a short shelf-life (24 hours).

3.2.2.2 NaOH method

As mentioned before, NaOH acts a digestant-decontaminant. A final concentration of 2% is toxic for contaminants, but affects also Mycobacteria.

Pro: easy to use.

Contra: toxicity for Mycobacteria.

3.2.2.3 NALC-NaOH-OxA method

The NALC-NaOH-Oxalic acid method is mainly used in the CF population, where specimens are known to be consistently contaminated with *P. aeruginosa*. However, some reports suggest that this method affects the viability of Mycobacteria, with false-negative results since some species, such as *M. abscessus* when present in low concentration, may be killed by this decontamination step.

Pro: high decontamination activity against *P. aeruginosa*

Contra: toxicity for Mycobacteria

3.2.2.4 Chlorhexidine method

Ferroni *et al.* showed that sputum samples treated with chlorhexidine (1%) yielded twice as many NTM-positive cultures as those treated by the NALC-NaOH-OxA method, despite a higher contamination rate[24].

Pro: high NTM yield

Contra: chlorhexidine is incompatible with the Mycobacterial growth indicator tube (MGIT) culture

3.2.2.5 New or alternative methods

OMNIgene sputum reagent is a non-toxic reagent that liquefies and decontaminates sputum samples, with a 1-year shelf life. When added to sputum samples (either in the point of collection or in the laboratory), *M. tuberculosis* survives up to 8 days at ambient temperatures. This simplifies the transportation step in the process. Furthermore, less contamination has been seen using OMNIgene and more *M. tuberculosis* have been detected in samples decontaminated with OMNIgene sputum reagent in comparison with the NALC-NaOH method[25].

Currently this reagent is in evaluation in the Laboratory of the university Hospital Saint-Luc Brussels.

Prolongation of the decontamination time is another option to decontaminate sputum samples of CF patients. In order to obtain a lower contamination proportion (mainly Gram negative rods) the decontamination time is prolonged from 15 min to 45 min. This allows avoiding the use of oxalic acid (because of its toxicity).

The Laboratory of the university Hospital Saint-Luc Brussels uses this decontamination method with good results.

3.2.3 How should samples be cultured for NTM screening?

Cultures should preferably be processed after collection within 2 hours at room temperature in order to optimize the detection of NTM in respiratory samples. If a delay in processing is expected, refrigeration of samples is advised (with a maximum of 24 hours)[10,26].

The CFF and the ECFS recommend that respiratory tract samples should be cultured using both solid and liquid media (using an automated growth detection system, such as MGIT) for a minimum of 6 weeks.

In practice, the laboratory of the university Hospital Saint-Luc Brussels only uses liquid media, i.e. MGIT (Mycobacteria Growth Indicator Tube) as the experience shows that solid media (Lowenstein–Jensen medium) doesn't provide added value.

CF patients should be screened annually for NTM. Sputum or BAL specimens are preferred and must be transported within 2 hours at ambient temperature or 24 hours at 2-8°C.
The most widely used digestion–decontamination method for Mycobacteria culture is the NALC-NaOH method. For CF patients, whose sputa are generally massively contaminated with *P. aeruginosa*, other gram-negative rods and yeasts, this method may be insufficient, leading to a high proportion of contamination. Alternative solutions include adding oxalic acid (NALC-NaOH-OxA method). Because NTM may be sensitive to oxalic acid, another option is to use two-step decontamination with oxalic acid only for those specimens overgrown by bacteria other than NTM or prolongation of the decontamination time (45 minutes instead of 15 min).
The laboratory of the university Hospital Saint-Luc Brussels, rather prefers to prolong the decontamination time with NALC-NaOH instead of adding oxalic acid (NALC-NaOH-OxA method).
It is very difficult for a laboratory to choose the optimal decontamination method that fits the most to the laboratory, as it depends on external factors including the population (CF patients or no CF patients).

4 QUESTION 3: IS THERE EVIDENCE FOR DIRECT OR INDIRECT TRANSMISSION PERSON-TO-PERSON TRANSMISSION IN CF PATIENTS?

4.1 THEORETICALLY DIFFERENT TRANSMISSION ROUTES OF NTM

4.1.1 Direct person-to-person transmission

Direct person-to-person transmission happens when NTM are spread between people by direct contact.

4.1.2 Indirect person-to-person transmission

Indirect transmission occurs when NTM are transmitted to new hosts through intermediates such as objects, substances in the environment or clinic equipment. As Mycobacteria are able to survive several weeks to months on surfaces, transmission can occur in absence of the initial host[27].

4.1.3 Environmental exposure (of highly genetically related strains)

Environmental exposure of *M. abscessus* (and other NTM) is a third route of NTM acquisition. Sometimes patients are infected by highly genetically related strains due to environmental exposure.

4.2 HISTORY

Until a few years ago it was supposed that NTM infections were exclusively due to environmental exposure and not to person-to-person transmission. Even in CF patients, where organisms (such as *P. aeruginosa*) are clearly passed between patients, there was no evidence of person-to-person NTM transmission. NTM are ubiquitous and infection was suspected to be acquired from environmental exposures, although the specific source usually could not be identified. This belief was based on several studies that showed a great diversity within *M. abscessus* group strains among CF patients, even siblings living in the same household for more than 10 years, suggesting independent acquisitions of NTM from the environment, ruling out a common environmental reservoir or person-to-person transmission[4-9]. Since several years, suspicion of person-to-person transmission arises.

4.3 FIRST SUSPICION OF PERSON-TO-PERSON TRANSMISSION (2012)

In 2012, Aitken *et al.* reported an outbreak of lung infection due to *M. abscessus subsp. massiliense* in a CF center in Seattle (Washington, USA)[28]. 5 patients were infected with multidrug-resistant isolates. All 5 strains were indistinguishable by repetitive unit sequence–based PCR (rep-PCR, see section 5.4.) patterns and pulsed-field gel electrophoresis analysis (PFGE, see section 5.5.). The authors concluded this outbreak could have occurred following direct person-to-person respiratory spread or indirect person-to-person transmission (due to contamination by a common source, i.e. clinic equipment).

4.4 PARTIAL CONFIRMATION OF THE SUSPICION (2013)

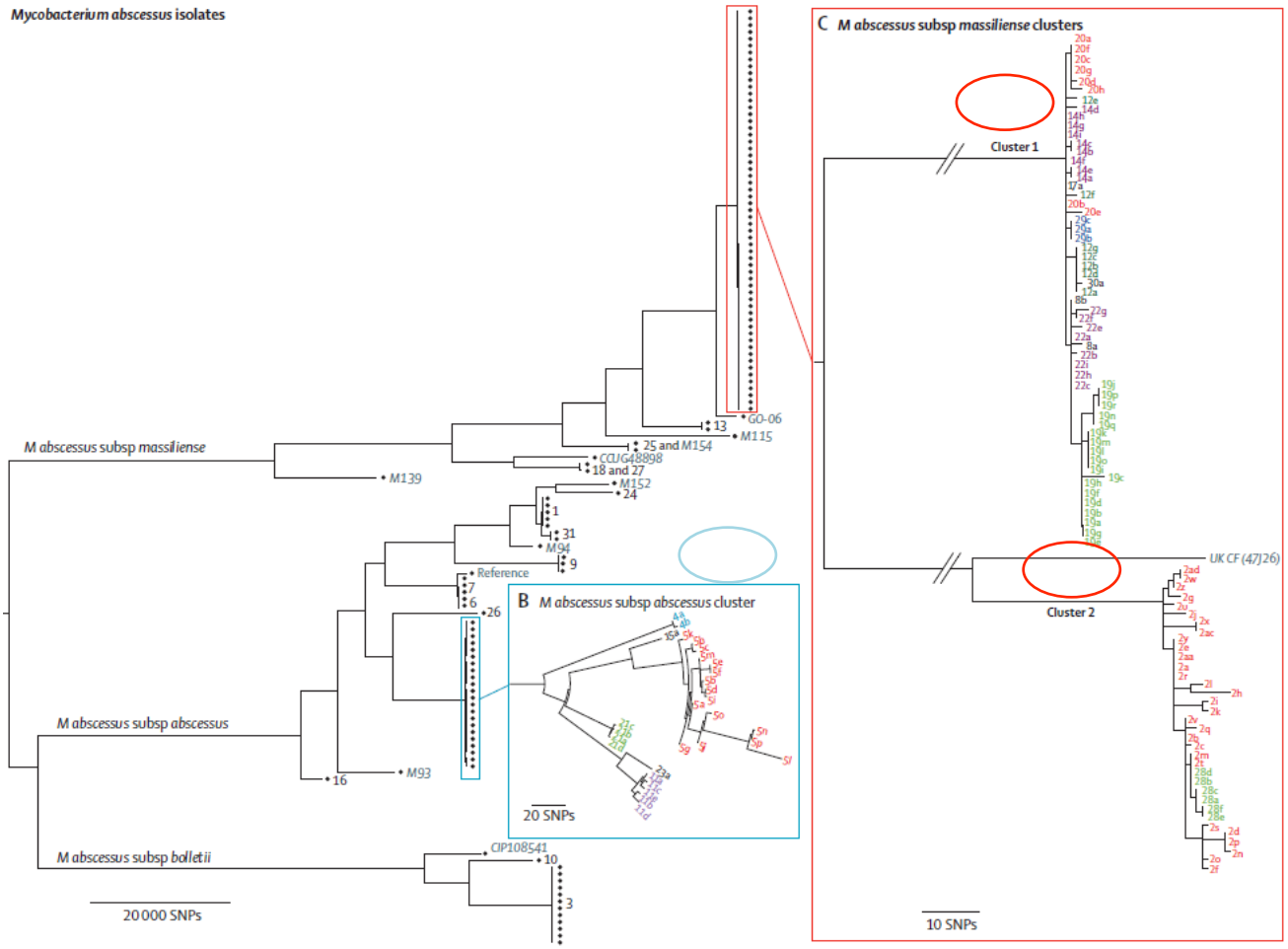
Bryant *et al.* performed whole genome sequencing (WGS, see section 5.1.) and drug susceptibility testing (DST) on 168 NTM isolates coming from 31 CF patients in the UK:

- 13 patients were infected with *M. abscessus subsp. abscessus*
- 15 patients were infected with *M. abscessus subsp. massiliense*
- 2 patients were infected with *M. abscessus subsp. bolletii*
- 1 patient was co-infected with both *M. abscessus subsp. abscessus* and *M. abscessus subsp. massiliense*

Between different *M. abscessus* strains single nucleotide polymorphisms (SNPs) were calculated in order to make a phylogenetic tree (A, figure 2). This tree revealed 3 clusters:

- 2 *M. abscessus subsp. massiliense* clusters (cluster 1 and 2, red box, figure 2).
- 1 *M. abscessus subsp. abscessus* cluster (cluster 3, blue box, figure 2)

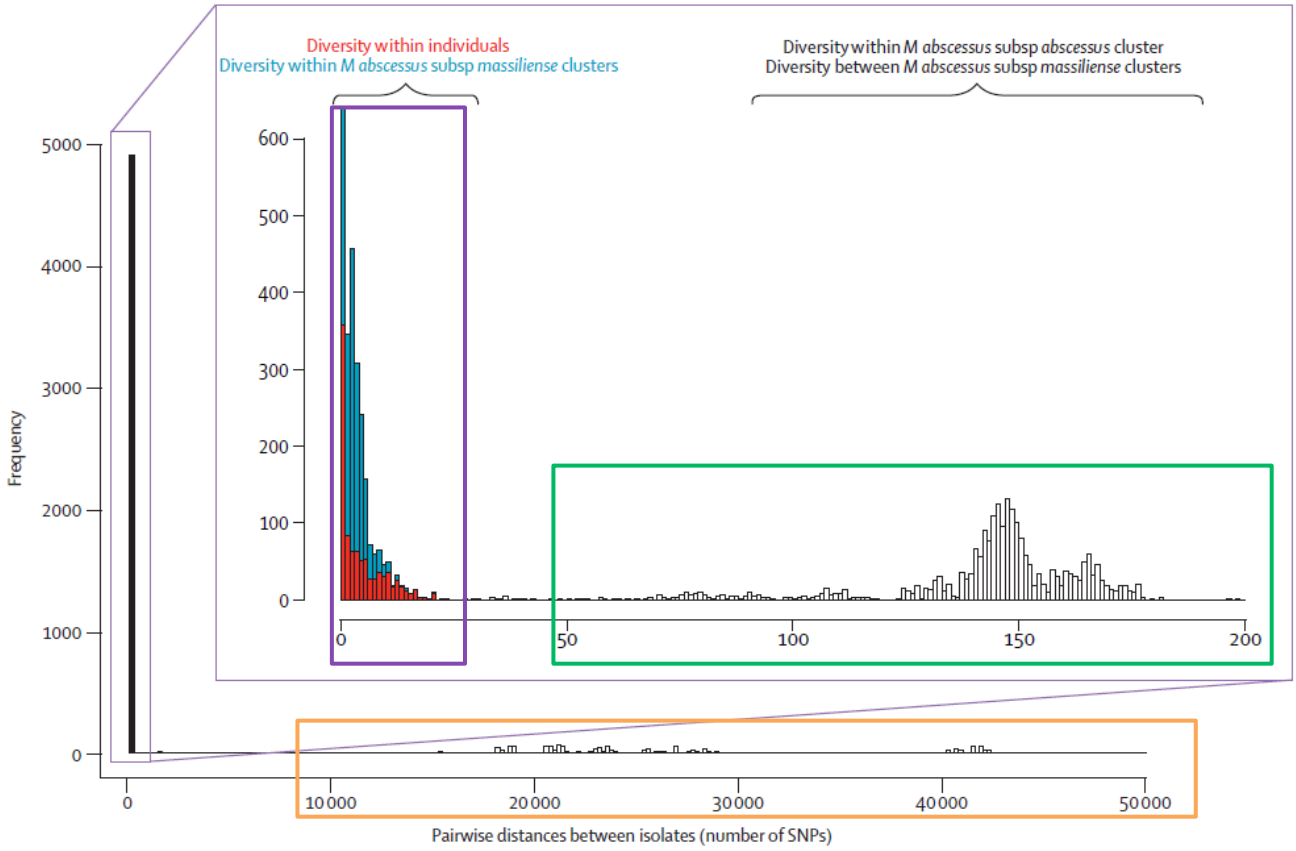
Figure 2: Phylogenetic tree of M. abscessus isolates (Bryant *et al.*, 2013)



They examined the distribution of genetic similarity between individual isolates, expressed as base pairwise distances:

- Isolates with > 10.000 base pair differences (orange box, graphic 3) consisted of isolates from different *M. abscessus* subspecies and from non-clustered *M. abscessus* strains.
- Isolates with 50 - 200 base pairs differences (green box, graphic 3) included isolates within the *M. abscessus subsp. abscessus* cluster (cluster 3, blue box, figure 2) and within the *M. abscessus subsp. massiliense* clusters (cluster 1 and 2, red box, figure 2).
- Isolates with < 25 base pair differences (purple box, graphic 3) represent the diversity of isolates from single individuals (red bars, graphic 3) and the similarity of isolates from different patients within each *M. abscessus subsp. massiliense* cluster (blue bars, graphic 3).

Graphic 3: Histograms of SNP pairwise distances between isolates of the same subspecies



Based on these results Bryant *et al.* could make some conclusions:

- *M. abscessus subsp. massiliense*: although some patients acquired strains that were not clustered, most have been infected with isolates from cluster 1 or cluster 2 (figure 2). The genetic difference between isolates from different individuals (blue bars, graphic 3) was often less than the variation of isolates seen within one person (red bars, graphic 3), strongly indicating person-to-person transmission. Furthermore, the authors identified clear opportunities for cross-infection within the hospital for all patients within *M. abscessus subsp. massiliense* cluster 1 and 2. In addition, isolates within *M. abscessus subsp. massiliense* cluster 1 and 2 had high level, constitutive clarithromycin and amikacin resistance in individuals without a history of relevant antibiotic exposure. Generally this is only seen in isolates from patients receiving long term macrolide and aminoglycoside therapy. Since extensive environmental sampling within the hospital CF center failed to detect any potential point source of NTM infection, there was strong evidence for person-to-person transmission. Given that the CF center adopted strict infection control policies, which included care for CF patients in individual rooms and advice against meeting socially, transmission probably occurred indirectly.

- *M. abscessus subsp. abscessus*: patients must have independently acquired either genetically diverse strains (non-clustered isolates, orange box) or a dominant circulating clone (loosely-clustered isolates, green box). Furthermore, patients infected with the clustered *M. abscessus subsp. abscessus* isolates had no clear opportunities for cross-infection.

4.5 STUDY OF HARRIS *ET AL.*: NO CONFIRMATION

Harris *et al.* analyzed 27 *M. abscessus* isolates coming from 20 pediatric CF patients (in the UK) by WGS and Variable number tandem repeat profiling (VNTR, see section 5.2.)[29]. The data obtained with WGS have provided a more accurate basis for assessing the degree of genetic similarity between isolates and have confirmed that VNTR profiling is an accurate method for identifying genetically related strains.

M. abscessus strains were clustered by VNTR profile: *M. abscessus subsp. abscessus* isolates belonged to VNTR cluster I, VNTR cluster II or had unique VNTR profiles and *M. abscessus subsp. massiliense* belonged to VNTR cluster III or had unique VNTR profiles.

Of the 20 pediatric patients, 12 patients acquired *M. abscessus* the first time after initial contact with the CF center:

- 11 patients were infected with *M. abscessus subsp. abscessus*:
    - 3 patients belonging to the VNTR cluster I: minimal exposure to other patients with *M. abscessus subsp. abscessus* VNTR cluster I
    - 3 patients belonging to the VNTR cluster II: 2 out of 3 patients (siblings) had multiple exposure occasions
    - 5 patients with unique VNTR profiles
- 1 patient was infected with *M. abscessus subsp. massiliense*

Patients who acquired *M. abscessus subsp. abscessus* VNTR cluster I strains, had minimal exposure to patients already infected with a *M. abscessus subsp. abscessus* VNTR cluster I strain (either as an outpatient or as an inpatient). Furthermore, these patients were exposed several times to other patients already infected with VNTR cluster II strains and strains with unique VNTR profiles.

2 patients (siblings) who acquired VNTR cluster II strains, had multiple exposure occasions (outpatient and inpatient settings), but they logically also had multiple contacts with the same environments.

Based on these observations, Harris *et al.* could not demonstrate person-to-person transmission. The main limitation was the insufficient numbers in each VNTR-defined outcome group to make statistically robust comparisons.

There are some recent studies that suggest (indirect) person-to-person transmission of *M. abscessus* (in the report of Bryant *et al.* only *M. abscessus subsp. massiliense* was suspicious for person-to-person transmission) in CF patients. However, Harris *et al.* couldn't confirm these findings. One possibility is that *M. abscessus subsp. massiliense* is more transmissible than other *M. abscessus* subspecies. Another possibility could be that adults (in contrast to children in the study of Harris *et al.*) experience more intense exposures or shed a higher load of NTM into the environment, making cross-transmission more likely.
Other possibilities to explain the discrepancy between the studies include the difference in infection control practices between centers, the limited number of patients and samples,… We also must take in consideration that the three types of acquisition may co-exist.

One main limitation in some studies includes not taking samples of the environment to exclude environmental exposure as transmission route. Another limitation is the low number of CF patients and consequently the low number of *M. abscessus* isolates. This will be difficult to overcome since even in large CF centers, there are only a few NTM infected CF patients. Therefore it is important CF centers work together and a database with the available information on NTM isolates from different countries must be created. This could simplify further investigation on the transmission of NTM within CF patients.

5 QUESTION 4: WHICH TECHNIQUES ARE AVAILABLE TO INVESTIGATE THE NTM TRANSMISSION IN CF PATIENTS?

Identification of Mycobacteria is usually performed using genotypic methods. Two commercial line probe assays are employed by many laboratories: the first assay is the INNO-LiPA Mycobacteria v2 (Innogenetics, Ghent, Belgium), a reverse hybridization DNA probe assay that has the capability to identify up to 16 Mycobacterial species. The second assay is the Genotype Mycobacterium CM/AM kit (HAIN Lifescience GmbH, Nehren, Germany) that accurately identifies 23 Mycobacterial species.
In recent years Matrix-Assisted-Laser-Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI - TOF MS) has showed to be a suitable, reliable, and fast technique to identify NTM up to species level, especially given its simplicity and the large number of species included in its database[13].

Another method for the identification up to species level is 16S rRNA sequencing.

None of above mentioned methods is able to differentiate between the three *M. abscessus* subspecies.

Until a few years ago, more accurate identification (until subspecies level) was achieved by performing *rpoB* sequencing. Recently the inaccuracy of single-target sequencing, including *rpoB* sequencing, for distinguishing between the three subspecies was highlighted[30-31]. Therefore nowadays *hsp65* gene and *erm* gene are sequenced together or WGS is used. These methods remain limited to specialized laboratories due to their cost and complexity.

Further differentiation of Mycobacteria subspecies, in order to investigate cross-transmission events and/or outbreaks, has been achieved using a number of different molecular methods (table 6). These methods include WGS, Multiple Loci Variable number tandem repeat Analysis (MLVA), Multilocus Sequence Analysis (MLSA), Rep-PCR and Pulsed-field gel electrophoresis (PFGE).

Table 6: Characteristics of the most commonly used molecular methods

	WGS	**VNTR (MLVA)**	**MLST/MLSA**
ADVANTAGE	- PCR amplification isn't required - high degree of resolution	- rapid - inexpensive	
DISADVANTAGE	- price	- only a small part of the entire genome is analyzed	- only a small part of the entire genome is analyzed - high cost
REQUIREMENTS	sequencing system	standard PCR and gel electrophoresis/capillary electrophoresis	sequencing system
REQUIRED TIME	< 24h	5h (6 loci)	4-10h
COST	$100	$6 (6 loci)	$40 (7 loci)
PRACTICAL INFORMATION	most used device: Illumina Hiseq platform	primers for loci: attachment 1 and 2	primers for housekeeping genes: attachment 3
REFERENCE	Bryant *et al.* and Harris *et al.*	Wong *et al.* and Harris *et al.*	Kim *et al.*

5.1 Whole-genome sequencing (WGS)

This technique can obtain sequence data of the entire genome. This approach is the ultimate typing tool, yielding genetic information on the (sub)species, the genetic diversity and

variation on strain level. This serves the research on the etiology, transmission, and even antibiotic susceptibility.
Bryant *et al.* and Harris *et al.* performed WGS on NTM isolates from CF patients.

We must pay attention as Davidson *et al.* have demonstrated highly similar genome sequences in *M. abscessus* isolates, coming from different patient populations at different global locations. For example, near-identical genome sequences in *M. abscessus* isolates were found, coming from cutaneous infections in Brazilian patients and pulmonary infections in UK CF patients[32]. Therefore, it is very important to interpret closely related genome sequences carefully, especially when investigating potential person-to-person transmission.

The high degree of resolution offered by WGS permits much finer typing than any other method and it has the capacity to provide valuable information about transmission events.

5.2 Multiple Loci Variable number tandem repeat Analysis (MLVA)

Tandemly repeated sequences are repetitions of one or more nucleotides that are directly adjacent to each other. These repetitions are called loci and show hypervariability in their repeat numbers (4-50 repeats). Therefore, they are called variable number tandem repeat (VNTR) loci (see figure 3).

Figure 3: Example of tandem repeat (tandemly repeated sequences): 7 repeats

TGATGCATACATACATACATACATACATACATAGGACT

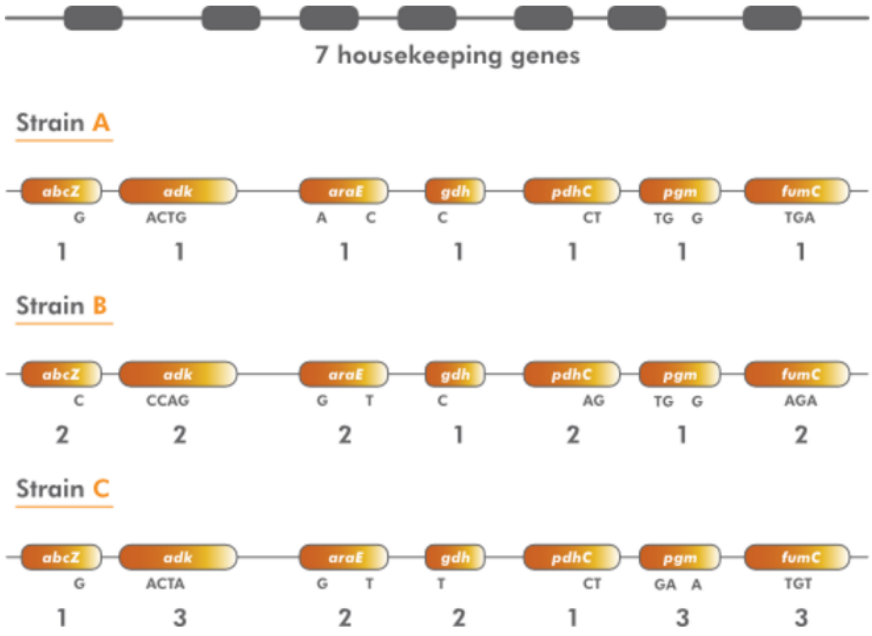
The first step of MLVA is performing a PCR of the VNTR loci. Subsequently these amplification products must be separated on agarose gels or by capillary electrophoresis. Next, the size of the amplification products is used to calculate the number of repeat units in each locus. The calculated numbers of repeats of the VNTR loci are represented as the MLVA profile, which consists of a combination of numbers, e.g. 14-4-6-4-15-7-12-6 (in this example 8 loci were analysed). Each unique MLVA profile is given a MLVA type (e.g. MT 1) or a VNTR type (e.g. VNTR 1). Wong *et al.* and Harris *et al.* used this technique to differentiate *M. abscessus* strains for epidemiological reasons. The primers used in these studies are listed in attachment 1 and 2.

5.3 Multi Locus Sequence Typing (MLST)

MLST is a technique where PCR amplification of 6-8 housekeeping genes is followed by DNA sequencing. Housekeeping genes typically are constitutive genes that are required for the maintenance of basic cellular function. For each housekeeping gene (or MLST locus),

each different sequence is assigned a distinct allele number regardless of how many nucleic acids are different. For each isolate, the combination of alleles obtained at each housekeeping gene (or MLST locus) determines the sequence type (ST) or strain (as in figure 4).

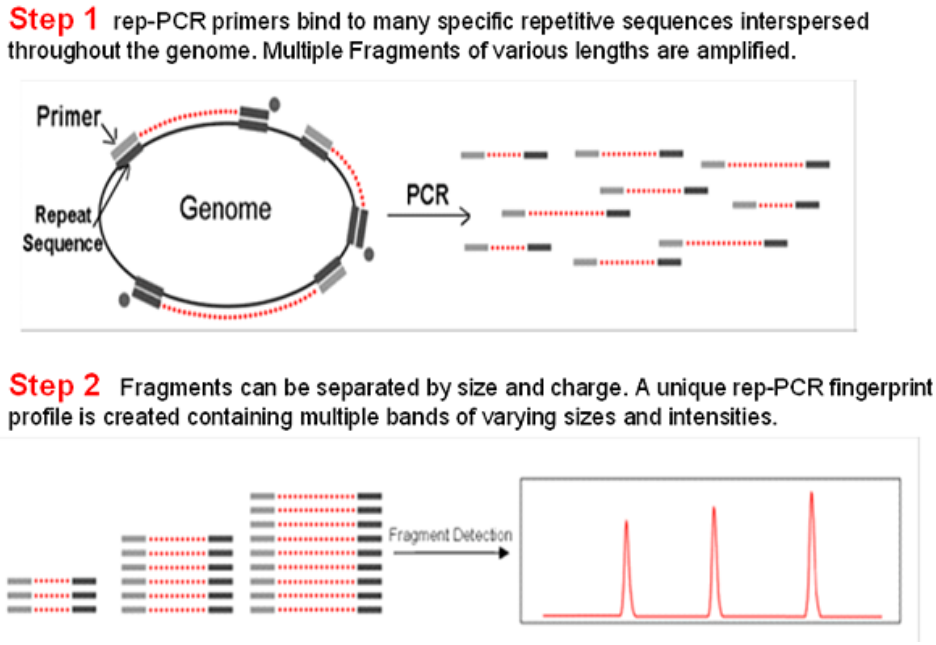
Figure 4: Sequencing of 7 housekeeping genes (7 MLST loci) resulting in different alleles



5.4 Repetitive sequence-based PCR (Rep-PCR)

The Rep-PCR method uses primers that target noncoding repetitive sequences interspersed throughout the bacterial genome to produce a profile of peaks or bands (see figure 5). It is an established approach for subspecies classification and strain delineation of bacteria. A commercial rep-PCR method, Diversilab, (BioMérieux, Marcy l'Etoile, France) has been employed in a number of studies for typing *M. abscessus* isolates.

Figure 5: Principle of Repetitive sequence-based PCR (www.nyas.org)



5.5 Pulsed-field gel electrophoresis (PFGE)

PFGE allows the separation of large DNA fragments by use of a 'pulsed' voltage gradient, which permits much greater resolution than the resolution achieved by conventional electrophoresis. PFGE has been used to type many bacterial species to strain level, and is routinely used to investigate cross-transmission events and outbreaks. However, this technique is largely confined to reference laboratories because it's laborious and technically demanding.

There is a variety of techniques available which allows – with different levels of precision – the detection of epidemiological analysis of NTM transmission within CF patients. Until now, no 'gold standard' has been established. Most studies have been performed using WGS and MLVA. It's certain that WGS is the most sensitive technique, but cautious interpretation of closely related strains is required. Harris *et al.* demonstrated non-inferiority of MLVA compared to WGS.

The main limitation of all techniques mentioned above is the inability to definitively prove person-to-person transmission versus same environmental exposure as transmission route. Therefore it is very important that environmental samples are taken and analyzed for the presence of NTM.

The laboratory of the university Hospital Saint-Luc Brussels has chosen to use the MLVA method, since they already have built up experience with this method. Furthermore, it is less expensive than WGS.

Until now it is still unclear whether person-to-person NTM transmission occurs, as well if the risk of transmission is only restricted to *M. abscessus subsp. massiliense* or whether it may be generalized to all *M. abscessus* subspecies. Furthermore, we don't know the proportion of person-to-person transmission in the NTM lung infections within CF patients. But even, if the proportion only is 10%, we still must pay a lot of attention as the treatment is very difficult and intensive for patients. Therefore, close collaboration with the infection control team and abiding by infection control procedures in CF and bronchiectasis clinics, including respiratory isolation for patients with *M. abscessus* is warranted in outpatient as well as inpatient settings to prevent transmission of *M. abscessus* in high risk situations.

The laboratory of the university Hospital Saint-Luc Brussels will soon evaluate the MLVA method.

Belgian CF centers (and European centers) should create a database with the available information of *M. abscessus* isolates collected in CF patients. This could contribute to a better epidemiological follow-up and infectious disease control, and furthermore it would be easier to make statistically robust conclusions.

Attachment 1: Primers used for MLVA (Wong *et al.*)

Locus	Primer sequence (5′→3′)		Period size (bp)[a]
	Forward	Reverse	
TR2	AATGGGTTCTTACGCAGGTT	GAGGGCACACACCAAAGG	33
TR28	GAGACCGAACAACGACTGCT	CCGGTAATGAATTGGGTTGA	27
TR45	CGAACTGCCTCGTGATCG	CACTCTCCTGACGCCAGAC	32
TR86	GCGCGTATCTTGAACCAATC	GGCGTACTCGTCGTAAAAGG	33
TR101	CCAGTGAACGACGCGATAC	ACAGCTTCAGTTGGCATGTG	33
TR109	GCGTGTGGGCATATCAATTA	CAATCTCGAGGTGGATGTGA	32
TR116	GAACACCTCAACCGCAGTG	ATTAGCGCGATAGGCTCACC	33
TR131	CGACAAAGCCTGGAAGGAC	AGGCATCCAGATCCACTGAT	30
TR137	ACAAGGTGGTGGTGCAGTC	GGGGAGGTCAAAGAAAGAGG	33
TR139	ATCTCGAGCAGACCAGCATC	GTCAACTGGATCCGGAGAAA	32
TR149	CTTCGGTCATCAAACAGCTTC	AGGGTGACCTGTGCGATATG	33
TR150	ACGTGGCATCTCGATTGG	TCCCACGAGACCATCAGAAT	30
TR155	CAACGTGGAATCTCAATACGC	CCCTTGAACAATTCGAGGAA	31
TR163	AGGGCAAGGTTGTCGACTC	GCGAAGTCCTCGGCACTC	30
TR167	CGGTCGTCACGATTACCAG	GAATAGAGCGTCGTGGTGGA	33
TR172	CGTGTAGTCGCTTTGTGCTC	ACTAACCATCCCCCACGAC	30
TR179	CCGAACGGTATAGGAGGTCA	TTCGTCATCAACGTGGTCAT	33
TR200	ACATGACACGAACCCTCTGG	GCTATCTGGTGAGCGATGGT	27

Attachment 2: Primers used for MLVA (Harris *et al.*)

TABLE 1 VNTR primer sequences and corresponding *Mycobacterium abscessus* genomic locations

Locus no.	Locus name	Primer	Sequence	Repeat unit size (bp)	Flanking sequence (bp)	Genome location[a]
1	3416	3416F 3416R	5′-CGT TCA TGG TCA GCA AGG AT-3′ 5′-TCC CAC GAG ACC ATC AGA AT-3′	30	228	3416798-3416904
2	4356	4356F 4356R	5′-CCA TCG AAG AAC CAA ACG AC-3′ 5′-AGT GGT GCC ATT GGT GGT A-3′	126	180	4356558-4357020
3	3163	3163F 3163R	5-CGC AAC ATC ATC GGA GAA C-3′ 5′-GCA TCC AGA TCC ACT GAT CC-3′	30	217	3163695-3163780
4	4038	4038F 4038R	5′-GAA GAC CCC CAC TCC AAT TT-3′ 5′-AAT AGA GCG TCG TGG TGG AT-3′	33	200	4038904-4038975
5	4093	4093F 4093R	5′-TCG TGT AGT CGC TTT GTG CT-3′ 5′-CCC GTA TAC GAG GAC GAT GT-3′	30	210	4093541-4093666
6	3320	3320F 3320R	5′-AAC CGT ATT CGT CGT CTG CT-3′ 5′-GTC AAC TGG ATC CGG AGA AA-3′	32	187	3320506-3320604
7	2177	2177F 2177R	5′-AAA ACG CGC GTA TCT TGA AC-3′ 5′-GTC GTA AAA GGC CCT CAT CA-3′	33	154	2177971-2178050
8	3398	3398F 3398R	5′-CGG TTT CAT GAC AAG CCA GT-3′ 5′-GTA GCT CTC GCC AAA AGT CG-3′	33	242	3398103-3398198
9	2220	2220F 2220R	5′-GGC AGA ATT ACC GAG TGC AG-3′ 5′-GAC ATG GGC ATC GAC AAA C-3′	33	224	2220890-2220983

[a] Loci were selected using the Tandem Repeat Finder (http://tandem.bu.edu) using the whole-genome sequence of *Mycobacterium abscessus* CIP104536T (14).

Attachment 3: Primers used for MLST (Kim *et al.*)

Gene	Primer	Primer sequences (5′ to 3′)	Amplicon size (bp)	Tm (°C)
argH	argH-F	GACGAGGGCGACAGCTTC	629	60
(argininosuccinate lyase)	argH-R	GTGCGCGAGCAGATGATG		58
cya	cya-F	TAAGGGTGATGACGTGCTGT	807	59
(adenylate cyclase)	cya-R	GTGAACGGCAACGCCTAC		60
glpK	glpK-F	AATCTCACCGGCGGTGTC	609	58
(glycerol kinase)	glpK-R	GGACAGACCCACGATGGC		60
gnd	gnd-F	GTGACGTCGGAGTGGTTGG	634	62
(6-phosphogluconate dehydrogenase)	gnd-R	CTTCGCCTCAGGTCAGCTC		62
murC	murC-F	TCAATGAAGCCGGTACCAAT	730	60
(UDP N-acetylmuramate-1-Ala ligase)	murC-R	GCGAATTCTGTAGCGAAAGC		60
pgm	pgm-F	CCATTTGAACCCGACCGG	596	60
(phosphoglucomutase)	pgm-R	GTGCCAACGAGATCCTGCG		66
pta	pta-F	GATCGGGCGTCATGCCCT	720	60
(phosphate acetyltransferase)	pta-R	ACGAGGCACTGCTCTCCC		66
purH	purH-F	CGGAGGCTTCACCCTGGA	634	64
(phoshoribosylaminoimiazolcarboxylase ATPase subunit)	purH-R	CAGGCCACCGCTGATCTG		60
rpoB	rpoB-F	TCCGATGAGGTGCTGGCAGA	940	68
(RNA polymerase, beta subunit)	rpoB-R	ACTTGATGGTCAACAGCTCC		68