Tuberculosis and its sequelae

Diagnostic, epidemiological and therapeutic studies

Onno W. Akkerman

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Promotores

Prof. dr. T.S. van der Werf
Prof. dr. H.A.M. Kerstjens
Prof. dr. H.W. Frijlink

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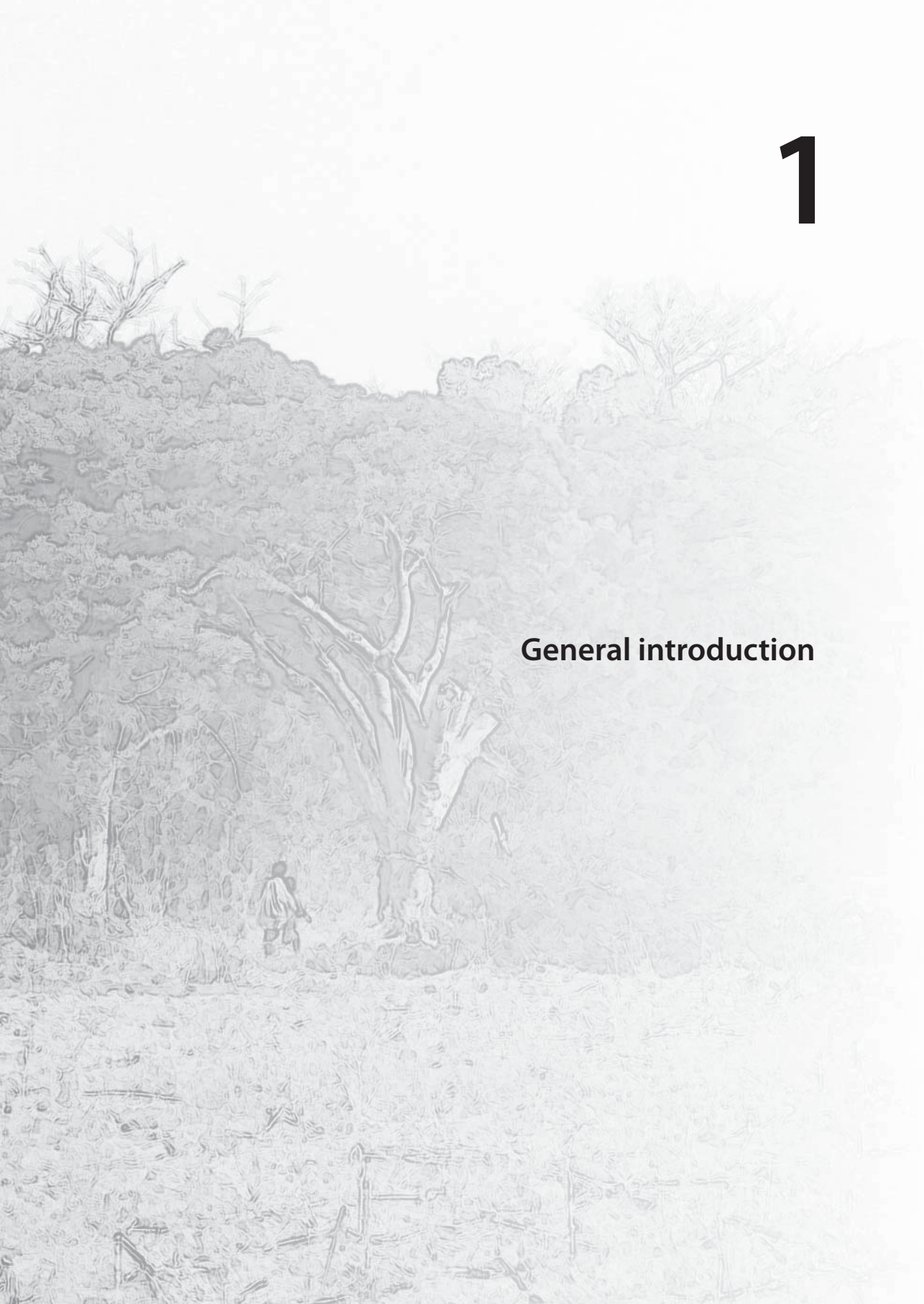

General introduction

Tuberculosis

Tuberculosis (TB) is an infectious disease that has haunted mankind for thousands of years. TB is caused by *Mycobacterium tuberculosis* (Mtb) [1]. Around one third of the world population today is infected with Mtb, of which around 9 million people develop active TB annually, and the prevalence of TB has been estimated at around 12 million. In 2014 alone, approximately 1.3 million people died from TB [2]. Moreover, those who survive often have impaired quality of life and reduced life expectancy due to severe sequelae – an effect of TB that is not captured by any statistical estimate [3].

In the Netherlands, an affluent country, the decline in prevalence and in incidence of TB started in the past century, long before modern drug treatment was introduced after World War II. After the introduction of drug treatment, the downward slope of the incidence and prevalence curves became steeper, and between the year 2000 and 2014, the number of newly detected cases has gradually dropped to below 850 per annum. Like in most other countries in the affluent parts of the world, TB has become an import disease; the vast majority of current patients were born outside the Netherlands, in regions of the world where TB is still endemic [4].

Mycobacterium tuberculosis complex

The causative organism of TB, Mtb, does not survive for more than a few hours in most environments; the human host is its main obligatory reservoir. Our current understanding is that both Mtb and mankind originated from ancestral populations in sub-Sahara Africa [5].

Mtb accompanied its host out of Africa, especially during the Neolithic Demographic Transition and less during earlier human expansion events [5]. Mtb, like men, differentiated into seven different lineages. These lineages are correlated with the migration of men to different parts of the world. Mtb strains of these lineages had a kind of co-evolution together with man. The new lineages of Mtb are less well adapted to their natural host and are perhaps therefore likely more virulent than the old lineages; for Beijing strains however, increased virulence is still debated [5–7].

Apart from TB caused by Mtb, humans may develop TB as a result of infection with closely related organisms from the species Mtb – referred to as Mtb complex. The mycobacteria from the Mtb complex probably have the same ancestor, referred to as the most recent common ancestor (MRCA) [8]. This MRCA of the whole Mtb complex was probably a human

Mtb strain, which evolved to animal-adapted strains; for example *M. bovis* has a smaller chromosome than Mtb [9]. The MRCA of the different species of mycobacteria may change over time as lineages die out and the population evolves. Most of the mycobacteria species within the Mtb complex do not have humans as their natural reservoir. If humans become infected and subsequently develop TB disease, their infecting organisms are considered zoonotic: their infection is then acquired from an animal reservoir. As these organisms are so closely related to Mtb, and the organisms are so well adapted to human hosts, patients infected with these organisms may in turn transmit the organisms to other human hosts, and their disease is therefore considered TB [8]. Table 1 shows the different species of Mtb complex, with their natural hosts.

Table 1 Mtb complex species and natural hosts

Mycobacteria species	Natural host
M. tuberculosis	Human
M. africanum	Human
M. bovis	Cattle
M. microti	Vole
M. caprae	Cattle and goat
M. canetti	Unknown
M. pinepedii	Seal
M. mungi	Mongooses
M. orygis	Antilopes

TB is a notifiable disease under Dutch law, irrespective of the species within Mtb complex [10], implicating that the authorities must be notified. This contrasts with infections caused by non-tuberculosis mycobacteria (NTM). NTM reflect a wide range of other – environmental – mycobacteria that may occasionally infect humans. This can be because of anatomical anomalies in affected organs – usually, the lungs, or as a result of severe immune compression, such as advanced stage HIV infection with extremely low CD4+ helper cell counts (typically, below 50/μL). Patients with bronchiectasis or other forms of structural lung disease may develop NTM infections that may mimic TB, with upper lobe cavities; or bronchiectatic abnormalities and nodular densities [11,12]. Although potentially detrimental for those affected, these environmental organisms are not transmitted among vertebrate hosts.

Pathogenesis and dormant status

Infection of Mtb is through inhaling Mtb containing aerosols. After inhalation, alveolar macrophages and bronchial dendritic cells phagocytize the mycobacteria.

These macrophages normally kill the Mtb intracellularly through fusion of the vacuole of the macrophage with lysozyme. If this fusion is blocked, Mtb survives and persists in the macrophage. The infected macrophage regulates formulation of granuloma via several pathways in a complex immune reaction [13–15]. Granulomas are considered the host sites where the host immune response controls the Mtb organisms that persist in a low metabolic and slowly replicative state, called dormancy [16]. Dormancy can be triggered by several different stress factors including hypoxia and host immune surveillance. A genetic program of Mtb called the dormancy survivor R regulon operates this process; the dosR regulon gene was discovered at the time the Mtb genome was first sequenced [17]. This state is usually called latent tuberculosis infection (LTBI) [18,19]. If the host immune surveillance fails, Mtb organisms switch back into a metabolically active state and the organisms start replicating, resulting in reactivated TB. Around 10% of the people with LTBI will get active TB during their lifetime – around 5% will develop active disease within the first two years after initial infection and approximately 5% for their remaining lifetime [20]. The risk of reactivation of TB increases if a person is immune compromised [21], such as persons with HIV co-infection or persons with rheumatoid arthritis or Crohn's disease [21]. The treatment for the two latter conditions, like TNF alpha inhibitors, can cause severe immune compression as well [22]. However most people that develop active disease in the affluent world do not have an inborn or acquired defect in their cell-mediated immunity; they are genetically susceptible to develop TB [23,24]. To detect LTBI there are 3 immunological tests nowadays [19]. The first is the tuberculin skin test (TST), a diagnostic tool developed more than a century ago. The TST contains a crude mixture of antigens present in *Mtb* complex, *M bovis* BCG and several NTM, causing cross reactivity with the latter species [25,26]. The two other tests are both interferon gamma release assays (IGRAs), which are commercially available as the QuantiFERON-TB Gold In-Tube [27] and the T-SPOT-*TB* [28]. Both were introduced early in the 21st century. The IGRAs targets the ESAT6 and CFP10 proteins (and TB7.7 for the QuantiFERON-TB Gold In-Tube), which are more specific for the MTB complex, and are therefore supposed to be more specific for detecting LTBI compared to the TST [25,29,30]. The immunological tests are used for several indications. Mostly they are used to detect possible LTBI [26,30,31], including patients who will be immune compromised due to future treatment [29,32–35] or within health care workers [36], or

for tracing of contacts of an index case of TB [37]. The tests are not developed nor fit to detect active TB, though occasionally they are used as circumstantial evidence [38–40].

Mtb is able to travel within the body of its host through the lymphatic and circulatory system, both directly after infection but also after reactivation. Manifestations of TB can be pulmonary (PTB), extra-pulmonary TB (EPTB) or both PTB and EPTB. In immune competent individuals, the ratio between PTB and EPTB is between 2/3 to 1/3. In immune compromised individuals EPTB is more common. TB commonly affects the lungs, the pleurae and the lymph nodes. TB of the spine and the central nervous system is less common. The latter manifestation gives serious sequelae in high percentages [41–43].

Genome of Mtb and its importance in transmission of Mtb

In 1998 Cole et al. published the whole genome sequence of Mtb. They used the best-characterized strain, H37Rv. They showed that Mtb consists of around 4,000 genes and contains 4,411,529 base pairs with a Guanine + Cytosine content of 65.6% [17].

The technique of whole genome sequencing (WGS) has now gradually become more affordable and attainable for tracing of transmission of Mtb. Through the technique of WGS it became apparent that most strains have small variations in the genome referred to as Single Nucleotide Polymorphisms (SNPs). With this approach of WGS and through the variations in SNPs it is not only possible to better understand the cluster and route of transmission but also its timeline [44,45].

Understanding transmission of Mtb is of imminent importance in TB control strategies. WGS is superior to conventional genotyping techniques for Mtb tracing and investigating outbreaks or epidemics [45]. However, the conventional genotyping techniques ('genetic fingerprinting') are more widely available and much more affordable than WGS. Conventional genotyping techniques still in use are spoligotyping, IS*6110* Restriction Fragment Length Polymorphism, and Variable Number of Tandem Repeats (VNTR) [46–49]. People (and therefore patients) travel around the world; the index case can be an alive or dead person from all around the world. Index sources are not necessarily only humans [50,51] and thus worldwide databases will be necessary for storage of genotyping results.

Diagnosis of TB

Diagnosing TB starts with a clinical suspicion. Subsequently, diagnostic confirmation is required, and diagnostic sampling from the affected site is critically important for the correct diagnosis, and for susceptibility testing. Several microbiological techniques for the diagnosis of Mtb are are available. Direct microscopy can be performed by using either a staining technique distinguishing mycobacteria from other bacteria, using a decolouration with acid alcohol, e.g., Ziehl-Neelsen staining or using a fluorescence staining which can be detected by a light-emitting diode (LED) microscope, this technique is preferred by the WHO since a couple of years [52]. Culturing of Mtb needs decontamination techniques to stop rapidly growing bacteria and fungi. Liquid media have replaced solid culture media with considerable improvement of laboratory return time and improved sensitivity compared to solid media. If there is a suspicion of non-tuberculosis mycobacterial, solid media should always be used [53].

TB media enriched with nutrients that allow mycobacteria to grow. After its invention in the 1980s the polymerase chain reaction (PCR) became available for diagnosing TB usually targeting a non-coding insertion sequence (IS) with multiple copies present in most strains of Mtb, called IS*6110* [54–56]. A disadvantage is that Mtb strains can have zero copies of IS*6110*, which has already been described in India and Vietnam [57,58]. As far as we know, data of the number of copies of IS*6110* in the strains in China is lacking. Today, many different commercial and in-house PCRs are available [59–63]. Neither the microscopy techniques showing presence of acid-fast bacilli, nor PCR techniques showing the presence of Mtb genome are able to distinguish alive from dead bacilli. Though recent results studying viability staining or using mRNA to discriminate between viable and dead bacilli are promising, further research needs to be done [64,65]. Interpretation of the microscopy and the PCR requires prudent critical review of the patients' treatment history, careful interpretation of clinical and imaging data, to distinguish active TB from old, healed disease; culture of Mtb is still the gold standard for diagnosing TB. A cultured isolate can subsequently be tested for drug susceptibility. Today two molecular techniques are available for testing drug susceptibility as well. One is a line-probe assay which test the sensitivity to the two most important anti-TB drugs, isoniazid and rifampicin [66,67] and the other test, a PCR assay, can detect molecular resistance against rifampicin [68–71]. This test is called the Xpert MTB/RIF and is currently distributed worldwide which makes the Xpert MTB/RIF more and more frequently used in diagnosing (resistant) TB all over the world [72]. The first studies have now been published using WGS to detect genetic resistance patterns before phenotypic susceptibility is known [73–75].

Anti TB treatment

Patients harbouring drug-susceptible Mtb can be treated with the most potent drugs against TB, the so-called first line anti TB drugs (FLD). By definition, patients with Mtb resistant for isoniazid and rifampicin have multidrug resistant TB (MDR-TB) [76,77]. Treatment of MDR-TB consists of second-line anti TB drugs (SLD). The WHO categorizes anti TB drugs in 5 groups; group 1 consists of the first-line anti TB drugs. SLD are categorized in groups 2 to 5. Group 2 are the so-called injectables, amikacin, kanamycin and capreomycin. Group 3 are the fluoroquinolones, including the potent agent moxifloxacin [78]. The group 4 drugs include the oral, weak SLD, e.g. ethionamide, prothionamide and cycloserin. Group 5 consists of SLD with less known efficacy, toxicity and tolerability and they count for half a drug. Examples are linezolid, clarithromycin, co-trimoxazole, clofazimine and meropenem. In its programmatic treatment against MDR-TB the WHO advises that the first choice regimen should include at least pyrazinamide, a fluoroquinolone, an injectable and two group 4 drugs [79]. In the Netherlands we use a slightly different guideline based on susceptibility results [80]. Our intention is to use drugs that are proven to be effective for the strains. We also measure the levels of the drugs in the serum, called Therapeutic Drug Monitoring (TDM). TDM as standard practice has not been included in the WHO TB treatment recommendations to date. TDM addresses the combination of pharmacokinetics (PK) and pharmacodynamics (PD) where PK describes the absorption, distribution, metabolism and elimination of the drug in the human host and PD, in case of TB, describes how the drug in the human host targets Mtb. By optimizing treatment, the risk of toxicity in individual patients is decreased while efficacy has been maintained [81], or even improved [82]. However, there is a strong need for generally accepted target values for PK/PD for TB or MDR-TB treatment.

Bronchiectasis

Infamous sequelae of pulmonary TB are bronchiectasis (BE). BE due to TB is becoming rare in the Netherlands due to decreasing incidence of TB, but worldwide TB is still a very important aetiology of BE. The most common cause of BE in the developed world nowadays is cystic fibrosis (CF). BE is a condition of the lungs which is associated with recurrent infections with a wide variety of microorganisms. One of these microorganisms is *Pseudomonas aeruginosa*, which colonizes the airways. *Pseudomonas aeruginosa* is known to cause more exacerbations of the BE thereby causing increased impairment and deterioration of lung function and deterioration of quality of life [83–92]. Preventing these exacerbations by

inhalation of antibiotics is current practice nowadays both for patients with CF BE and non-CF BE. Preventing exacerbations has proven its efficacy in patients with CF BE; the mean survival of this population increased from the 1980s mostly due to strict antibiotic treatment having become more standard practice, including inhalation of tobramycin and colistin. This exacerbation reduction by strict antibiotic regimens has not yet been as firmly positioned in the guidelines for patients with non-CF BE.

Inhalation of antibiotics

Inhalation of medicine is well known and it has been used as a route of drug treatment for more than 4,000 years. Inhalation of an anti TB-drug was described for the first time in 1950. This study was performed in young children and blood levels were measured after inhalation. These blood levels were not considered sufficiently high to influence the course of the disease [93].

Nowadays, inhalation of antibiotics is most common among patients with CF. Starting in the 1980s, most of the research on inhalation of antibiotics has been conducted in this population [94–96]. Patients with non-CF bronchiectasis have increasingly been treated with inhaled antibiotics as well. The patient group with non-CF bronchiectasis is perhaps more heterogeneous, with many different aetiologies [84,88].

Inhalation of the antibiotics can be done either by nebulisation or by dry powder inhalation. The latter system has increasingly been explored in the last decade [97–104], and these devices have now become commercially available for treatment. The benefits of inhalation by dry powder are shorter time of intake, possibly less risky for contamination compared to using nebuliser, and perhaps more efficacious due to a higher deposition of aerosolised drug at the site of infection. The difference in drug deposition between individuals using the same device is unclear. However, the individual inhalation profile is repeatable after one inhalation per person [unpublished data, personal communication Paul Hagedoorn]. This quality makes it easier to predict both the benefits and the side effects for a single person. Side effects of inhalation of aminoglycosides are both local, like cough or an unpleasant taste in the mouth, as well as those of systemic treatment [101,104,105].

Aims and outline of the thesis

In **chapter 2** we perform diagnostic studies of tuberculosis. In the first study of chapter 2 we look for the most sensitive polymerase chain reaction (PCR) detection method by comparing 14 different assays for the detection of Mtb. In the second study of chapter two we evaluate a new method of using variable number of tandem repeats (VNTR) in the molecular epidemiology and its sensitivity compared to PCR assays.

In **chapter 3** we study epidemiologic aspects of tuberculosis. In the first part of the third chapter transmission between a non-human primate held in captivity to human contacts is studied. We aim to provide evidence of this route of transmission using spoligotyping results. In the second part of the third chapter a case with human *M. bovis* is described. The route of transmission is studied using both epidemiological and fingerprinting results and furthermore we also explore the utility of an IGRA for contract tracing in an outbreak around this case of human *M. bovis*.

In **chapter 4** we look at pharmacokinetic aspects of tuberculosis. In the first part of the fourth chapter we study the blood- and lung tissue concentrations of the second-line anti-TB drugs of a patient with MDR-TB.

In a letter to the editor in the second part of the fourth chapter we discuss other potential contributing beneficial factors to treatment results of TB meningitis.

Finally, in another letter to the editor as the third part of the fourth chapter we discuss optimized use of PK/PD as parameters to predict treatment outcome in clinical studies.

Chapter 5 is about dry powder inhaled antibiotics. In the first study we aim to develop a model that can predict the systemic absorption after inhalation of dry powder aminoglycosides. We propose the use of this model for drug dosing in future studies in conjunction with an optimized inhalation device. In the second study of this chapter we study a novel device, called the Cyclops, especially developed for inhalation aminoglycosides. This study is the first with the Cyclops and we test the local tolerability and the pharmacokinetics of dry powder tobramycin using the Cyclops in patients with non-CF BE.

Chapters 6 and 7: in these chapters a summary of the studies presented in the thesis is provided and the clinical impact with future perspectives are discussed.

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2A

Comparison of 14 molecular assays for detection of *Mycobacterium tuberculosis* complex in broncho-alveolar lavage fluid

OW Akkerman
TS van der Werf
M de Boer
JL de Beer
Z Rahim
JW Rossen
D van Soolingen
HA Kerstjens
AG van der Zanden

J Clin Microbiol. 2013;51(11):3505-3511

Abstract

We compared 14 molecular assays for their ability to detect the *Mycobacterium tuberculosis* complex in brochoalveolar lavage fluid. Three approaches were followed. First, by using DNA from *Mycobacterium bovis* BCG, we determined the detection limits of the assays in routine molecular methods. Second, in order to determine the analytical sensitivity of the assays, we added one of four *M. tuberculosis* isolates with various numbers of the IS*6110* to N-acetyl-L-cysteine (NaLC)-NaOH treated bronchoalveolar lavage fluid samples in dilutions of 1:10 up to 1:10,000,000. Third, intertest variabilities were measured and defined by standard deviation for the quantitation cycle (Cq) values of three positive test results per dilution per assay. The 14 assays tested had similar analytical sensitivity, except for GeneXpert, which had an analytical sensitivity that was 10- to 100-fold lower than that of the other assays. The MP MTB/NTM test and the in-house-Taqman-10 revealed the best performances for the detection limit and had the highest analytical sensitivities. Most of the tests performed well regarding detection limit and analytical sensitivity for the detection of *M. tuberculosis* complex in serial dilutions, and differences were small. The MP MTB/NTM and the in-house-Taqman-10 revealed the best, and GeneXpert the worst, overall performance.

Introduction

In 2011, 6.2 million patients were diagnosed with tuberculosis (TB) worldwide and reported to the national tuberculosis control programmes (NTP) [1]. Control of this high-burden disease heavily depends on improved, rapid diagnosis and optimal treatment. Real time (RT)-PCR is the standard DNA amplification technique currently used in TB laboratories. In a short time span, a substantial number of commercial nucleic acid amplification tests for *Mycobacterium tuberculosis* complex (MTC) infections have become available. However, these assays have not been compared in a systematic manner. The clinical sensitivity of a part of the assays has been reported in only a few studies, using culture positivity and/or clinical diagnosis as the "gold standard" [2-6].

The insertion sequence IS*6110* has long been appreciated as a target in the molecular detection of *M. tuberculosis* complex in clinical material, and is the most abundant IS element in the genome of *M. tuberculosis*. However, among clinical isolates worldwide, the number of IS*6110* copies in the genome of *M. tuberculosis* however varies from 0 to 25, potentially influencing detection limits of related assays [7-10].

In this study, we compared the analytical performance of 14 assays for the molecular detection of MTC in three different approaches. We hypothesized that not all assays would have equally good analytical sensitivities and that the analytical sensitivity of assays targeting IS*6110* would depend on the number of target copies in the genome of *M. tuberculosis* strains studied. We therefore compared IS*6110*-targeting tests with those not targeting this element, or that explored unknown or undisclosed targets.

Material and methods

Assays

Fourteen assays were compared, 9 of which are commercially available (Table 1). The assays were performed according to the instructions of each manufacturer. The cutoff values of the assays applied were those provided by the manufacturer.

Table 1 Molecular assays tested, in order of their target

Target	Assay	Manufacturer (short version used in the manuscript)
IS*6110*	5 µl DNA input and Roche master mix	Experimental RT-PCR (with 5 µl input and Roche mastermix, Roche Diagnostics Nederland BV, Almere, the Netherlands)[11] (in-house-Roche-5)
IS*6110*	5 µl DNA input and TaqMan Universal PCR Master Mix, +AmpErase UNG	In-house RT-PCR IS*6110* (with 5 µl DNA input and TaqMan Universal PCR Master Mix, +AmpErase UNG, Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) [11] (in-house-Taqman-5)
IS*6110*	10 µl DNA input and TaqMan Universal PCR Master Mix, +AmpErase UNG	in-house RT-PCR IS*6110* (with 10 µl DNA input and TaqMan Universal PCR Master Mix, +AmpErase UNG, Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) [11] (in-house-Taqman-10)
IS*6110*	MTB Q PCR Alert kit detection of *Mycobacterium tuberculosis* DNA	Lucron ELITechGroup, Dieren, The Netherlands (Lucron)
IS*6110*	RealAccurate™*Mycobacterium tuberculosis* PCR Kit	PathoFinder B.V., Maastricht, The Netherlands (PathoFinder)
IS*6110*	MTB Real-Time kit for detection of *Mycobacterium tuberculosis* complex	MP MTB kit, Sacace Biotechnologies, Como, Italy, provided by MP Products, The Netherlands (MP MTB)
IS*6110*	Multiplex MTC Real-Time PCR kit: AmpliSens MTC-FRT PCR kit	MTB/NTM kit, Interlabservice (ILS), Moskou, Rusland, provided by MP Products, The Netherlands (MP MTB/NTM)
IS*6110*	Myco Direct 1.7, DNA based identification of *Mycobacterium tuberculosis* complex (MTUB) and other Mycobacteria (MOT) LCD array kit The target specific capture probes and the capture probes for the external control sequence 'Ctrl-EX1' together form a cross or a 'plus' sign.	Chipron, GmbH, Berlin, Germany (Chipron)

Table 1 continues on next page

Table 1 ***Continued***

Target	Assay	Manufacturer (short version used in the manuscript)
16S-23S rRNA gene	Internal Transcribed Sequence High Resolution Melting (ITS-HRM) and Roche HRM master mix.	In-house RT-PCR for ITS-HRM[12] (in-house ITS-HRM)
16S rRNA of 584 bp	COBAS® TaqMan® MTB Test CTM MTB	Roche, Roche Diagnostics Nederland BV, Almere, The Netherlands (Cobas)
Direct repeat region of *M. tuberculosis*	Spoligotyping Recorded positive if 3 or more spacers are present	Home made[13] (Spoligotyping)
rpoB gene	GeneXpert MTB/RIF Assay	Cepheid, Cepheid Benelux, Apeldoorn, The Netherlands[14] (GeneXpert)
Not disclosed by the manufacturer	Accupower MTB Real-Time PCR kit	Bioneer, South Korea provided by Goffin, The Netherlands (Goffin MTB)
Not disclosed by the manufacturer	Accupower MTB&NTM Real-Time PCR kit	Bioneer, South Korea provided by Goffin, The Netherlands (Goffin MTB/NTM)

Internal transcribed sequence high resolution melting

Amplification of *M. tuberculosis* complex DNA was done using a real-time PCR with a high-resolution melting (HRM) mix (HRM Master mix, Roche, Roche Diagnostics Nederland BV, Almere, The Netherlands). The amplification of a part of the internal transcribed spacer (ITS) spacer of 200–330 base pairs, which is located between the 16S and 23S gene spacer, was performed with primers tb-ITS-fw en tb-ITS-re-rev1. For this amplification, no probe is used, so the sensitivity of this test solely depends on the primers. The melting curve results of the *M. tuberculosis* complex control were recorded as positive if the DNA denatured between 89 and 90. Therefore, when testing for *M. tuberculosis*, the results were considered positive when melting curves were between 89 and 90. Subsequently, the quantitation cycle (Cq) values were used for further calculation [11].

M. tuberculosis complex control

The *Mycobacterium bovis* BCG strain is normally used as internal control for the in-house PCR assay, which is used in our laboratory and contains a single copy of IS*6110* [12,13]. One colony of this *M. bovis* BCG was added to Prepman solution (Prepman® Ultra Sample Preparation Reagent, AB Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) to prepare a turbid suspension. This suspension was incubated for 10 min at a temp of 100°C to release DNA. Thereafter, a 1:10 dilution was prepared in Tris-EDTA (TE) buffer. This extracted DNA was used in all the different PCRs for determining the detection limits (DLs).

Isolates

Four *M. tuberculosis* isolates with a known number of IS*6110* copies were selected from the *M. tuberculosis* isolate collection at the National TB reference laboratory, each containing 1, 5, 10 and 20 copies of the IS*6110* element, respectively. The four isolates were cultured to prepare a large set of identical bronchoalveolar lvage fluid (BALF) samples with quantified numbers of mycobacteria. Culture was done in the MGIT BACTEC 960 system (Becton Dickinson, Sparks, MD).

Specimen processing

We pooled redundant BALF specimens obtained from routine bronchoscopy procedures performed at several hospitals on various patients not suffering from any *Mycobacterium* infection. The BALF specimens were combined to a total volume of 800 ml and mechanically liquefied and homogenized with N-acetyl L-Cysteine-sodium hydroxide (NaLC-NaOH) [14]. After centrifugation, the treated BALF was concentrated, and this resulted in a total volume of 80 ml. The concentrated NaLC-NaOH treated BALF mixture was checked to be negative for MTC using the in-house IS*6110* RT-PCR.

Strain processing

Cultures were prepared and heat-killed (40 min 115°C). Heat killing of the strains was performed before and after mixing the bacteria with the NaLC-NaOH-treated BALF mixture. The difference in quantitation cycle (Cq) values in the in-house IS*6110* RT-PCR for one strain between both processes was never higher than 0.57 (dilution 1:10 from a *M. tuberculosis* isolate with one IS*6110* copy).

Serial dilution and DNA isolation

A fixed number of heat-killed *M. tuberculosis* organisms was added to the NaLC-NaOH-treated BALF. Dilutions of 1:10 to 1:10,000,000 were made of this suspension, and the dilutions were frozen at -80°C in portions of 250 µl. From these diluted *M. tuberculosis* suspensions, 200 µl was extracted with NucliSens easyMAG (bioMérieux, Boxtel, The Netherlands). The extracted DNA was tested in the in-house IS*6110* RT-PCR for reproducibility and was further used in a comparison of all the assays.

The reproducibility of the DNA extraction and the in-house IS*6110* RT-PCR was tested on the frozen material at three different time points, in duplicate. In this way, the means and coefficients of variance (CV) for the Cq values of the eight different dilutions from the four isolates in duplicate were calculated. The difference in mean Cq value was never higher than 1.08, and the CV never higher than 2%.

Assay comparison

The analytical sensitivities of the assays were compared in two different ways. First, we determined the detection limit (DL). For this purpose, we used a dilution series of the DNA of the *M. bovis* BCG strain that is used as control in routine diagnostics. The DNA was diluted in TE buffer. The assay was tested in triplicate for each dilution. A higher dilution of the suspension was recorded as positive only if it tested positive three times in the respective assay. After a first negative result, the dilutions between the first negative dilution and the last positive dilution were tested as well.

Second, we determined the analytical sensitivity that captures both the process of DNA extraction and the use of the extracted DNA from the *M. tuberculosis* serial dilutions in BALF mixture in RT-PCR. Again, the test of a higher dilution was recorded as positive only if the three results were all positive.

Additionally, as third method of comparison, the intertest variability of the assays was assessed. The intertest variability of each assay was defined by standard deviation (SD). The SD was calculated for the Cq values of the three positive test results per dilution per assay, using the results from the *M. tuberculosis* serial dilutions in BALF samples and not from results using *M. bovis* BCG stain diluted in TE buffer for detection limit determination.

Statistics

Descriptive statistical methods have been provided in the methodological sections above.

Results

Detection limit

We used serial dilutions of *M. bovis* BCG DNA that is used routinely as a positive control in molecular assays and that contains one copy of IS*6110* per genome. When comparing the detection limits, we considered the assay with a positive result at the greatest dilution to have the lowest detection limit. The results for assays targeting IS*6110* and for assays using other genetic targets, or for tests with a target not disclosed by the manufacturer, are shown in Figure 1. Four tests had the same, high detection limit: these assays positively identified MTC at up to a 1:100 DNA dilution. The detection limit, independent of the type of target, was highest with the Lucron, Pathofinder and in-house ITS-HRM. These assays were able to amplified up to a 1:10 DNA dilution.

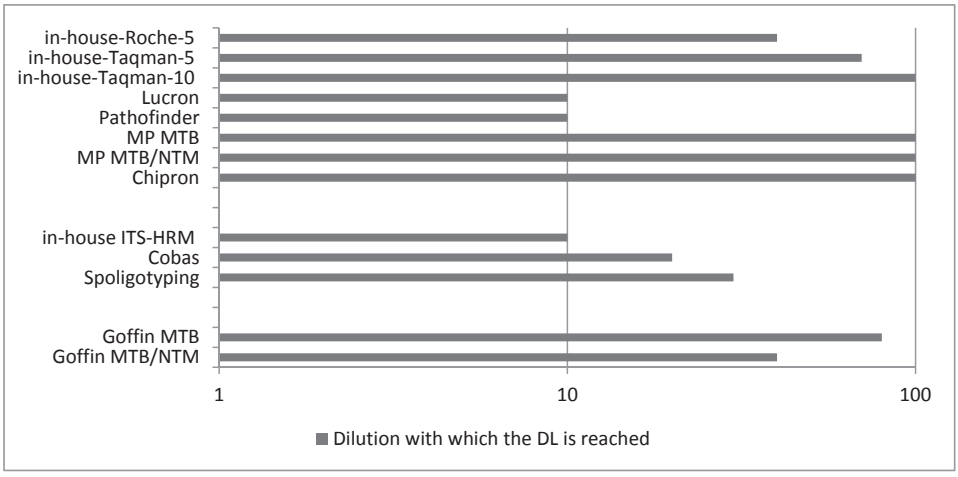

Figure 1 Comparison of detection limits using dilution series of the DNA of *M. bovis* BCG that is used as positive control in routine diagnostics and contains one copy of IS*6110*.
The *x* axis shows the dilution, which was recorded as positive if the assay tested positive three times; the longer the black bar, the better the test performed. The *y* axis shows the assays tested. The first eight assays target IS*6110*. Three other assays do not target this insertion sequence, but another known target (see Table 1). For the two Goffin assays, the manufacturer does not disclose the target.

The detection limit of GeneXpert assay could not be calculated because this assay can be performed only with clinical intact material and not with purified DNA.

Analytical sensitivity in serial dilutions with different number of IS*6110* elements

The second approach for comparing the assays was to analyze the DNA from the different *M. tuberculosis* strains with various numbers of IS*6110* copies, serially diluted in a BALF mixture. The results are shown in Figure 2.

Of the assays targeting IS*6110* in the dilutions with the strains carrying one copy of IS*6110*, the in-house Roche-5 assay was able to detect *M. tuberculosis* DNA to a 1:10,000 dilution.

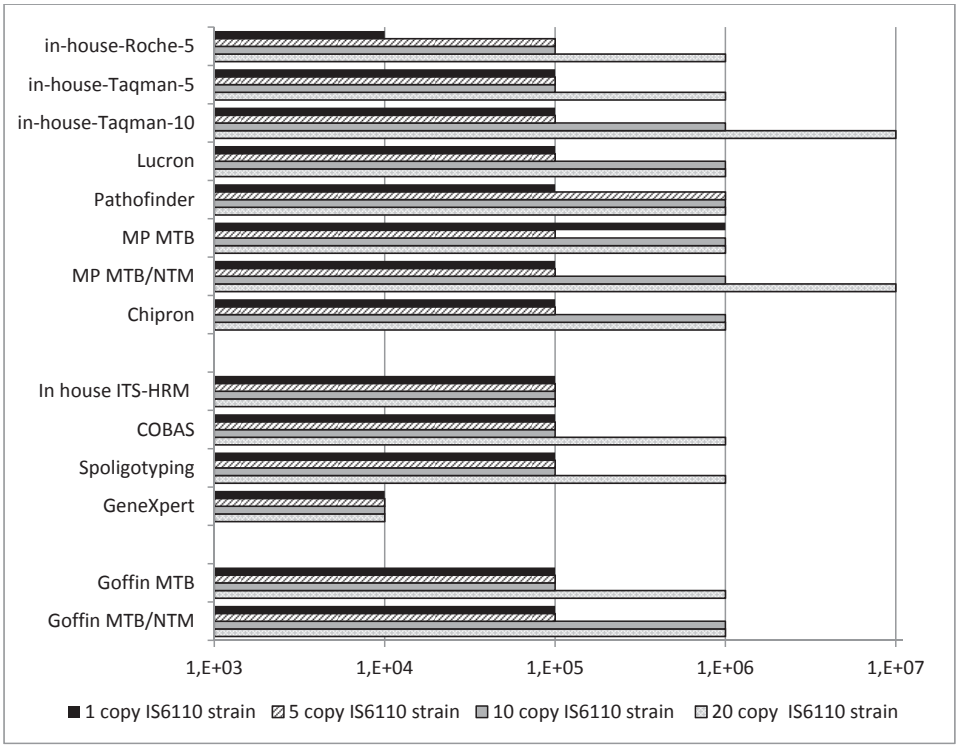

Figure 2 Comparison of analytical sensitivities of the assays tested with dilutions of DNA isolated from strains with various numbers of IS*6110* copies in BALF samples.
The *x* axis shows the dilution, which was recorded as positive if an assay tested positive three times for the specific assay. The *y* axis shows the assays tested. The first eight assays target IS*6110*. The next four assays are known not to target IS*6110*. For the two Goffin assays, the manufacturer does not disclose the target.

All other assays were able to detect *M. tuberculosis* DNA to a 1:100,000 dilution, and the MP MTB was even able to detect *M. tuberculosis* DNA to a 1:1,000,000 dilution.

The highest analytical sensitivity with the dilution of the five-IS*6110*-copy strain was found for the PathoFinder assay, which detected *M. tuberculosis* DNA to a 1:1,000,000. All other assays detected *M. tuberculosis* DNA to a 1:100,000 dilution.

With dilutions of the ten-IS*6110*-copy strain, both the in-house Roche-5 and the in-house Taqman-5 assays detected *M. tuberculosis* DNA to a 1:100,000 dilution. All other assays detected *M. tuberculosis* DNA to a 1:1,000,000 dilution.

With dilutions of the twenty-IS*6110*-copy strain, the MP MTB/NTM and in-house Taqman-10 assays were able to detect *M. tuberculosis* DNA up to a 1:10,000,000 dilution. All other assays were able to detect *M. tuberculosis* DNA to a 1:1,000,000 dilution.

All assays except MP MTB had increased analytical sensitivities for multicopy IS*6110* strains.

The analytical sensitivities of the assays not targeting IS*6110* yielded comparable results for the assays and were, as expected, not dependent on the number of IS*6110* copies per genome in the strains. An exception was the GeneXpert, which had an analytical sensitivity that was 10-fold lower than that of the other assays. The analytical sensitivity of Goffin MTB/NTM was higher if ten IS*6110* copies were present in the *M. tuberculosis* strain tested. For the dilutions of the twenty copies IS*6110* strain, the Cobas, Goffin MTB and spoligotyping tests were able to detect *M. tuberculosis* DNA at a one-log-higher dilution than for strains containing fewer copies of IS*6110*.

Inter-test variability of the molecular assays

The inter-test variability of each assay was defined by standard deviation (SD). The SD was calculated for the Cq values of the three positive test results per dilution per assay, using results from the *M. tuberculosis* serial dilutions in BALF mixture.

Results of three different dilutions are depicted in Table 2 to 4. The first results are for the undiluted series. The second results are for a dilution of 1:10,000, the dilution in which the first assay (in-house Roche-5) reached its analytical sensitivity (Figure 2). The last results are for the dilutions at which the separate assays reached the analytical sensitivity (AS) (which was a different level depending on the assay).

Table 2 Intertest variability of assays targeting IS*6110*, calculated by standard deviation (SD) of the Cq values

Assay	Copies / Dilution	1	5	10	20
Experimental-5[a]	Undiluted	0.19	0.29	0.06	0.07
	1:10,000	0.26	0.41	0.06	0.16
	AS[b] (=1:10,000)	0.26	1.53	0.46	1.08
In-house Taqman-5	Undiluted	0.04	0.39	0.04	0.01
	1:10,000	0.12	0.08	0.07	0.05
	AS	0.77	0.48	0.18	0.25
In-house Taqman-10	Undiluted	0.07	0.05	0.02	0.13
	1:10,000	0.10	0.02	0.07	0.03
	AS	0.60	0.29	0.36	0.4
Lucron	Undiluted	0.05	0.03	0.04	0.26
	1:10,000	0.5	0.33	0.07	0.2
	AS	1.21	0.14	0.44	0.61
Pathofinder	Undiluted	0.03	0.01	0.08	0.02
	1:10,000	0.43	0.10	0.05	0.04
	AS	0.47	0.39	0.40	0.23
MP MTB	Undiluted	0.4	0.30	0.07	0.07
	1:10,000	0.09	0.22	0.16	0.10
	AS	0	0.06	1.03	0.23
MP MTB/NTM[a]	Undiluted	0.03	0.03	0.06	0.04
	1:10,000	3.2	1.00	0.07	0.04
	AS	0.57	0.27	0.69	0.39

[a] Assays with highest intertest variability according to the standard deviation (SD). [b] AS, analytical sensitivity.

Table 3 Intertest variability of assays not targeting IS*6110*, calculated by standard deviation (SD) of the Cq values

Assay	Copies / Dilution	1	5	10	20
In-house ITS-HRM	Undiluted	0.04	0.06	0.06	0.06
	1:10,000	0.25	0.61	0.64	0.15
	AS	1.36	0.53	0.73	0.20
Cobas	Undiluted	0.1	0.25	0.1	0.06
	1:10,000	0.25	0.2	0.31	0.21
	AS	0.46	1.06	0.72	1.93

Table 4 Intertest variability of assays with a target not disclosed by the manufacturer, calculated by standard deviation (SD) of the Cq values

Assay	Copies / Dilution	1	5	10	20
Goffin MTB	Undiluted	0.03	0.02	0.06	0.03
	1:10,000	0.18	0.04	0.08	0.11
	AS	0.97	0.43	0.23	0.20
Goffin MTB/NTM	Undiluted	0.05	0.05	0.09	0.42
	1:10,000	0.29	0.14	0.14	0.15
	AS	1.15	0.40	0.82	0.47

For all assays, the intertest variability was within a standard deviation of 2. Only a disproportionately high inter-test variability (SD 3.2) was found for MP MTB/NTM assay for the 1:10.000 dilutions containing one copy of IS*6110*.

Calculating the intertest variability was not possible for GeneXpert as a sole test, due to its five probes. All probes had their own Cq value and had to give a positive result to score the overall result as positive. After the results for the GeneXpert assay in this laboratory became known, we retested GeneXpert in another laboratory. We did this to calculate intertest variability of GeneXpert with isolates in dilutions of 1:1,000 and 1:10,000 in triplicate. The results of the intertest variability of the probes themselves from both laboratories are shown in Table 5.

Discussion

This is the first study to describe a direct comparison of 14 different PCR assays for the molecular detection of *M. tuberculosis* complex in clinical material. We assessed the sensitivities of the assays using two different approaches, as well as studied intertest variability. All assays performed equally well regarding detection limit and analytical sensitivity for the detection of serial dilutions of *M. tuberculosis* suspensions with various numbers of IS*6110* elements; the positive exceptions were the in-house Taqman-10 and the MP MTB/NTM assays. A more clear discrepancy between results in both approaches was observed for the Lucron and PathoFinder tests. The inter-test variability of all assays tested was within 2 standard deviations, and their performance was thus comparable.

For the first comparison approach, assays targeting IS*6110* showed the lowest detection limits. In a comparison for the overall detection limits for all assays with the analytical sensitivities,

Table 5 Intertest variability of the probes of GeneXpert, calculated by standard deviation (SD) of the Cq values

GeneXpert probe and laboratory testing location	Copies / Dilution	1	5	10	20
A	Undiluted	1.2	2.0	2.8	1.1
Lab 1	1:1000	1.0	0.7	1.0	1.4
Lab 2	1:1000	NC[a]	2.7	1.5	8.2
Lab 1[b]	1:10,000	0.9	0.8	2.8	1.8
Lab 2	1:10,000	1.3	1.3	ND[c]	1.1
B	Undiluted	1.4	2.1	2.9	1.6
Lab 1	1:1000	1.0	0.7	1.1	1.3
Lab 2	1:1000	NC	2.5	1.3	7.3
Lab 1	1:10,000	1.0	0.7	2.3	1.9
Lab 2	1:10,000	1.0	1.8	ND	1.0
C	Undiluted	1.1	2.1	3.0	1.0
Lab 1	1:1000	1.0	0.7	1.1	1.4
Lab 2	1:1000	NC	2.7	1.4	8.3
Lab 1	1:10,000	0.9	0.8	2.3	1.7
Lab 2	1:10,000	1.3	1.6	ND	0.9
D	Undiluted	1.1	1.7	2.8	1.0
Lab 1	1:1000	1.1	0.6	1.0	1.3
Lab 2	1:1000	NC	2.5	1.4	8.0
Lab 1	1:10,000	0.9	0.8	2.0	1.7
Lab 2	1:10,000	1.1	1.5	ND	1.1
E	Undiluted	1.3	2.3	3.0	0.8
Lab 1	1:1000	1.1	0.7	1.0	1.3
Lab 2	1:1000	NC	2.8	1.6	ND
Lab 1	1:10,000	0.9	0.9	2.3	1.8
Lab 2	1:10,000	1.6	1.4	ND	1.2

[a] NC, one of the three runs gave an error and thus the interest variability could not be calculated.
[b] GeneXpert reached its analytical sensitivity with a dilution of 1:10,000 in lab 1.
[c] ND, at least one run could not be detected and thus the analytical sensitivity was not.

all assays performed similarly, except for Lucron and PathoFinder. An explanation might be the lower DNA input in both these assays (5 µl DNA) compared to that of the best performing assays (10 µl DNA). Another possible explanation is that the DNA extraction method (PrepMan for the DL and EasyMAG for the analytical sensitivity) yields differences in the purity of the DNA, and less pure DNA might yield a higher detection limit [15]. To our knowledge, no direct comparison of the PrepMan and EasyMAG extraction procedures for *M. tuberculosis* has been performed so far.

All assays targeting IS*6110* had increasing analytical sensitivity with an increasing number of IS*6110* copies in the strains tested, which is in accordance with our hypothesis. Only the MP MTB assay yielded unexpected results. This kit had an analytical sensitivity with the dilution of the one-copy IS*6110* strain, which was better compared to the five-copy IS*6110* strain. Both in-house Taqman-10 and MP MTB/NTM had the lowest detection limit as well as the highest analytical sensitivity at the dilution of the twenty-copy IS*6110* strain. These were the only assays yielding fully concordant results between the detection limit analysis and the analytical sensitivity. Assays not targeting IS*6110* yielded comparable results with both approaches. All assays had a better analytical sensitivity at the dilutions of the twenty-IS*6110*-copy strain, except for the In-house ITS-HRM and GeneXpert assays. Since the analytical sensitivity of Goffin MTB/NTM, which has an undisclosed molecular target, performed better if more IS*6110* copies were present in the test strain, this assay probably also targets IS*6110*.

Intertest variability was the third study subject and was tested on basis of triplicate results from the serial dilutions in BALF mixture. Overall, no assay was found to under- or outperform another. Nevertheless, the highest intertest variability of the assays, independent of their target and the tested dilution, was found with MP MTB/NTM assay at the 1:10,000 dilutions. One of the three runs of this assay had a Cq value at this dilution, which is used as cutoff value by the manufacturer. The result was still positive, but had a difference of 5 Cq values compared to those of the other two runs. After the results were analyzed, this was the only divergent run of all runs tested for each PCR assay included, for which we found no obvious laboratory-related explanation. Tests that have higher inter-test variability obviously are more prone to produce more divergent results.

We checked our methodology in this study regarding the even distribution of *M. tuberculosis* in the BALF samples, the procedure of heat killing of the strains and the efficacy of DNA isolation. The homogenized and concentrated NaLC-NaOH-treated BALF mixture was checked to be negative for MTC using the in-house IS*6110* RT-PCR. Comparing the amplification of DNA before and after heat killing tested the influence of this crucial step on DNA isolation and the even distribution of *M. tuberculosis* in BALF samples. Furthermore, the reproducibility of the DNA isolation was tested at three different time points. All differences in Cq values of the heating procedure and the reproducibility were below the 0.5-log^{10} difference. A difference above this value is considered to be clinically relevant [16,17]. We therefore conclude that our methodology of DNA isolation of the strains by using heat-killed bacilli, the even distribution of MTC, and the reproducibility was appropriate.

The focus of this study was on the analytical sensitivity of the assays. A comparison of the clinical sensitivities was not performed on routine clinical samples, such as sputum, because the quantity of these samples is normally low, especially after NaLC-NaOH treatment and homogenization of this material. Also, the distribution of *M. tuberculosis* DNA in sputum is generally not homogeneous. Therefore, a comparison of fourteen PCR assays using sputum samples is virtually impossible and would have yielded irreproducible sensitivity results for each assay. A benefit of our comparison with one pool of BALF samples is that this is highly comparable for each assay. This material was used to add cultured bacteria in serial dilutions, in order to mimic as much as possible the clinical context for patients with difficult-to-diagnose TB. For future evaluation of novel molecular assays to be compared within the same conditions, this material has been stored at -80°C.

In our current analysis comparing 14 assays, the GeneXpert assay showed a lower analytical sensitivity than all other assays. However, it is likely that after the suspension was heated, freezed and thawed, some of the bacterial cells were already lysed. As this is the only assay purifying whole bacterial cells from clinical material, this may have negatively influenced its performance in comparison to the methods that directly isolate and purify DNA. This hypothesis is subject for further research, which is planned for the near future. As the individualized probes of the GeneXpert sometimes revealed intertest variability exceeding two standard deviations, we retested the GeneXpert intertest variability in another laboratory with isolates which were kept at -80°C. In this second laboratory, the intertest variability for the individualized probes was even greater than in the first laboratory.

Our study also evaluated the dependence of the assays on the number of IS*6110* copies in the genome of MTC species detected in the BALF samples. It is known that especially in Asia IS*6110* is absent in a considerable part of the *M. tuberculosis* isolates [18]. In the Netherlands, over a 5-year period, the IS*6110* element was absent in only 3 isolates of a total of 3,884 isolates. As expected, our study showed that assays targeting the IS*6110* have a higher sensitivity with larger numbers of copies of IS*6110*. In any case, the results in PCR targeting IS*6110* in MTC will vary according to the number of targets present in the detected bacteria, as now has been proven in this study.

Clinical sensitivity is widely used as a measure for evaluating the performance of a PCR assay. The analytical sensitivity as used in our study has, in contrast to clinical sensitivity, hardly been used for comparing the performance of PCR assays [2-6]. However, a comparison of data regarding clinical sensitivity must be interpreted with caution because clinical sensitivity

is calculated by comparing results of with culture results or with the clinical diagnosis. Comparing the clinical sensitivities of assays is important in smear-negative culture-positive samples, as smear-positive culture-positive samples already are known to have high clinical sensitivity due to a higher bacterial load [19,20]. Research focused on comparing clinical sensitivity of selected assays in smear-negative culture-positive samples, using the same clinical specimens, is therefore needed.

In conclusion, comparing the sensitivities of PCR assays is most reliable by analyzing the analytical sensitivities and the detection limits. The detection limits were nearly identical compared to the results of the analytical sensitivity in this study, except for the results of Lucron and PathoFinder assays. As expected, the analytical sensitivity was better for strains with more copies of IS*6110* elements. GeneXpert had the lowest analytical sensitivity and was at least 10-fold less sensitive than the other assays. In our study, the in-house Taqman-10 and MP MTB/NTM assays had the lowest detection limits and the highest analytical sensitivities while testing dilutions of the twenty-IS*6110*-copy strain. Overall, intertest variability was within 2 standard deviations and was comparable for all assays, except for the individualized probes of GeneXpert, which sometimes had intertest variability exceeding 2 standard deviations.

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2B

Optimization of standard in-house 24-locus variable-number tandem-repeat typing for *Mycobacterium tuberculosis* and its direct application to clinical material

JL de Beer
OW Akkerman
AC Schürch
A Mulder
TS van der Werf
AGM van der Zanden
J van Ingen
D van Soolingen

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Abstract

Variable-number tandem-repeat (VNTR) typing with a panel of 24 loci is the current gold standard in the molecular typing of *Mycobacterium tuberculosis* complex isolates. However, because of technical problems, a part of the loci often cannot be amplified by multiplex PCRs. Therefore, a considerable number of single-locus PCRs have to be performed for the loci with missing results, which impairs the laboratory work flow. Therefore, the original in-house method described by Supply et al. in 2006 was reevaluated. We modified seven primers and the PCR master mixture and obtained a strongly optimized in-house 24-locus VNTR typing method. The percentage of instantly complete 24-locus VNTR patterns detected in the routine flow of typing activities increased to 84.7% from the 72.3% obtained with the typing conducted with the commercially available Genoscreen MIRU-VNTR typing kit. The analytical sensitivity of the optimized in-house method was assessed by serial dilutions of *M. tuberculosis* in bronchoalveolar lavage fluid. A 1:10 dilution of the different strains tested was the lowest dilution for the detection of a complete 24-locus VNTR pattern. The optimized in-house 24-locus VNTR typing method will reduce the turnaround time of typing significantly and also the financial burden of these activities.

Introduction

Since the introduction of standardized variable-number tandem repeat (VNTR) typing in 2006 by Supply et al. [1], laboratories worldwide have implemented this method. Compared to the level of discrimination of the formerly used restriction fragment length polymorphism (RFLP) typing method, that of 24-locus VNTR typing has proven to be sufficient to trace the trans-mission of tuberculosis (TB) in low-burden settings [1,2]. The advantages of VNTR typing over RFLP typing include simplified comparison of the results and applicability to small amounts of DNA, by which the turnaround time decreased from an average of 44 days to 15 days at our laboratory. However, in the first worldwide proficiency study of VNTR typing, the level of interlaboratory reproducibility was only 60% and intralaboratory reproducibility was only 72%. A second worldwide proficiency study revealed important improvements after the adjustment of some technical elements in the methodology and a higher degree of standardization [3]. Still, laboratories applying VNTR typing face several technical challenges. First and foremost, some of the 24 loci may not be amplified in the multiplex PCRs and have to be amplified with a single-locus PCR; this holds true both for the commercially available MIRU-VNTR typing kit (Genoscreen, Lille, France) and for the in-house methods. In practice, this involves a significant increase in the workload and turnaround time. The need for an optimized, fast, and high-quality VNTR typing method is high, especially for municipal health services and clinicians. The results of typing are used to steer the direction of source case finding and eventually to support the activities of the elimination of TB transmission. For the clinician, the most important information extracted from the results of typing is whether the patient has a TB relapse or an exogenous TB reinfection.

Given the common use worldwide of the standardized VNTR typing method, we have attempted to improve the original in-house VNTR method described by Supply et al. [1]. In addition, we have determined the minimum amount of DNA required for successful VNTR typing of *Mycobacterium tuberculosis* in clinical material.

Materials and methods

Samples

For optimization of the in-house 24-locus VNTR typing technique, we used the DNA of two different *M. tuberculosis* strains, control strain H37Rv and a strain from the National

Tuberculosis Reference Laboratory (NLA000901369). For the final quality check of the optimized in-house method, we used the panels used in the first [4] and second [3] proficiency studies on VNTR typing.

To detect the effect of the implementation of the optimized in-house technique rather than the commercial method used, we included the results of routine typing of *M. tuberculosis* isolates as part of the national surveillance in The Netherlands conducted at the National Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The percentage of complete 24-locus VNTR patterns obtained with the commercial typing kit from January 2010 to November 2011 was compared with that obtained with the optimized in-house method from November 2011 to July 2013.

DNA isolation

DNA isolation was performed with the QIAamp DNA Minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for DNA purification from blood and body fluids. From a positive culture medium, 1 ml was centrifuged for 15 min (11,800 *g*). The pellet was used as the input for the DNA isolation procedure. From a solid medium, 1 colony was suspended in MilliQ water to serve as the starting material. The DNA was eluted from the column in 30 l of elution buffer and diluted to a final concentration of 10 ng/ l.

Twenty-four-locus VNTR typing by the commercial kit

The commercial 24-locus VNTR typing kit from Genoscreen (Lille, France) was used in accordance with the manufacturer's instructions. This technique was used in a diagnostic setting until November 2011.

Optimized in-house method for 24-locus VNTR typing

The original set of primers described by Supply et al. was evaluated by the optimized in-house method for 24-locus VNTR typing. Seven primers affecting the amplification of 4 loci of the complete 24-locus VNTR set were replaced with new primers designed with Primer3Plus [5]. The sequences of these new primers are in bold in Table 1; the other primers used are identical to those described by Supply et al. [1]. The final concentrations of the for-ward and reverse primers for a particular locus were identical.

Table 1 Primer sequences used in the optimized 24-locus VNTR typing method[a]

Locus	Genome position	Mix nr.	Final primer conc.[b] (nM)	Primer sequence (5'– 3' with labeling)
MIRU 04, ETR-D	580	1	400	GCGCGAGAGCCCGAACTGC (6FAM)[c] GCGCAGCAGAAACGCCAGC
MIRU 26	2996	1	400	CATAGGCGACCAGGCGAATAG (VIC) TAGGTCTACCGTCGAAATCTGTGAC
MIRU 40	802	1	400	GGGTTGCTGGATGACAACGTGT (NED) GGGTGATCTCGGCGAAATCAGATA
MIRU 10	960	2	400	GCCACCTTGGTGATCAGCTACCT (6FAM) GTTCTTGACCAACTGCAGTCGTCC
MIRU 16	1644	2	320	CCCGTCGTGCAGCCCTGGTAC (VIC) TCGGTGATCGGGTCCAGTCCAAGTA
MIRU 31, ETR-E	3192	2	400	GTGCCGACGTGGTCTTGAT (NED) ACTGATTGGCTTCATACGGCTTTA
Mtub 04	424	3	400	GGCAGCAGAGCCCGGGATTCTTC (6FAM) CTTGGCCGGCATCAAGCGCATTATT
ETR-C	577	3	320	CGAGAGTGGCAGTGGCGGTTATCT (VIC) AATGACTTGAACGCGCAAATTGTGA
ETR-A	2165	3	400	AAATCGGTCCCATCACCTTCTTAT (NED) CGAAGCCTGGGGTGCCCGCGATTT
Mtub 30	2401	4	400	CTTGAAGCCCCGGTCTCATCTGT (6FAM) ACTTGAACCCCCACGCCCATTAGTA
Mtub 39	3690	4	400	CGGTGGAGGCGATGAACGTCTTC (VIC) TAGAGCGGCACGGGGGAAAGCTTAG
QUB 4156	4156	4	400	**GATGTGCGGTACGTGCATC (NED)** **TGACCACGGATTGCTCTAGTC**
QUB 11b	2163b	5	800	**GTCGAAGTGAATGGTGGCAT (6FAM)** **GTAAGGGGGATGCGGGAAAT**
Mtub 21	1955	5	400	AGATCCCAGTTGTCGTCGTC (VIC) CAACATCGCCTGGTTCTGTA
QUB 26	4052	5	640	AACGCTCAGCTGTCGGAT (NED) **GCCAGGTCCTTCCCGAT**
MIRU 02	154	6	400	TACTCGGACGCCGGCTCAAAAT (6FAM) TGGACTTGCAGCAATGGACCAACT
MIRU 23	2531	6	400	CTGTCGATGGCCGCAACAAAACG (VIC) AGCTCAACGGGTTCGCCCTTTTGTC

Table 1 continues on next page

Table 1 *Continued*

Locus	Genome position	Mix nr.	Final primer conc.[b] (nM)	Primer sequence (5′– 3′ with labeling)
MIRU 39	4348	6	400	CGGAAACGTCTACGCCCCACACAT (NED) CGCATCGACAAACTGGAGCCAAAC
MIRU 20	2059	7	400	**GGAGAGATGCCCTTCGAGTTAG (6FAM)** **GGAGACCGCGACCAGGTA**
MIRU 24	2687	7	400	GGGCGAGTTGAGCTCACAGAA (VIC) CGACCAAGATGTGCAGGAATACAT
MIRU 27	3007	7	400	GCGATGTGAGCGTGCCACTCAA (NED) TCGAAAGCCTCTGCGTGCCAGTAA
Mtub 29	2347	8	400	GCCAGCCGCCGTGCATAAACCT (6FAM) AGCCACCCGGTGTGCCTTGTATGAC
ETR-B	2461	8	800	ATGGCCACCCGATACCGCTTCAGT (VIC) CGACGGGCCATCTTGGATCAGCTAC
Mtub 34	3171	8	320	GGTGCGCACCTGCTCCAGATAA (NED) GCTCTCATTGCTGGAGGGTTGTAC

[a] Primers that differ from those used in the standardized method described by Supply et al. [1] are in bold.
[b] The final concentrations of the forward and reverse primers for a particular locus were identical.
[c] FAM, 6-carboxyfluorescein.

Three different commercially available PCR master mixtures were used to amplify the DNA control samples, i.e., PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, United Kingdom), Multiplex PCR 5 Master Mix (Westburg Benelux Office, Leusden, The Nether-lands), and AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA).

A DNA input of 2 l was used to perform the amplification reactions. The PCR products obtained with the optimized in-house method of 24-locus VNTR typing were visualized by gel electrophoresis (2% agarose).

All VNTR analyses were performed according to standard laboratory procedures by three experienced technicians.

We considered implementation only when a specific combination of PCR master mixture and primers met the following criteria. (i) Twenty-four loci in monoplex reactions had to produce the expected amplicon sizes for the DNA controls used, and (ii) triplex PCRs had to produce identical amplicon sizes, as detected with a capillary electrophoresis (CE) DNA analyzer (ABI 3730).

Implementation in a diagnostic setting

For final implementation in a diagnostic setting, national surveillance, the lengths of PCR products were detected with a CE DNA analyzer (ABI 3730) with the addition of an internal lane size standard, the GeneScan 1200 Size Standard (Applied Biosystems, Foster City, CA).

Samples used for analytical sensitivity testing

In a recent publication by Akkerman et al. [6], 14 molecular assays were compared for *M. tuberculosis* complex detection in pooled bronchoalveolar lavage fluid (BALF). Serial dilutions of four *M. tuberculosis* strains with various numbers of copies (1, 5, 10, and 20) of the IS*6110* element were used to deter-mine the analytical sensitivity of the optimized 24-locus VNTR typing method in BALF. All VNTR analyses were performed in triplicate.

Statistical analysis

The statistical significance of differences between the two tests performed was determined by Pearson's chi-square test on a two-by-two table of method versus result.

Results

Optimization of the in-house 24-locus VNTR typing method

The results of amplification by PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, United Kingdom) did not meet the criteria because multiple nonspecific amplicons with unexpected lengths were obtained. The results of amplification by the Multiplex PCR 5 Master Mix (Westburg Benelux Office, Leusden, The Netherlands) also did not meet the expectations. In this case, the optimum annealing temperatures for the different loci were too far apart to be useful in a multiplex PCR. The amplification results of the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA) yielded the amplicon sizes expected for all of the 24 loci. Even in a multiplex reaction, the amplicons were clear in the analysis of the results in the CE DNA analyzer. Thus, the final PCR mixture of the optimized 24-locus VNTR typing method consisted of 12.5 l of AmpliTaq Gold 360 Master Mix for each reaction with a total volume of 25 l. Table 1 depicts the loci combined in the multiplex PCR mixtures and the final primer concentrations. The PCR program used was 10 min at 96°C;

40 cycles of 1 min at 96°C, 1 min at 60°C, and 1 min at 75°C; and a final step of 7 min at 72°C.

Quality of the optimized in-house 24-locus VNTR typing method

The interlaboratory reproducibility of the optimized in-house 24-locus VNTR typing method was tested before the implementation of this method in the daily routine. The results obtained with the panels used in the first and second worldwide studies of VNTR typing proficiency [3,4] organized by the RIVM were good; the interlaboratory reproducibility was 100%, and the intralaboratory reproducibility was 97%.

Implementation of the optimized in-house VNTR typing method

In a 20-month period (November 2011 to June 2013) after the introduction of the optimized in-house 24-locus VNTR typing method, 1,401 *M. tuberculosis* strains were typed by this method. The percentage of instantly complete VNTR patterns detected was 84.7% (n=1,186). The performance of the commercial 24-locus VNTR typing method was mapped retrospectively. In a 22-month period (January 2010 to October 2011), 1,638 *M. tuberculosis* strains were typed in the daily routine. The percentage of complete 24-locus VNTR patterns detected by this test was 72.3% (n=1,184). The percentage of initial complete results of the optimized in-house 24-locus VNTR typing method was shown to be significantly higher (P=0.001) and was related to better yields for the loci for which new primers were designed.

The incomplete VNTR patterns detected were caused by missing results for one or more alleles because of amplification failure or because of the false detection of two different numbers of repeats for one or two alleles. Of the incomplete patterns detected by the commercial 24-locus VNTR method, 91.6% (n=416) were due to missing results and 8.4% (n=38) were due to the detection of double alleles. By the optimized 24-locus VNTR method, these were 80.9% (n=174) and 19.1% (n=41), respectively.

The analytical sensitivity of VNTR typing by the optimized 24-locus VNTR method was tested with DNA from serial dilutions of the four *M. tuberculosis* strains in BALF. The 24-locus VNTR patterns of the four *M. tuberculosis* strains used to prepare dilutions are given in Table 2. Undiluted samples and 1:10 dilutions of all four strains yielded a complete 24-locus VNTR pattern by the optimized in-house method. With the 1:100 dilution of BALF with a strain with a single copy of IS*6110*, one of the three VNTR analyses yielded results for

Table 2 VNTR patterns of the four strains used to test the analytical sensitivity of the optimized in-house VNTR method

Locus	Genome position number	No. of repeats in the strain with following no. of IS*6110* copies:			
		1	5	10	20
MIRU 04	580	5	2	2	2
MIRU 26	2996	2	5	5	6
MIRU 40	802	4	4	4	2
MIRU 10	960	4	4	4	3
MIRU 16	1644	3	3	3	3
MIRU 31	3192	4	3	2	5
VNTR 42	424	2	4	3	4
VNTR 43	577	4	3	2	4
VNTR ETR-A	2165	6	3	2	4
VNTR 47	2401	1	4	1	4
VNTR 52	3690	4	3	2	3
VNTR 53	4156	1	3	2	2
VNTR QUB11b	2163b	3	3	1	5
VNTR 1955	1955	6	3	3	5
VNTR QUB-26	4052	6	5	6	8
MIRU 02	154	2	2	1	2
MIRU 23	2531	6	5	6	5
MIRU 39	4348	3	2	2	3
MIRU 20	2059	2	2	2	2
MIRU 24	2687	2	1	1	1
MIRU 27	3007	3	3	3	3
VNTR 46	2347	3	4	4	4
VNTR 48	2461	1	2	2	2
VNTR 49	3171	3	3	1	3

only 22 loci instead of 24; for the other three strains with higher IS*6110* copy numbers, this dilution showed the complete VNTR pattern thrice. Also, with the 1:1,000 dilution of BALF with the strain with a single copy of IS*6110*, one of the triplicates showed an incomplete VNTR pattern of 23 loci. The 1:1,000 dilution of the other samples tested showed a triplicate of complete patterns for the strains with 5 and 10 IS*6110* copies but three times showed an incomplete VNTR pattern with 10 missing loci for the strain with 20 IS*6110* copies. At dilution one step higher, 1:10,000, incomplete VNTR patterns were obtained for all of the strains. Table 3 shows the results in quantitation values of the *M. tuberculosis* detection tests for the analytical sensitivity of VNTR typing determined at a 1:100 dilution.

Table 3 Analytical sensitivity of VNTR typing performed with 1:100 dilution[a]

Assay	Cq with strain with following no. of IS6110 copies:			
	1	5	10	20
In-house-Roche-5	31.20	27.19	27.35	26.21
In-house-Taqman-5	30.43	27.50	26.44	25.30
In-house-Taqman-10	29.86	26.24	25.61	24.40
Lucron	30.31	27.29	26.92	25.57
Pathofinder	29.81	26.77	25.72	24.68
MP MTB	32.66	29.48	28.77	27.81
MP MTB/NTM	32.14	28.95	28.19	27.50
In-house ITS-HRM	24.62	28.33	29.24	28.22
Cobas	32.03	33.00	34.87	31.40
GeneXpert	20.90	24.77	24.57	19.43
Goffin MTB	32.66	29.48	28.77	27.81
Goffin MTB/NTM	32.14	28.95	28.19	27.47

[a] Associated with the quantitation cycle (Cq) values of the diagnostic tests performed in the study of Akkerman et al.

Discussion

We have optimized the VNTR typing method. The combination of seven redesigned primers and the use of a suitable master mixture contributed to the high percentage (84.7%) of complete 24-locus VNTR typing profiles in the first multiplex PCR run. This practical improvement is considerable, as fewer strains have to be reamplified by single-target PCRs, and this reduces the workload and turnaround time.

Another important aspect of this improvement is cost efficiency. The high cost of the commercially available VNTR kit and a lack of access to a CE DNA analyzer hamper its use in many laboratories. The optimized in-house method may be an important alternative, because the products of single-locus PCRs can be analyzed on a gel without the need of a sophisticated DNA analyzer, and the yield of complete VNTR profiles obtained by this inexpensive approach is better than that obtained by the commercial method. In a recent proficiency study of VNTR typing, the sizing of VNTR PCR products yielded almost the same degree of reliability as the commercial method [3].

For the daily practice of molecular epidemiological studies of TB and to reduce the laboratory turnaround time, an adequately performing 24-locus VNTR typing method is of the utmost importance. Failing loci hamper the interpretation of VNTR typing results in many countries,

and this has introduced a bias into the international comparability of VNTR typing results. Failing loci are not the result of a natural absence of VNTRs, as often assumed, but merely the result of technical problems in the typing technique used [4].

In the meantime, the commercial 24-locus VNTR method was changed from an eight-reaction system to a six-reaction system. The manufacturer did not disclose information about the technical adjustments.

In this study, we also analyzed the analytical sensitivity of the improved in-house VNTR typing method. The detection of the 24-locus VNTR pattern of the *M. tuberculosis* complex in BALF showed an analytical sensitivity of 1:10 for the optimized in-house method. A slightly more lenient definition of analytical sensitivity changes the interpretation to an analytical sensitivity of 1:100. An analytical sensitivity of 1:100 can be used for VNTR typing. With the risk of missing a single locus, VNTR typing can still exclude the possibility of transmission and relapses.

In theory, this sensitivity provides the ability to perform typing of *M. tuberculosis* directly in BALF samples, even with a minimal bacterial load. This may increase the applicability of typing results in the early stage of examinations of TB outbreaks or, in an earlier diagnostic phase, permit better discrimination of whether a patient has a relapse or a new infection. The moment VNTR typing can be performed depends on the bacterial load of the sample, shown as quantitation cycle values, and can be read from Table 3 for different diagnostic tests. In practical diagnostics, the number of IS*6110* elements is unknown; therefore, the results of the 10-copy IS*6110* strain can be used and are most representative of the samples collected in The Netherlands.

This study has some important limitations. First, we could not use a single set of samples to investigate the performance of the standard versus that of the improved VNTR methodology. Nonetheless, the sets used to assess the performance of both methods are representative of the epidemiology in The Netherlands and were similar with regard to genotype distributions. Second, analytical sensitivity should ideally be determined with prospectively gathered clinical specimens for which quantitative cultures are performed in parallel. In our national reference laboratory setting, these materials cannot be gathered. In summary, optimization of the in-house 24-locus VNTR method resulted in 84.7% complete VNTR patterns in a diagnostic setting. This improves the laboratory workflow because of the reduction of the number of reamplification reactions. In addition, this technique is much cheaper than the commercial 24-locus VNTR method and is useful for laboratories without a CE DNA analyzer.

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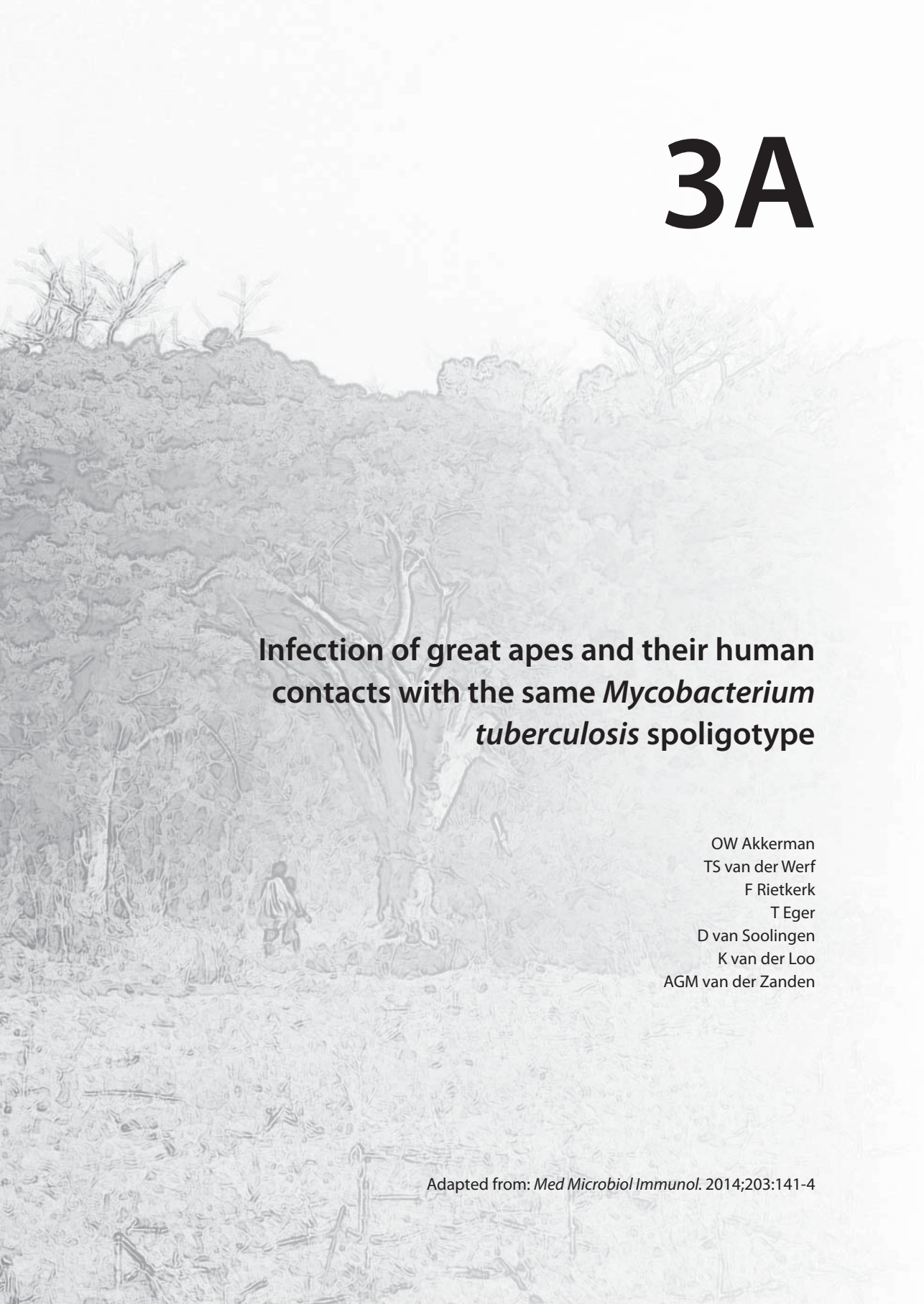

Infection of great apes and their human contacts with the same *Mycobacterium tuberculosis* spoligotype

OW Akkerman
TS van der Werf
F Rietkerk
T Eger
D van Soolingen
K van der Loo
AGM van der Zanden

Adapted from: *Med Microbiol Immunol.* 2014;203:141-4

Abstract

A human was diagnosed with pulmonary tuberculosis (TB) after bi-annual screening for latent TB infection among zoo employees. In the same period, several bonobos of the zoo were suffering from TB as well. Based on *Mycobacterium tuberculosis* spoligotyping, the patient and the bonobos shared an identical outbreak strain. We provide evidence that the animals infected the human.

Introduction

Tuberculosis (TB) is a global threat with more than nine million cases reported annually [1]. In the Netherlands with its 16 million inhabitants, the incidence of pulmonary TB (inclusive both pulmonary and extrapulmonary) is 3,0/ 100,000, and for extrapulmonary TB, the incidence is 2,7/ 100,000. The common route of transmission of TB is by inhalation of aerosols containing viable *Mycobacterium tuberculosis* bacilli. Typically, transmission of TB between humans and/or animals occurs before the index case has been diagnosed [2]. Outbreaks of TB at schools, homeless shelters, nursing homes and prisons are common [3–9]. Outbreaks with transmission from animals to humans in zoos and circuses have been described infrequently [10]. Transmission of *M. tuberculosis* from animals to people is rare [3,4,11,12]. Here, we describe an outbreak of TB among bonobos (Pan Paniscus) living in captivity.

Report

In a primate's zoo in the Netherlands, the regional municipal health center (RMHC) performed a standard bi- annual screening for latent TB infection among zoo employees in June 2002. Sixty-one employees were tested by the tuberculin skin test (TST), and 7 other employees were tested through chest radiograph. None of the employees solely tested by radiology had evidence of TB. Seven employees tested positive in the TST, all with indurations of more than 15 mm. Six of them had normal chest radiographies. One of them showed signs suggesting active TB, with small infiltrates in the left upper lobe, but had no cough or sputum production. In this person, a bronchoscopy was performed. Microscopy with Ziehl-Neehlsen staining and direct in house IS*6110* PCR tests of the bronchoalveolar lavage remained negative. Culture revealed *M. tuberculosis*, which was confirmed with the GenoType Hain MTBC, Hain Life science GmbH. Conventional anti-TB treatment with isoniazid, rifampicin, ethambutol and pyrazinamide was started. The isoniazid and rifampicin were given for 6 months, the ethambutol was stopped when the results of drug susceptibility test revealed resistance only to streptomycin, and the pyrazinamide was stopped after 2 months. The employee made a full recovery. Of the seven employees with a positive TST result, five were animal keepers working with bonobos, including the employee with active TB. These animal keepers had been regularly in close contact with the infected animals. One of the seven employees with a positive TST result worked for the technical department of the zoo, and the last of the employees testing positive was an administrative co-worker, both without close contact to the infected animals. The population of bonobos in the zoo originally consisted of eleven monkeys. The bonobos' outdoor residence was large

Table 1 Overview of the bonobos, including their origin, year of entrance in the zoo, health status, date of first signs of disease and the location of affected organs, body sites

Bonobos	Origin	Year of entrance in the zoo	Date of first signs of disease	Died	Location of specimen with a positive culture result with *M. tuberculosis*
Hani	Institut National de Recherche Biomedical (INRB), Kinshasa, Democratic Republic Congo (DRC)	1998	August 2001 (cough and malaise)	October 2001	2004 (after reassessment by the first laboratory, Central Veterinary Institute, Lelystad, the Netherlands), lung, bronchial and mediastinal lymphnodes and abcesses of the abdomen
Rosi	INRB	1998	August 2001 (malaise, bad appetite, without cough)	March 2002	Lung and tracheobronchial lymphnodes (not cultured from liver, kidneys, spleen and mesenterial lymphnodes).
Molasso	INRB	1998	December 2001 (malaise) May/June 2002 Visible abcesses and diffuse infiltration on the chest radiograph	Survived	Lung, pleura, liver, kidneys, peritoneum, glandibulair lymphnodes, mediastinal lymphnodes, mesenterial lymphnodes
Tarishi	This zoo Parents: Mobishi/Jill		June 2002 (malaise, normal chest radiograph)	August 2002	Lung, bronchial lymphnodes and mediastinal lymphnodes. Culture positive with MTB of an abscess of the lung
Mobikisi	Zoo in Belgium	1996	Healthy		
Jashari	Zoo in Belgium	1996	Healthy		
Jill	Zoo in US	1997	Healthy		
Lomela	Zoo in Germany	1997	Healthy		
Zuani	INRB	1998	Healthy		

Table 1 continues on next page

Table 1 ***Continued***

Bonobos	Origin	Year of entrance in the zoo	Date of first signs of disease	Died	Location of specimen with a positive culture result with *M. tuberculosis*
Mwindu	INRB	1998	Healthy		
Liboso	INRB Parents: Zuani/Hani	1998	Healthy		
Kumbuka	This zoo Parents: Molassi/Hani	1999	Healthy		
Animal keeper	Netherlands		June 2002	Survived	July 2002 (BAL fluid)

and surrounded with a large canal. Distance to the public was more than 10 m. They had their own dormitory with an entrance only accessible for their keepers. For a description of the bonobos, their origin and their medical history, see Table 1. In the 12 months prior to the screening, four bonobos fell ill. One died in 2001, and retesting of the postmortem specimen of different body parts in 2004 showed *M. tuberculosis* in culture results. Two of the bonobos died during 2002 with signs suggesting TB and with *M. tuberculosis* recovered by culture of abscesses or specimens taken post-mortem from different body sites and organs. For a complete overview of the timeline and the affected organs, see Table 1. Drug susceptibility testing of the isolates of the bonobos was not done. Spoligotyping showed a similar pattern of all *M. tuberculosis* from the bonobos and the animal keeper (Figure 1a) [13]. The spoligopattern was identified as SIT2526. The octal code of the spoligopattern is 007777606000031 and was unique in the SpolDB4 database (personal communication N. Rastogi, Unité de la Tuberculose et des Mycobactéries, Institut Pasteur de Guadeloupe, Guadeloupe) [14]. Another laboratory (Laboratorium Medische Microbiologie en Infectiepreventie, Gelreziekenhuis Apeldoorn, the Netherlands) showed that one spacer in the spoligopattern was missing for two bonobos (Figure 1b), while the spoligopattern of the other two bonobos and the keeper was the same compared to the first laboratory (Central Veterinary Institute (CVI), Lelystad, the Netherlands).

The eight bonobos in the zoo, who were without active TB, were treated preventively with a combination of isoniazid and rifampicin. All of these eight bonobos have remained without evidence of active TB.

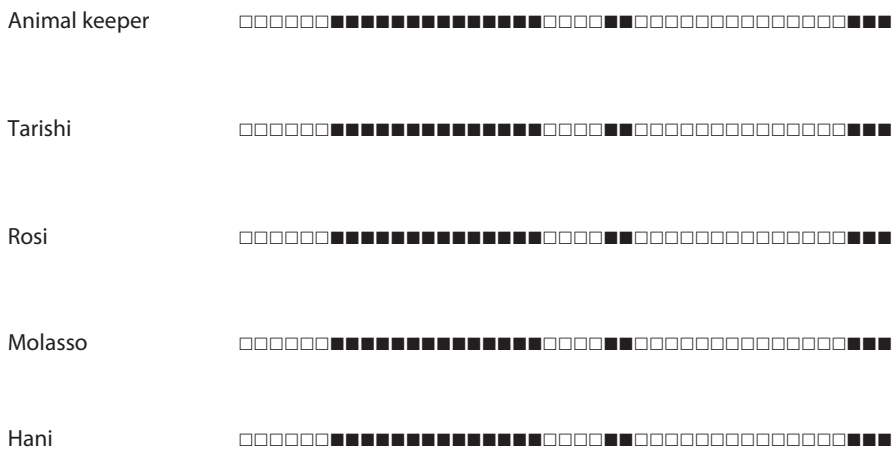

Figure 1a Spoligotyping of the bonobos and the animal keeper performed by the Central Veterinary Institute, Lelystad, the Netherlands.

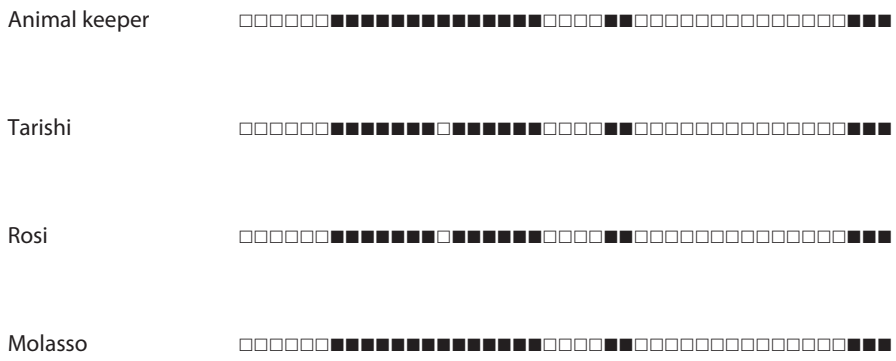

Figure 1b Spoligotyping of the bonobos and the animal keeper performed by the Laboratorium Medische Microbiologie en Infectiepreventie, Gelreziekenhuis Apeldoorn.

Discussion

TB transmission occurs through coughing, sneezing or other expiratory maneuvers of a contagious index case, by inhalation of viable bacilli present in the infectious aerosol. The contagiousness depends on the bacterial load, i.e., the number of viable bacilli/ml of sputum that the index patient produces, the exposure time and intensity, plus behavioral factors such as the cough etiquette of the index case [15–17].

Transmission of TB between humans and animals, though uncommon, has been described earlier. However, there are no data about contagiousness of animals suffering from TB; the type of expiratory maneuvers in nonhuman primates that results in transmission of TB is largely unknown, as is the route of transmission. In the outbreak we describe here, the ill bonobos likely caused the animal keeper to become infected and develop active disease. Transmission from zoo visitors to bonobos was highly unlikely as the distance from public to the residence of the bonobos was always more than ten meters. Also, the investigated specimens, both pulmonary and extrapulmonary, of the bonobos were smear and culture positive, while the animal keeper had smear negative, culture positive sputum. Lastly, the affected bonobos were more ill, and their TB was more widespread compared to the single human patient. In contrast, the positive TST results of the zoo employees do not prove transmission as two of the employees were without close contact with the bonobos.

The results by spoligotyping of the isolates of the bonobos and this animal keeper supported the route of transmission from bonobos to the animal keeper. The isolates showed identical spoligopatterns. Another laboratory did the spoligotyping of the isolates as well and found the absence of a single spacer in the isolates of two bonobos as compared to the pattern of the animal keeper. Changes in one spacer can be due to inter-laboratory differences that are coincidental. But they are also a hallmark of genetic drift in clonal subpopulations [18]. Both make that an extra manner of genotyping, like variable number of tandem repeats, is not contributable to understanding the route of transmission. As the spoligopattern of both isolates contained the spacers 8 and 9, this spoligopattern made these isolates more likely to be *M. tuberculosis* than *M. africanum*, as the latter misses spacers 8 and 9. The isolates were confirmed to be *M. tuberculosis* after testing them with the GenoType Hain MTBC. In a previous study by Gibson et al. [19], these isolates were identified as *M. tuberculosis* as well.

The source of the infection of the bonobos could not be found because no other spoligopattern was found in the global database SpolDB4. We therefore assume that the bonobos had already been infected in the Democratic Republic Congo, where they had resided in a biomedical research center in Kinshasa, before their translocation to the zoo, even though TB tests of the animals before they left the DRC had been negative.

In conclusion, TB may occur in captive bonobos, and as we described, the animals can infect their keepers even without extremely close contact.

Acknowledgement

We would like to thank Nalin Rastogi, Unité de la Tuberculose et des Mycobactéries, Institut Pasteur de Guadeloupe, Guadeloupe, for the correspondence over the spolDB4 database.

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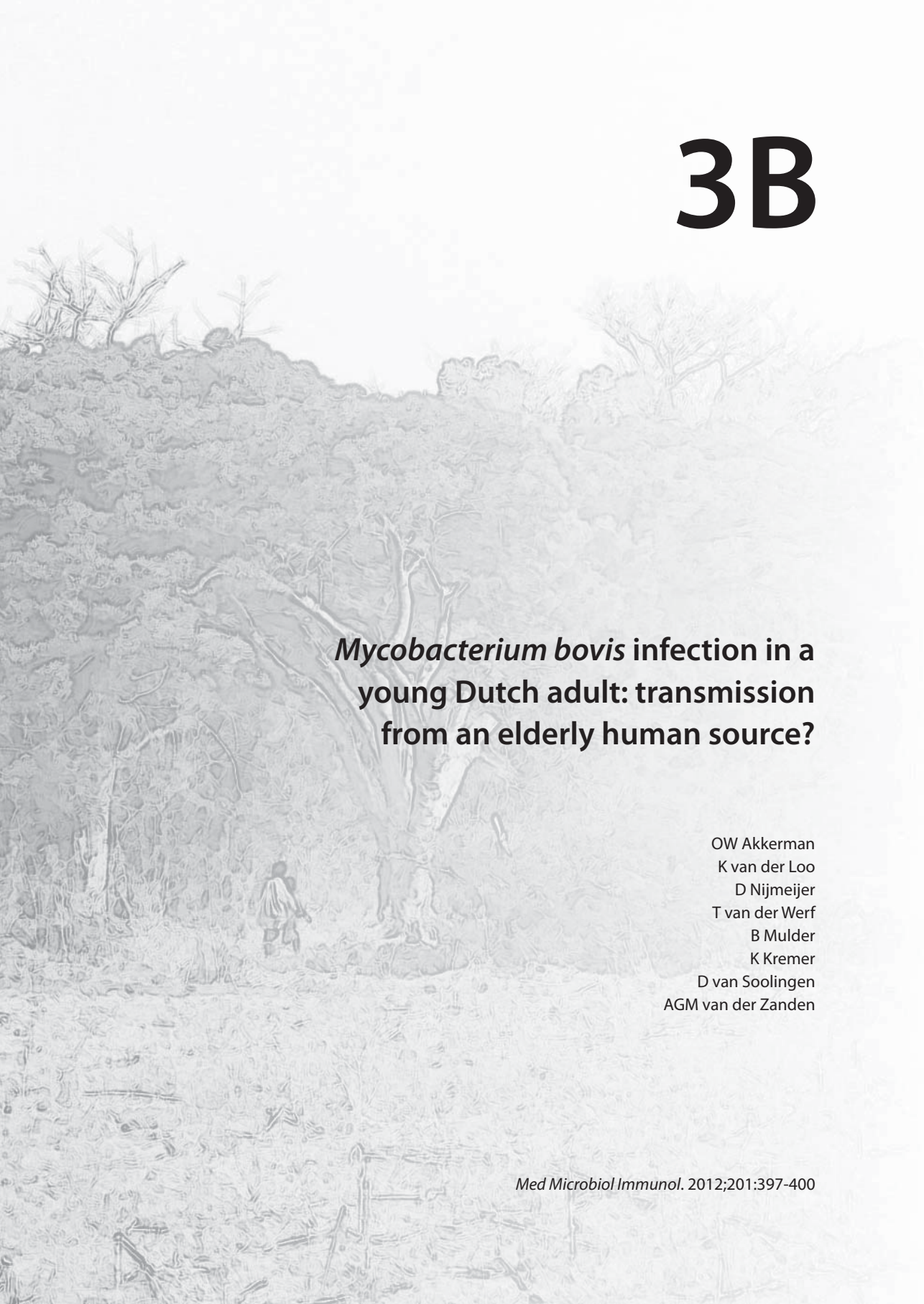

Mycobacterium bovis infection in a young Dutch adult: transmission from an elderly human source?

OW Akkerman
K van der Loo
D Nijmeijer
T van der Werf
B Mulder
K Kremer
D van Soolingen
AGM van der Zanden

Med Microbiol Immunol. 2012;201:397-400

Abstract

A young female health professional was diagnosed with pulmonary tuberculosis caused by *Mycobacterium bovis*. Source finding and contact tracing was initiated by the regional municipal health service using both tuberculin skin test and QuantiFERON-TB Gold (QFT-GIT (IGRA). The strain appeared near-identical to that of an elderly Dutch patient.

Introduction

Between 1993 and 2007, 231 cases of *Mycobacterium bovis* infections in humans were detected in the Netherlands and this counted for 1.4 % of all culture-positive tuberculosis cases. *M. bovis* infection in humans can be acquired by drinking unpasteurized milk [1], or animal to human transmission is possible through infected cattle and zoo animals [2]. Only rarely, *M. bovis* is transmitted between humans [3–5]. In the Netherlands, most indigenous patients with *M. bovis* infection are >60 years suffering from endogenous reactivations, whereas immigrants with *M. bovis* infection are usually younger [6]. We report a case of *M. bovis* infection in a young health professional in a low-endemic country. We show evidence that the infecting strain belongs to a cluster of infection affecting elderly people in the Netherlands. An Interferon Gamma Release Assay (IGRA) was useful in the diagnosis and contact screening in *M. bovis* infection and was not affected by BCG vaccination status [7,8].

Report

A 23-year-old woman with a history of 18 months' cough, not responding to antibiotics and weight loss of 10 kg was diagnosed with tuberculosis (TB). Although she lived on a cattle farm, she had not been exposed to animals, and the livestock was well. Her parents owned this farm and were both working at the farm. She worked as a nurse in a nursing residence; there were no reports of TB among the residents. She never travelled to a TB endemic country. Conventional and computed chest radiography showed bilateral, nodular interstitial infiltration and two small alveolar consolidations in the right upper lobe and a large bulla in the left upper lobe. Bronchoscopy was normal but bronchoscopic biopsies from the left upper lobe revealed granulomatous inflammation with central necrosis. Microscopy of Ziehl-Neelsen stained preparations showed acid-fast bacilli and IS*6110*-targeted PCR for *M. tuberculosis* complex of the broncho-alveolar lavage fluid was positive. Culture grew acid-fast bacilli and *M. bovis* was indentified with the HAIN Genotype MTBC (Hain Lifescience, Nehren, Germany) [9]. Drug sensitivity testing showed no resistance to standard drugs except for pyrazinamide, a standard feature of *M. bovis*.

Contact tracing

The Municipal Health Authority (MHA) conducted contact tracing according to the ring principle following the Dutch guidelines [10]. IGRA (Quantiferon-Gold in tube; QFT-GIT) [11]

was only performed in individuals testing tuberculin skin test (TST)-positive. During this investigation, contacts are classified into circles around the index case. In the first circle of contacts, 24/74 persons were TST-positive (induration ≥10 mm) with only 7 of these 24 testing positive with QFT-GIT. Among a second circle of 31 colleagues, 7 tested positive with TST (≥15 mm induration); none tested positive with IGRA. In a third circle, 2 of 30 colleagues working in other buildings tested TST-positive; one of these 2 tested positive with IGRA. Among 40 residents born before 1940, three had a positive IGRA. None of the people tested in contact tracing had any signs of active TB. Next, none of the 67 cows on the farm where our index patient lived skin-tested positive with a TST using bovine PPD, while one was slaughtered – on thorough postmortem investigation this cow tested negative. The Dutch database with IS*6110* and polymorphic guanine/cytosine-rich repeat sequence (PGRS) restriction fragment length polymorphism (RFLP) patterns of all *M. tuberculosis* complex strains isolated in the Netherlands since 1993 established within the framework of the national surveillance on tuberculosis was used for comparison with the IS*6110*/PGRS RFLP patterns of the *M. bovis* isolate of our patient [12,13]. For the comparison of human and animal *M. bovis* isolates also spoligotyping was performed [13–15]. The spoligopattern showed the presence of the spacers 1 and 22–38 and absence of the spacers 2–21 and 39–43. The octal code of the spoligopattern is 400000077777600 and matched in the SpolDB4 database with three strains from Spain and five strains from France (personal communication N. Rastogi, Unité de la Tuberculose et des Mycobactéries, Institut Pasteur de Guadeloupe, Guadeloupe) [16]. The isolate of our patient had two bands in its IS*6110* RFLP pattern, of which one had a size of 1.9 kb, which is characteristic of *M. bovis*. The combination of IS*6110*/PGRS RFLP and spoligotype pattern of the *M. bovis* isolate of the patient was unique in the Dutch national database containing all isolates collected since 1993 (Figure 1). However, one other *M. bovis* isolate (number 2) matched closely, exhibiting an identical spoligotype pattern, >96 % similarity in PGRS RFLP and one band less in IS*6110* RFLP. The deletion or insertion of one band in the IS*6110* RFLP has been described during transmission of *M. bovis* between humans or between animals and humans previously [14]. Thus, a transmission between these two patients or a common source is plausible. We assume that our patient and an elderly Dutch patient, whose isolate's DNA fingerprints match those of the one of our case, share the same infection cluster.

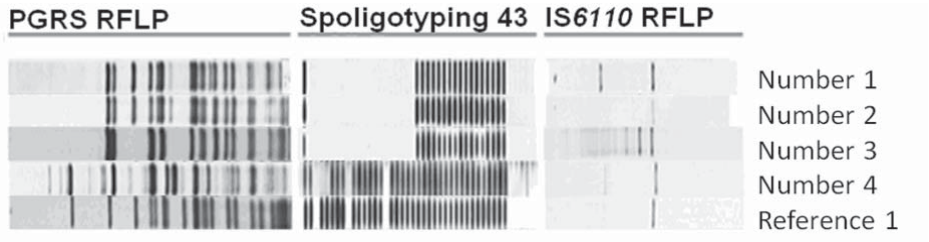

Figure 1 Comparison of PGRS RFLP, spoligotyping patterns and IS*6110* RFLP.
Number 1 shows the DNA fingerprint results of the *M. bovis* isolate of the index patient. *Numbers 2, 3 and 4* depict DNA fingerprint patterns of *M. bovis* isolates of Dutch patients, selected because of an almost identical pattern in PGRS RFLP and/or spoligotyping and/or IS*6110* RFLP. Reference 1 represents a *M. bovis* BCG reference isolate.

Discussion

In the Netherlands, the cattle herd has been officially declared free of bovine TB since 1999, with only occasional small outbreaks of bovine TB in imported cattle or in zoo animals [17,18]. The source of the bovine infection in our patient was either from infected cattle at the farm, from infected animals at the zoo she regularly visited, drinking unpasteurized milk in the past, or from patients in the nursing home she worked in. Neither the cattle at the farm where she had lived nor the zoo she regularly visited had been associated with any case of bovine TB in the last 20 years. We assumed, therefore, that an as yet unidentified Dutch resident of the nursing home might have been the source of infection in previous years. A large proportion of the elderly is latently infected by *M. tuberculosis* or *M. bovis* [6,17,19]. The high similarity of the DNA fingerprints of the isolate of our patient and those of the isolate of an elderly Dutch patient (number 2 in Figure 1), who had a lymph node *M. bovis* infection 5 years earlier, suggests that they belong to the same infection cluster. No epidemiological relation between both patients could be demonstrated by the MHA. During contact tracing in the nursing home among residents and personnel, no other potential sources were identified. Our results support the conceptually higher specificity of the IGRA compared with the TST for *M. bovis* infection [14,15]. This higher specificity helped to better target the prophylactic treatment strategy for latent *M. bovis* infections.

In conclusion, with thorough investigation and the use of several modern immunologic and molecular diagnostic tools, it was possible to identify a presumptive infection cluster on molecular methods but we could not identify the actual source of infection in this patient by conventional epidemiological methods.

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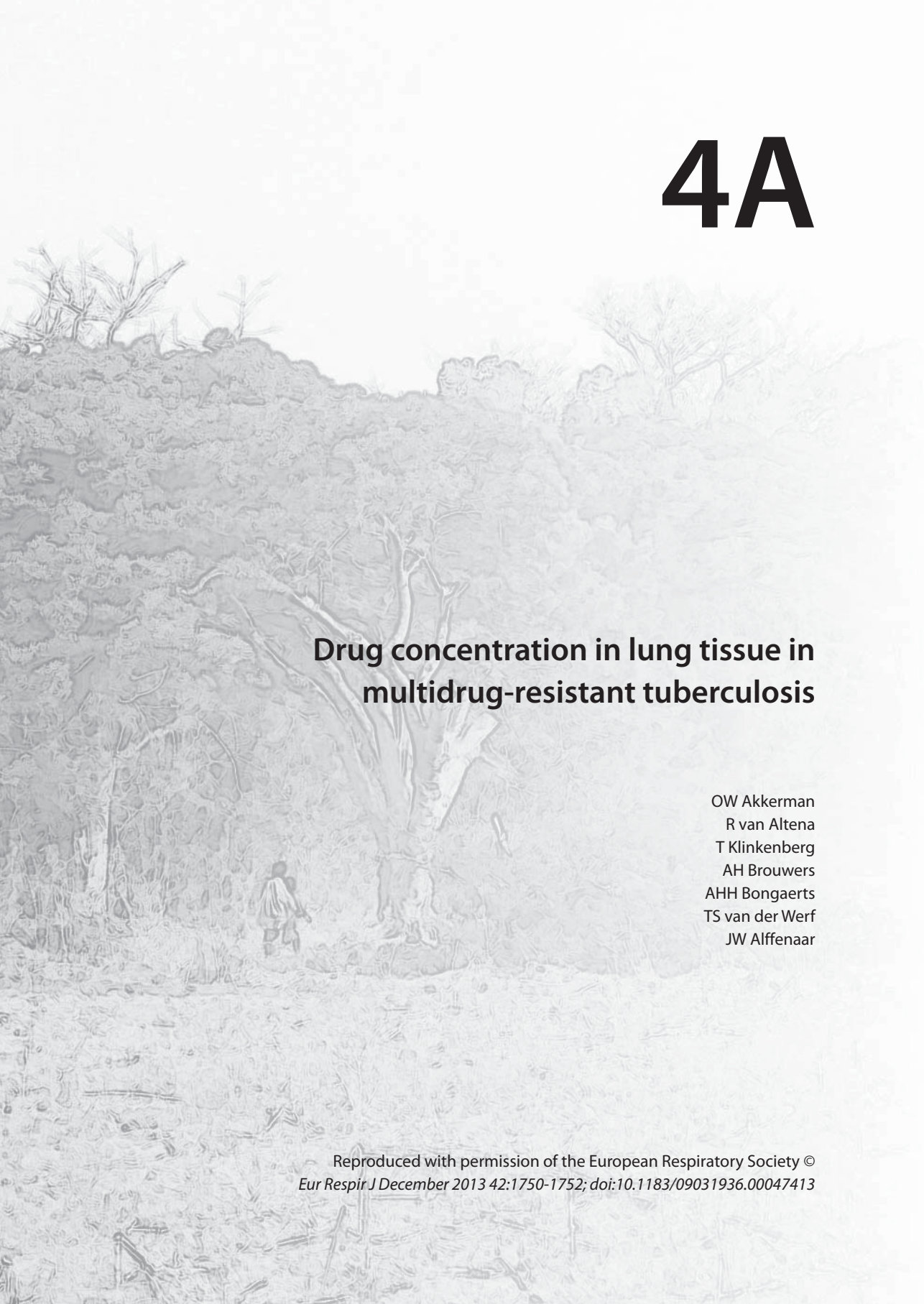

Drug concentration in lung tissue in multidrug-resistant tuberculosis

OW Akkerman
R van Altena
T Klinkenberg
AH Brouwers
AHH Bongaerts
TS van der Werf
JW Alffenaar

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To the Editor:

Multidrug-resistant tuberculosis (MDR-TB) is emerging worldwide, with 3.7% of new cases and 20% of previously treated tuberculosis (TB) cases having MDR-TB. Unfortunately, second-line TB drugs, used for MDR-TB treatment, are less effective than first-line drugs [1]. Sputum culture rather than sputum smear microscopy is recommended to monitor treatment response 1. Therapeutic drug monitoring (TDM), which may help optimize efficacy and minimize side-effects with the potential to safeguard intestinal absorption of drugs [2], is currently not recommended in World Health Organization (WHO) treatment guidelines [1]. Although TDM yields information on serum drug concentrations, penetration of second-line TB drugs into diseased tissues such as destroyed lung tissue has, to our knowledge, not been addressed in studies [3]. Herein, we report simultaneous blood and tissue concentrations of second-line TB drugs in lung tissue destroyed by MDR-TB.

A 13-year-old Somalian, HIV sero-negative female, residing in the Netherlands since 2010, was admitted to our TB Unit (TB Center Beatrixoord, University Medical Center Groningen, Groningen, the Netherlands) in September 2011 with a 3-month history of cough, fever, chest pain and unintentional 14 kg weight loss. TB contacts were denied and she had not received a bacilli Calmette–Guérin vaccination. Apart from almost absent breathe sounds and dullness to percussion over the left lung field her physical examination was normal.

Chest radiography showed infiltrates in the left lung, particularly in the left upper lobe. Sputum microscopy revealed acid-fast bacilli, and she was started on TB treatment with isoniazid, rifampicin, pyrazinamide and ethambutol. 2 weeks later, molecular diagnostic tests revealed mutations in both the katG and rpoB genes. MDR-TB was now considered and she was transferred to our hospital (University Medical Center Groningen).

We changed her treatment to pyrazinamide, ethambutol, kanamycin and moxifloxacin [4]. Drug susceptibility testing (DST) by the National Tuberculosis Reference Laboratory showed resistance to all first-line drugs, as well as protionamide. The isolate appeared susceptible to co-amoxiclav, amikacin, capreomycin, moxifloxacin and linezolid, as well as ertapenem and co-trimoxazole [5]. We started her on linezolid and ertapenem to replace ethambutol and pyrazinamide. Trough drug concentrations, part of TDM, as routinely performed in our centre, were 0.5 mg/L and 0.5 mg/L for linezolid and moxifloxacin, respectively. The minimal inhibitory concentrations for linezolid and moxifloxacin were 0.25 mg/L and 0.125 mg/L, respectively (D. van Soolingen, National Tuberculosis Reference Laboratory, National

Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands; personal communication). The area under the concentration–time curve (AUC) 0–24 for linezolid and moxifloxacin were 74.90 mg/h/L and 30.04 mg/h/L, respectively. As these AUCs were adequate, no dose adjustment of either drug was performed.

The first sputum microscopy was negative after 6 weeks, and the sputum culture turned negative after 2 months. Afterwards, culture was positive on a further two occasions; at 3 and 5 months after starting treatment. No radiological improvement occurred, with atelectasis of the left upper lobe. We were concerned that the drugs would not penetrate into the affected lung; therefore, surgery was now considered. 99mTc-macroaggregated albumin lung perfusion imaging (Figure 1), lung function testing and a computed tomography scan of the chest were performed to evaluate the relative contribution of the left lung for perfusion, and to assess her pulmonary reserve. It was concluded that the left lung hardly contributed to her pulmonary function and a left pneumonectomy was performed.

Immediately following surgery, samples of the left upper and left lower lobes were collected for microbiology and Prausnitz–Küstner testing. The left upper lobe was most affected and the left lower lung was less affected (Figure 1). Surgical specimens were all smear and culture negative. Samples from the left upper lobe tested positive by IS*6110* PCR, and samples of the left lower lobe remained negative. The drug concentration of moxifloxacin was 0.73 mg/g in the left upper lobe (most affected by TB), and the linezolid concentration was 3.87 mg/g.

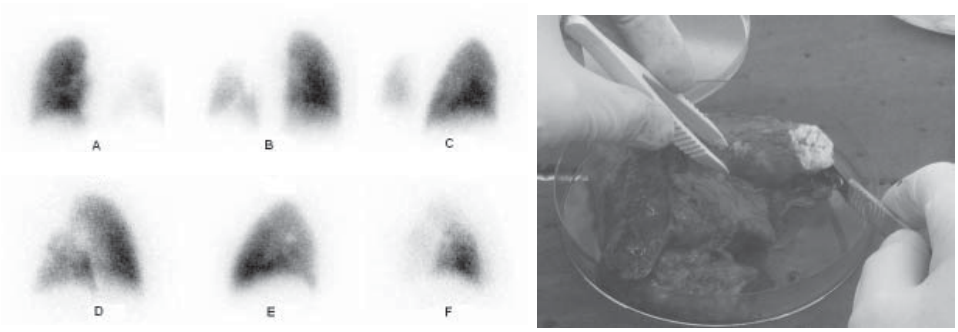

Figure 1 Lung perfusion scan with 72 MBq ^{99m}Tc-macoaggregated albumin 7.5 months after the start of the treatment: a) anterior, b) posterior, c) right posterior oblique, d) left posterior oblique, e) right lateral and f) left lateral views. g) A photo of the resected lung. The left upper lobe is the lobe most affected by tuberculosis.

The left/right perfusion percentage (90% versus 10%) was calculated from the geometric mean of the counts from both lungs in the anterior and posterior views, while the left upper/left lower lobe percentage (9% versus 91%) was calculated from the left posterior oblique image.

The concentrations in the left lower lobe (less affected) were 0.95 mg/g for moxifloxacin and 3.1 mg/g for linezolid. At the time of pulmonary resection, 36 h had lapsed after the last oral drug dosage.

Consecutive chest radiographs showed gradual fluid filling of the pleural cavity. Our patient was discharged 2 months after pneumonectomy in a good condition. Her TB treatment regimen was switched to an oral regimen, consisting of co-trimoxazole, moxifloxacin and clofazimine (five times per week, 100 mg) for another 10 months. She has been well, and at the time of writing this report, 4 months before completion of drug therapy, she reported being well with no signs of relapse and no side effects with improved exercise tolerance.

For the first time, we describe blood and tissue concentrations of second-line TB drugs. We showed that drug penetration was excellent, with similar drug concentrations in severely TB-affected lung tissue and in less affected lung tissue. Only one report, an overview by Dartois et al. [3], has addressed the question of how well second-line TB drugs penetrate into diseased lung and other tissues in relation to TDM but, unlike data on first-line drugs in resected lung tissue, no paired tissue and blood data have been reported on second-line drugs to date. Only data from rabbit models showed that moxifloxacin had a better AUC in lung and lesion tissue relative to plasma AUC compared to the first-line TB drugs [6].

Tailored regimens for MDR-TB treatment can be started after DST results are known. Efficacy of treatment for MDR-TB is solely based on sputum smear and culture status [1]. Studies with individualised treatment that studied treatment success have predominantly been based on DST results [7], without using pharmacokinetics with TDM. Although TDM may help evaluate TB drug absorption, even this aspect has not been extensively studied or practiced [2]. Therefore, to date, the full potential of TDM and drug penetration into TB-affected tissues has not been studied.

Surgery added to chemotherapy for MDR-TB has been described in case series but no randomised controlled trials have been conducted to evaluate this approach. In reported series, selection bias by indication is a potential flaw as patients who underwent surgery were perhaps younger or in a better clinical condition. However, even with these limitations, surgical resection has been suggested early in the course of treatment of MDR-TB [8,9]. A recent meta-analysis showed that the treatment effect of surgery added to chemotherapy was more pronounced in studies with extensively drug-resistant TB. This meta-analysis also showed that the treatment effect was stronger in MDR-TB studies with isolates showing resistance to more than 4.7 drugs [10].

Surgery added to chemotherapy was widely used in the pre-rifampicin era, even at our center as shown in the thesis of Mulder-De Jong [11], with 25% of samples being culture positive and 96% positive on microscopy. A limitation in our approach was that we took only one sample per resected lobe.

In our patient a pneumonectomy was performed because we feared that drugs would not reach the most severely affected parts of the destroyed lung, potentially resulting in persistent organisms that might subsequently cause a relapse or treatment failure if sub-therapeutic drug concentrations resulted in additional drug resistance. With a 36-h delay between the last dose of moxifloxacin and linezolid before surgery, both drugs were still detectable equally well. We have shown, although just in one patient, that treatment regimens yielding adequate blood concentrations as evidenced by TDM may provide similarly adequate penetration in affected tissue; non-resolving pulmonary infiltrates in TB patients do not necessarily preclude inadequate drug penetration.

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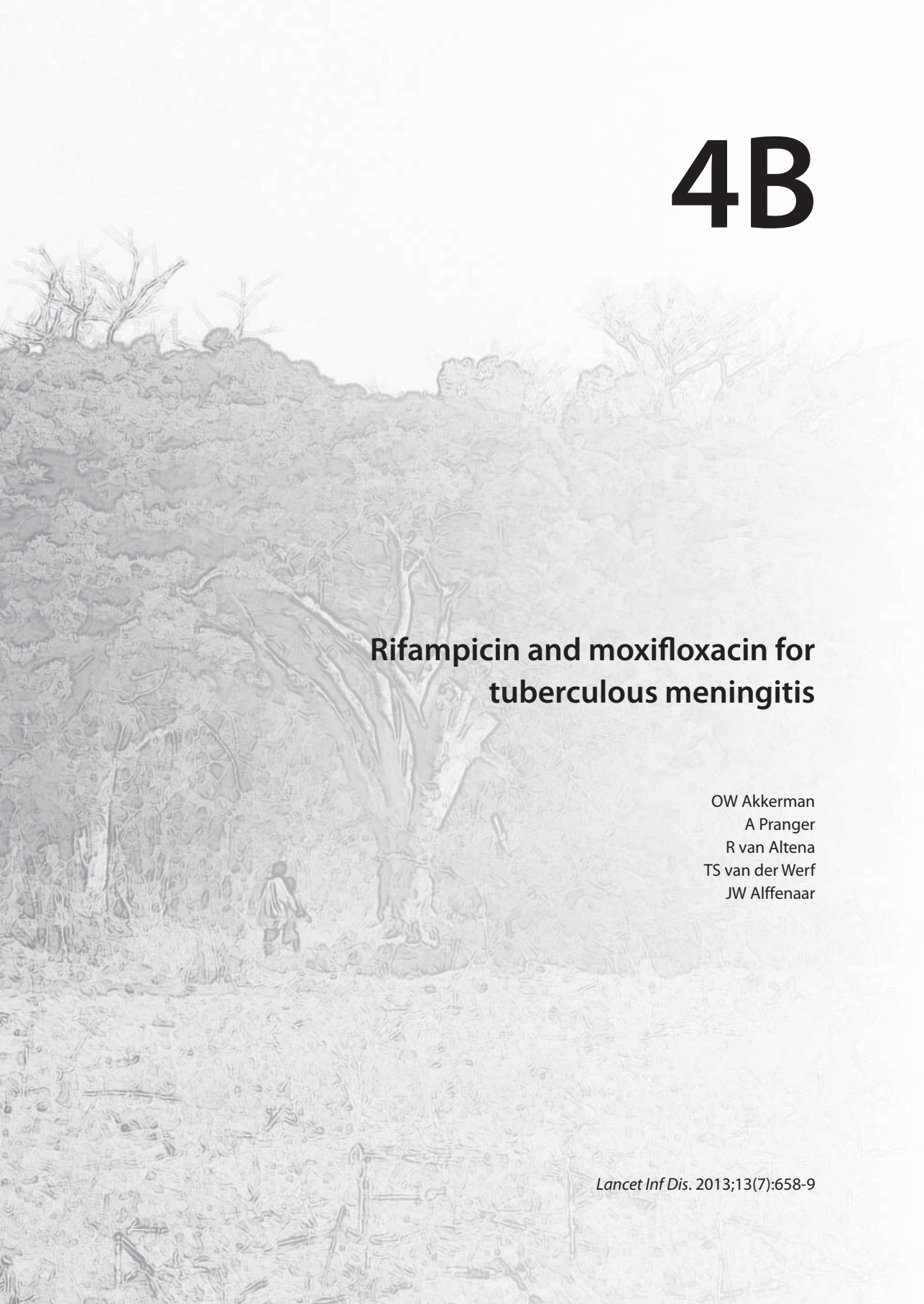

Rifampicin and moxifloxacin for tuberculous meningitis

OW Akkerman
A Pranger
R van Altena
TS van der Werf
JW Alffenaar

Lancet Inf Dis. 2013;13(7):658-9

Ravina Ruslami and colleagues presented a study assessing pharmacokinetics, safety, and survival benefit of different treatment regimens containing high-dose rifampicin and moxifloxacin in patients with tuberculous meningitis in a hospital setting [1].

Their findings that a treatment regimen containing a higher dose of rifampicin and standard-dose or high-dose moxifloxacin during the first 2 weeks is safe in patients with tuberculous meningitis, and that high-dose intravenous rifampicin could be associated with a survival benefit in patients with severe disease are important to note.

We agree with the authors that, on the basis of the small number of patients per group, clinical results should be interpreted carefully. To compensate for small group sizes, one could consider a different strategy with drug exposure as a continuous variable. Additionally, isoniazid concentrations should also be measured since isoniazid contributes to rapid culture conversion and penetrates well in cerebrospinal fluid. Receiver operating characteristic analysis could show the extent to which cumulative drug exposures of rifampicin, moxifloxacin, and isoniazid relate to outcome. Resultant potentially crucial values for positive treatment outcome could be detected and related to the antagonistic effect on cell kill as observed after co-administration of rifampicin and moxifloxacin in in vitro and in vivo studies [2,3].

Another consideration is the potential benefit of a higher oral dosage to reach similar drug exposure as achieved with intravenous dosing. The proposed alternative strategy to analyse the data would also compensate for the difference in drug exposure due to intravenous administration compared with oral dosing, especially in the presence of predisposing factors for poor drug absorption like HIV co-infection.

We therefore would like to encourage the authors to do further analyses of their data to generate additional hypotheses.

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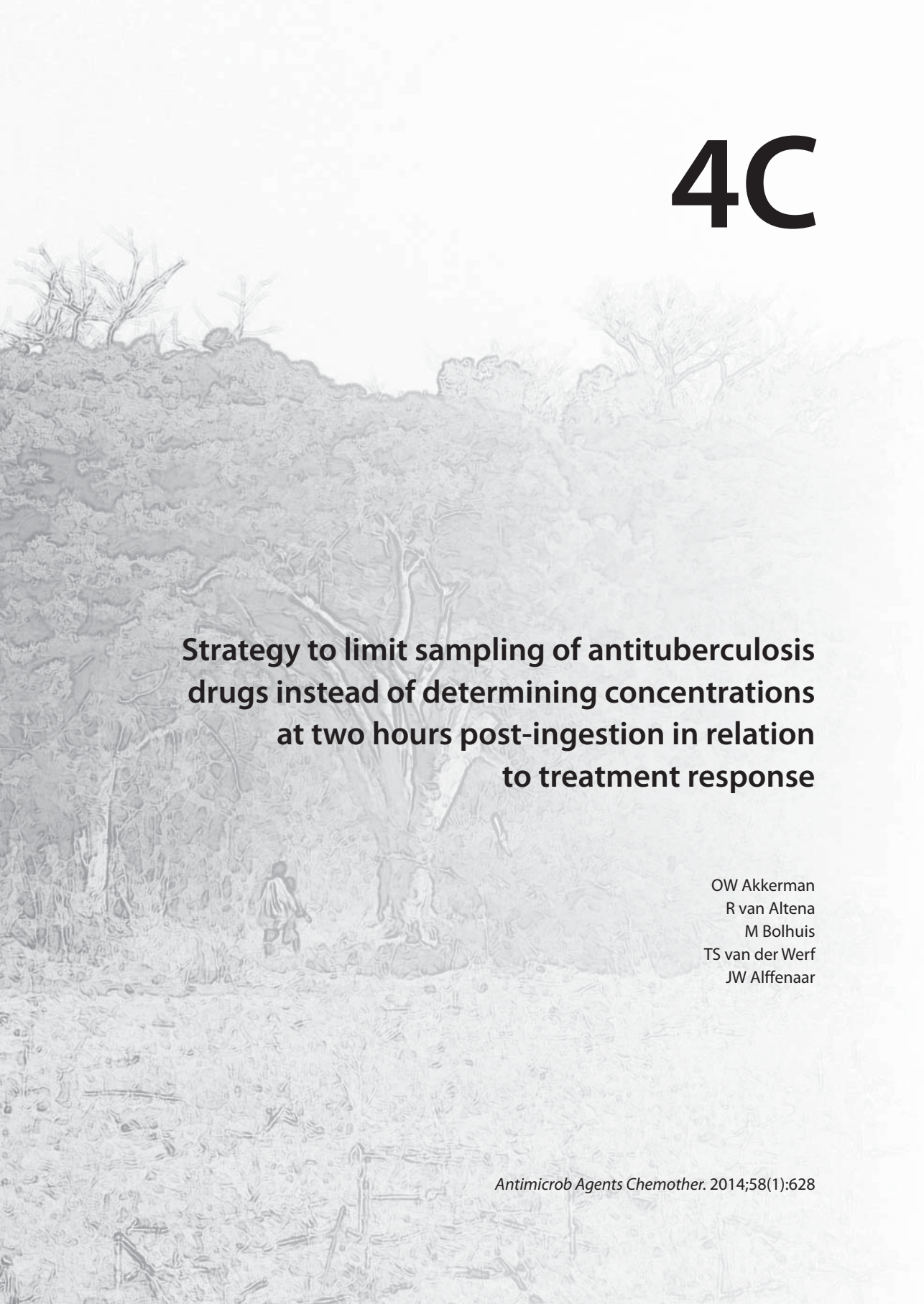

Strategy to limit sampling of antituberculosis drugs instead of determining concentrations at two hours post-ingestion in relation to treatment response

OW Akkerman
R van Altena
M Bolhuis
TS van der Werf
JW Alffenaar

Antimicrob Agents Chemother. 2014;58(1):628

We fully agree with Burhan et al. that "Numerous studies have reported low concentrations of antituberculosis drugs in tuberculosis (TB) patients, but few studies have examined whether low drug concentrations affect TB treatment response" [1]. The current anti-TB drug regimen of 2HRZE-4HRE (2 months treatment with isoniazid, rifampin, pyrazinamide, and ethambutol, followed by 4 months treatment with isoniazid, rifampin, and ethambutol) for drug-susceptible TB was based on numerous trials with similar favorable results, as this study shows [2]. Fortunately, the majority of patients who receive and finish the treatment for drug-susceptible TB will respond completely to treatment [2]. In cases of suspicion of inadequate exposure to anti-TB drugs and thereby a possibly suboptimal treatment response, indications for therapeutic drug monitoring (TDM) are those circumstances where the risk of treatment failure or toxicity is increased [3]. Burhan et al. showed that first-line anti-TB drug concentrations in plasma at 2 h postingestion (C2 h) were often low but that culture results after 4 and 8 weeks of treatment were nevertheless favorable, i.e., negative [1]. This raises the question of how much closer we are to predicting TB treatment response based on plasma concentrations of anti-TB drugs after this study. One important limitation of Burhan et al.'s study is that the measurement of the anti-TB drug concentrations were done just once during treatment and that only a single sample at 2 h postdose to estimate the maximum concentration of a drug in serum (Cmax) was used for the majority of patients. However, low 2-h concentrations do not rule out the possibility of delayed absorption. The relation between drug concentrations and efficacy has been determined in TB infection models, and it has been shown that the area under the concentration-time curve in relation to the MICs (AUC/MIC ratio) predicted efficacy best [4,5]. Therefore, we advocate measuring the AUC of each drug to evaluate a potential relationship between plasma concentrations of tuberculosis drugs and clinical outcome in TB patients. Besides, results of the MICs were lacking in the study of Burhan and coworkers. Not breakpoints but actual MICs are needed to calculate AUC/MIC values.

We are fully aware that full pharmacokinetic (PK) analysis is costly and difficult and that breakpoints are more frequently determined than actual MICs, but to be able to answer the question of whether TB drug concentrations influence clinical outcome, a strategy that assesses drug exposure (AUC) closely related to a full PK analysis should be considered. Limited-sampling strategies, using only 2 to 3 samples to predict AUC values with great accuracy and precision, may solve the problem of inadequate drug exposure assessment by C2 h monitoring [6]. Dried blood spot sampling, which is less expensive and more convenient for both patients and research teams, may help to collect the samples needed for predicting

AUC values [7]. The combination of limited-sampling strategies, dried blood spot analysis, and measuring actual MICs would give the information needed to deter- mine the relation between TB drug concentrations and clinical outcome [8]. In conclusion, we encourage investigators setting up clinical trials of TB patients to abandon classical C2 h monitoring and replace it with full PK monitoring or limited sampling.

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5A

Development of a semi-physiological pharmacokinetic model for aminoglycosides administered by dry powder inhalation

LHJ Keyzer
OW Akkerman
M Hoppentocht
AH de Boer
JWC Alffenaar
JH Proost

Submitted

Abstract

Purpose: Pulmonary administration of tobramycin with a dry powder inhaler (DPI) has shown to be effective in treating pulmonary infections by Pseudomonas aeruginosa in cystic fibrosis (CF) patients. The concentration of tobramycin in serum and in the lungs after dry powder inhalation depends on technical, physiological and individual patient properties. A pharmacokinetic (PK) model was developed to predict local and systemic drug exposure.

Methods: The deposition, absorption, distribution and elimination of pulmonary administered dry powder tobramycin were modeled using data from literature. The model was tested with simulations and compared with data from a non-CF bronchiectasis patient after inhalation of three different doses of tobramycin with the Podhaler© (28, 56 and 84 mg) DPI.

Results: The deposition of tobramycin in the alveoli and bronchioles and the apparent permeability coefficient are the most crucial parameters in the model. The agreement between the serum concentrations of the patient after inhalation of tobramycin and the computer simulation is promising.

Conclusions: The PK model developed in this study, based on literature data, was able to predict tobramycin concentrations in serum and lung fluids after inhalation by a DPI.

Introduction

Pulmonary administration of antimicrobial drugs, such as colistin sulphomethate sodium and the aminoglycoside tobramycin, has been shown to have a beneficial effect on the pulmonary condition of cystic fibrosis (CF) patients [1–4]. The inhalation of antibiotics in CF patients is used to oppose chronic infections with bacteria, especially *Pseudomonas aeruginosa*. Morbidity and mortality are high in CF patients due to pulmonary infections, which cause a more aggressive deterioration of their lung function [5]. Up to now, both colistin and tobramycin are nearly exclusively administrated with classic jet or ultrasonic nebulisers. However, the use of nebulisers is time consuming because of prolonged nebulization and preparation and cleanining of the nebuliser. In addition, re-usable nebulisers have to be disinfected on a regular basis, which eventually may damage the nozzle and change the size distribution of the aerosol [6]. These aspects negatively influence patient adherence to the therapy and for that reason there is a great interest in replacing the nebuliser technique. Dry powder inhalation could be a good alternative for aminoglycosides and other high dose drugs [6].

It has been recognised that both the inhaler design and the patient's inspiratory performance play an important role in successfully inhaling a dry powder drug. Most dry powder inhalers (DPIs) require a minimal inspiratory flow rate to reach sufficient emptying of the dose system and adequate deagglomeration of the dry powder dose [7]. If the threshold value for the flow rate at (and above) which the DPI performs satisfactorily is too high, substantial drug losses in the mouth and throat region may be expected [8]. To reduce the dependency on the inspiratory flow rate to a certain extent, a larger fine particle fraction should be delivered at higher flow rates to compensate for a shift in deposition of the dose to the upper airways at higher inspiratory flow rate [7]. Another key factor, with which the drug distribution in the lungs can be controlled, in addition to the flow rate, is the mass median aerodynamic diameter (MMAD) of the aerosol. A larger MMAD (range 3–6 μm) gives a higher deposition in the upper branches of the lungs whereas a finer aerosol (range 1–3 μm) is more appropriate for central and peripheral lung deposition [8]. Also, breath-holding time is clinically relevant as it promotes sedimentation, thereby increasing the amount of dry powder that deposits in the lower branches of the lungs [9].

The aim of this study is to develop a pharmacokinetic (PK) model, which takes into account the relevant aerosol properties, the key characteristics of the inspiratory maneuver and the patient's physiology and anatomy. Such a model may be used to predict the time course of

concentrations of an aminoglycoside (like tobramycin in this case) inhaled with a DPI, in the lungs as well as in serum.

Materials and methods

Block diagram

The block diagram was based on a model of Le Brun [10]. The model was modified to allow drug exchange between the conducting airways and the blood. Mucociliary clearance from the respiratory airways was excluded from the model, because of the limited occurrence of ciliated epithelium in the respiratory airways [11]. The peripheral compartment was also excluded from the model, because the pharmacokinetics of aminoglycosides is usually described by a one-compartment model [1,12]. Then, the variables of the DPI and the specific properties of the different compartments were added to the model. In the block diagram (Figure 1) the grouping of the different parts of the lungs was changed. The lungs have 23 generations, or branches, which can be subcategorized into conducting airways (generation 0–11), transitional airways (12–16), respiratory airways 1 or bronchioles (generations 17–21) and respiratory airways 2 or alveoli (generations 22–23). The surface area of the respiratory airways (generations 17–23) is 95% of the total surface area of the lungs and for that reason the respiratory airways are divided into the bronchioles and the alveoli, which account for 34% and 61% of the total surface area of the lungs respectively [13]. Another reason for dividing the respiratory airways is that the bronchioles and the alveoli have a difference in the thickness of the epithelial lining fluid (ELF). The conducting airways and transitional airways were combined as anatomical dead volume. A compartment for mouth and throat (oropharynx) has also been added to the model, because part of the inhaled dose is deposited in the mouth and throat of the patient. The variables of the DPI were elaborated and the deposition of the drug in different compartments was indicated by percentages of the dose.

Drug deposition model

To determine the percentage of the dose that reaches different compartments, the variables of the DPI play a significant role. In Table 1, the percentages of the dose that reach the different compartments are listed. The total surface area of the lungs can differ between individuals. Height, age and sex of the individual are factors involved in the difference in

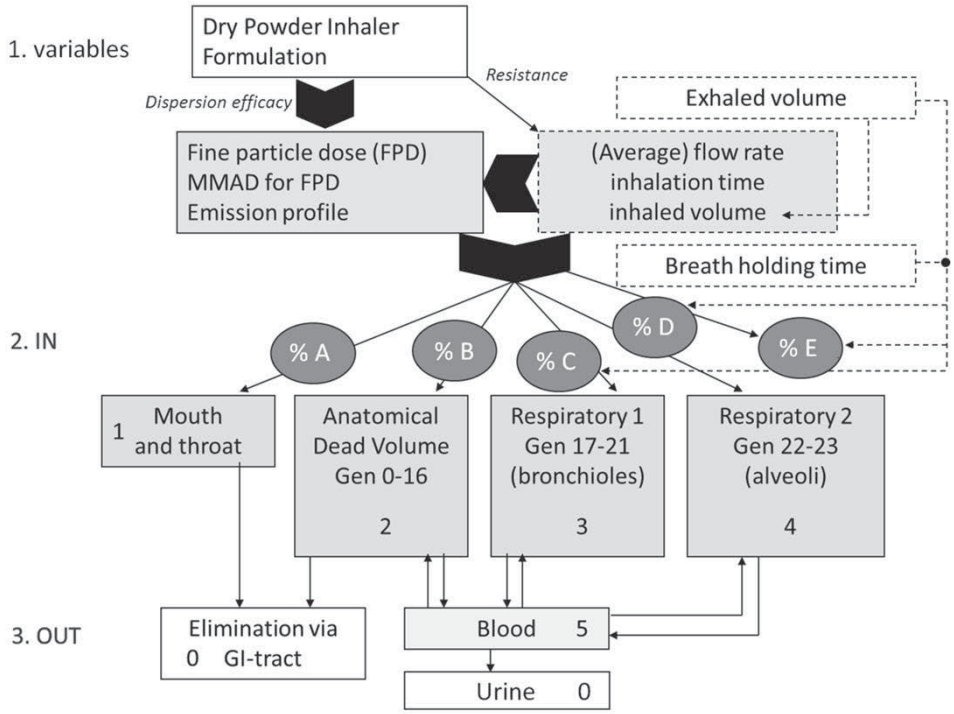


Figure 1 Block diagram of deposition of drug administered via dry powder inhalation.
The generations of the lungs are subcategorized in the anatomical dead volume (generation 0–16, compartment 2), respiratory airways 1 or bronchioles (generation 17–21, compartment 3) and respiratory airways 2 or alveoli (generation 22–23, compartment 4). Percentages A to D are part of the drug going IN to the body. Percentage E is the part of the drug that is exhaled and therefor part of the drug going OUT of the body. Percentages C, D and E are dependent on the breath holding time. Gen means generation, MMAD means mass-median aerodynamic diameter.

volume and surface area of the lungs [14,15]. Due to a lack of quantitative data, this was not included in the model.

The deposition of the fine particle dose in the lungs is mainly dependent on the flow rate, the MMAD and the breath holding time. The percentages listed in Table 1 are based on a MMAD of 3.0 μm and a flow rate of 60 L/min. These percentages were retrieved from planar gamma-scintigraphy and therefore it is possible that not 100% of the fine particle dose is recovered. Because the difference in deposition between the bronchioles and the alveoli has not been described, only an estimate of the amount deposited in either can be made [8,16–18]. Only a small volume of inhaled air can reach the alveoli and this volume can only reach

Table 1 Parameters used in the model

Parameter	Description	Dependence	Value	Reference
FSd	Fractional surface area of anatomic dead volume (gen 0–16)	Physiological	5%	[13]
FSr1	Fractional surface area of respiratory airways 1, bronchioles (gen 17–21)	Physiological	34%	[13]
FSr2	Fractional surface area of respiratory airways 2, alveoli (gen 22–23)	Physiological	61%	[13]
SA	Total surface area of two lungs	Physiological	140 m^2	-
Td	Thickness of ELF in gen 0–16	Physiological	8 µm	[20]
Tr1	Thickness of ELF in gen 17–21	Physiological	3 µm	[20]
Tr2	Thickness of ELF in gen 22–23	Physiological	0.07 µm	[20]
P_{app}	Apparent permeability	Drug/ physiological	0.43×10^{-7} cm/s	[25–27]
k10	Elimination constant from compartment 1 via GI-tract	Physiological/drug	3 h^{-1}	[16]
CL20	Elimination from compartment 2 via GI-tract	Physiological/drug	0.17 L/h	[16]
fr	Ratio of tobramycin renal clearance and creatinine clearance	Drug	0.77	[1,12]
CLm	Metabolic clearance	Drug	0.18 L/h	[1,12]
V1	Volume of distribution (per kg LBMc)	Drug	0.26 L/kg	[1,12]
f	Correction factor for distribution (used for calculating LBMc)	Drug	0.4	[41]
%A	Deposition in compartment 1	Physiological/drug	37% [a]	[8,16–18]
%B	Deposition in compartment 2	Physiological/drug	39% [a]	[8,16–18]
%C+D	Deposition in compartment 3+4	Physiological/drug	12% [a]	[8,16–18]
%E	Exhaled drug	Physiological/drug	7% [a]	[8,16–18]

[a] expressed as a percentage of the delivered fine particle dose, based on a MMAD of 3.0 µm and a flow rate of 60 L/min. Based on a series of known DPI deposition studies, using labelled drugs.

the alveoli if the patient exhales completely before inhalation. Because in practice this is very difficult for the patient, it can be assumed that no more than 25% of the total dose that reaches the respiratory airways reaches the alveoli, based on the amount of fresh air during an inhalation that reaches the alveoli in contrast to the bronchioles (unpublished results).

Elimination from mouth, throat and anatomical dead volume

The amounts of drug deposited in mouth and throat, and a fraction of the amount deposited in anatomical dead volume (gen 0–16) are eliminated via the gastrointestinal (GI) tract [10].

The exact amounts and rates of elimination are difficult to determine, because analytical determinations of the feces are not common. An estimate of the elimination from these sides had to be made, knowing that the mucociliary clearance at these sites is a fast process [19]. Tobramycin deposited in the anatomical dead volume can either be cleared via the GI-tract or diffuse to the systemic circulation (see below).

Lung model

The epithelium of the lungs is covered with a liquid film, ELF, which is suggested as the site of antimicrobial activity against lung infections caused by bacteria [20]. Several studies have been conducted to determine drug penetration into ELF and to compare serum and ELF concentrations of antimicrobial drugs. Because tobramycin is a small compound (467.5 Da) and easily dissolves in water [21,22], it can be assumed that all inhaled tobramycin is dissolved in the ELF. The ELF is in direct contact with the lung cells [23]. Different kinds of transport can occur across the lung barrier to the capillaries. For tobramycin, passive diffusion between the cells (paracellular transport) is the main mechanism of transport, because most exogenous macromolecules with a molecular weight less than 40 kDa are thought to be absorbed from the ELF through tight junctions by passive diffusion [24–26].

The absorption rate of hydrophilic compounds is inversely related to the molecular weight (range 60–75,000 Da) [27]. A way to determine the absorption rate from the lungs to the capillaries is by determining the apparent permeability coefficient (Papp) of the lung barrier in an in vitro transport assay system. Lucifer yellow, a hydrophilic compound with a similar molecular weight (MW) to tobramycin, is transported by paracellular transport with a Papp of $0.43 \pm 0.05 \times 10^{-7}$ cm/s based on studies in the Calu-3 model [28–30]. This value was used for tobramycin.

Model development

A short description of each parameter, its dependence, its value and reference(s) is listed in Table 1.

In order to determine the volume of the compartments in the lung, the following equation was used:

Equation 1 $Vx = Sax * Tx$

where SAx is the surface area of compartment x, Tx the thickness of the ELF of compartment x and Vx the volume of the ELF in compartment x. The clearance from different compartments of the lung to the blood was calculated according to the following equation:

Equation 2 $CLxb = Sax * Papp$

where SAx is the surface area of compartment x, Papp is the apparent permeability of tobramycin in the lungs and CLxb is the clearance of tobramycin from compartment x to the blood. The transport of the drug is dependent on passive diffusion, because tobramycin is a small, easily soluble compound. Therefore, the transport is bidirectional and the clearance from both compartments is the same.

Pharmacokinetic properties of aminoglycosides

The pharmacokinetic model for aminoglycosides was similar to that used in MW\Pharm© (Version 3.80; MwPharm, Zuidhorn, The Netherlands), a program for therapeutic drug monitoring [31].

In order to determine the distribution into fatty tissues, the corrected lean body mass is calculated (MW\Pharm manual version 3.15, volume 3, MwPharm, Zuidhorn, the Netherlands, 1995):

Equation 3 $LBMc = (W - LBM) * f + LBM$

where W is the actual body weight of the individual in kg, LBM is the lean body mass of the individual in kg and f is the correction factor for distribution into fat.

The LBM is calculated according the equation [32]:

Equation 4 $LBM = (H - 152) * 0.9 + G$

where H is the height of the individual in cm and G is a constant dependent on the gender of the individual (male is 50 and female is 45.5) in kg. If the calculated LBM is larger than W, LBM is set to W.

With the lean body mass corrected, the volume of distribution (Vd) in liters can be calculated for the individual by:

Equation 5 $Vd = V1 * LBMc$

where V1 is the volume of distribution in L/kg.

Because the renal function is not the same for every individual, a correction is made for the creatinine clearance in calculating the clearance of tobramycin (in L/h):

Equation 6 $CL = CLnr * (BSA / 1.85) + fr * CLcr$

where CLnr is the normalized non-renal clearance, fr is the ratio of the renal clearance of tobramycin and creatinine clearance, CLcr the creatinine clearance, BSA is the body surface area of the individual in m^2, estimated from [32]:

Equation 7 $BSA = W^{0.425} * H^{0.725} * 0.007184$

where W is body weight (kg), and H is body height (cm).

The value 1.85 is the body surface area of a standard patient of weight 70 kg and height 175 cm.

Patient data

A female, 63 years old patient with non-CF bronchiectasis suffered from side effects after administration of the standard dry powder tobramycin dose of 112 mg using the Podhaler©.

She gave oral informed consent to determine serum tobramycin concentrations after pulmonary administration of dry powder tobramycin for optimization this model. The patient was given three different doses over three different days. On day one the patient inhaled 28 mg, on day two 56 mg, and on day three 84 mg of tobramycin.

The patient's height was 160 cm and her weight was 66 kg. She had a serum concentration of creatinine of 65 µmol/L. The calculated creatinine clearance was 81.6 mL/min according to the Cockcroft-Gault formula [33].

The MMAD during the inhalations was 3 µm and the flow rate was (approximately) 60 L/min. The inhaler retention for the different doses was 1 mg (28 mg dose), 1.39 mg (56 mg dose) and 1.99 mg (84 mg dose), determined by weighing the capsules after the inhalation, corrected for the weight of the capsule itself. The delivered dose was calculated by subtracting the retention from the given dose. Blood samples were collected before and 0.25, 0.5, 0.75,

1, 1.25, 1.5, 1.75, 2, 4, 6 and 8 hours after the start of the inhalation. The samples were analyzed using a validated immunoassay method (Architect, Abbott Diagnostics, Chicago, IL) according to the FDA guidelines (with single determinations) in a clinical lab. The lower limit of quantitation of this assay is 0.2 mg/L.

Software

Berkeley Madonna™ (Version 8.3.18, BerkeleyMadonna.com, University of California at Berkeley, USA) and MW\Pharm© (Version 3.80; MwPharm, Zuidhorn, The Netherlands) were used for simulations. The simulated individual was a male, 30 years old, weight 75 kg, and height 180 cm, with a creatinine clearance of 120 mL/min. The variables during the inhalation were set on an MMAD of 3 μm and the flow on 60 L/min.

Results

Simulation model

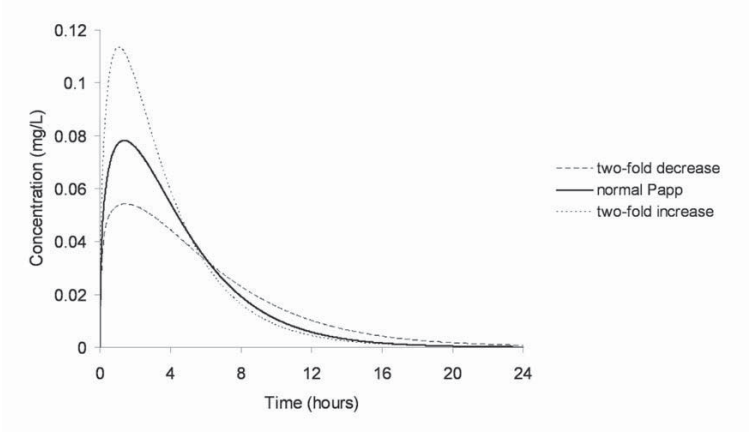
Using the parameters described in Table 1, a computer simulation of the described 'standard' individual was performed to create a typical serum concentration-time profile. All alterations or simulations of different parameters were made using this simulated individual.

Influence of critical parameters

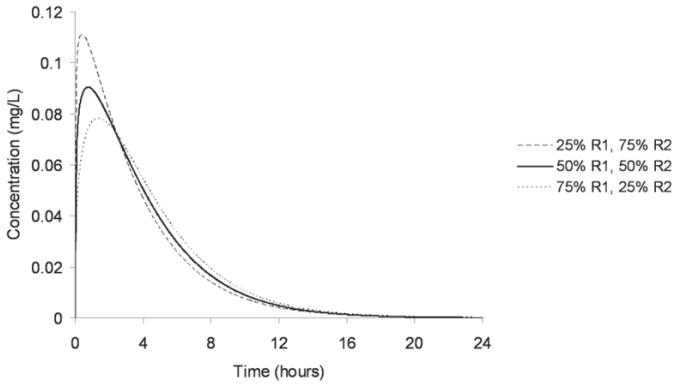
To determine the critical parameters of the model, the serum concentration of tobramycin was simulated for every parameter with a two-fold change in values. The effect of a two-fold change of the apparent permeability coefficient can be seen in Figure 2a.

The fraction that ends up in the respiratory airways 1 (generation 17–21) was also set as a variable. Because this fraction depends on the fraction that reaches the respiratory airways 2 (generation 22–23), both were set as a variable simultaneously in the simulation. In Figure 2b, the effect of the change in fraction deposited in the different respiratory airways is shown.

The effect of a two-fold change of the total surface area of the lungs is shown in Figure 2c.



2a



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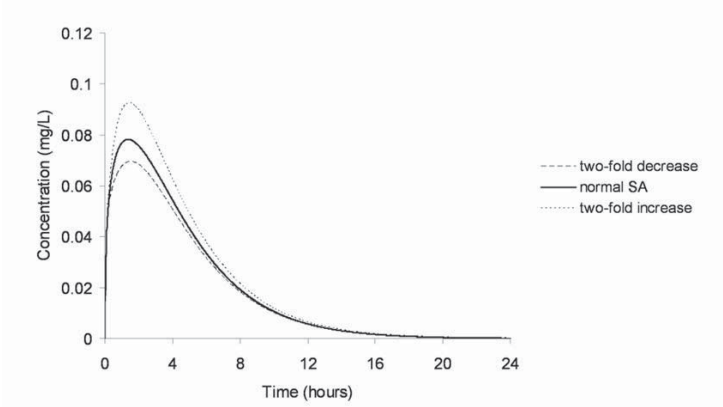


2c

Figure 2 Computer simulation of the serum concentration tobramycin for crucial parameters.
2a (Upper panel): Predicted serum concentration of tobramycin versus time for different values of the apparent permeability coefficient (P_{app}). **2b** (middle panel): Predicted serum concentration of tobramycin versus time for different values of the fraction deposed in the two respiratory airway compartments. R1 is respiratory airways 1 (bronchioles, generation 17–21) and R2 is respiratory airways 2 (alveoli, generation 22–23), expressed as fractions that reach the respiratory airways (12% of fine particle dose). **2c** (lower panel): Predicted serum concentration of tobramycin versus time for different values of the total surface area of the lungs (SA).

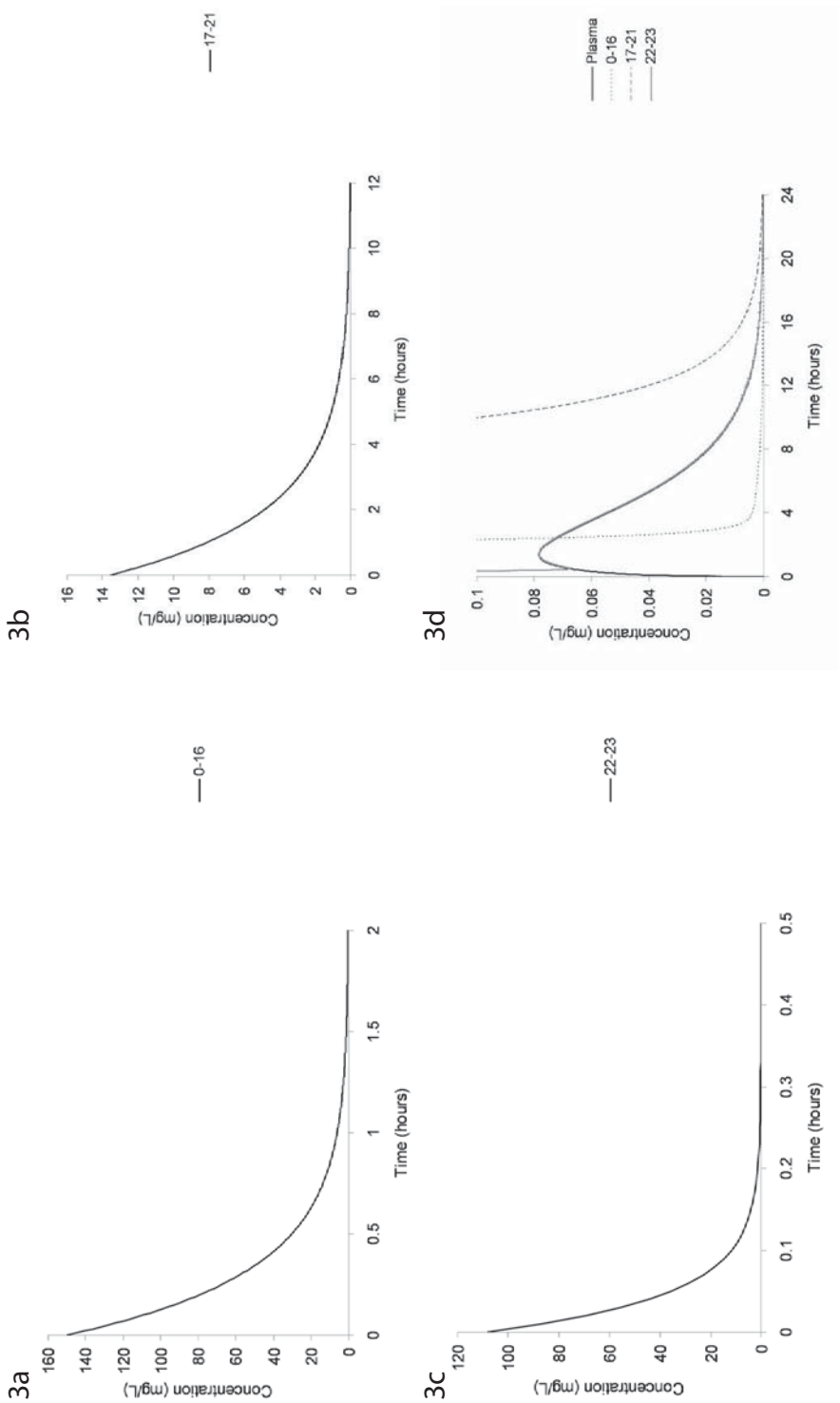


Figure 3 Computer simulation of the concentration tobramycin at different sites of the lung.
Predicted local concentrations and serum concentration after inhalation of 25 mg of tobramycin. The numbers in the legend represent the generations of the airways. Generation 0–16 is the anatomical dead volume, generations 17–21 are the respiratory airways 1 (bronchioles) and generations 22–23 are the respiratory airways 2 (alveoli).

Concentrations in different compartments

Using the model, simulations can be performed to determine the time course of the concentration of tobramycin in different compartments. The concentration profiles of the anatomical dead volume (generations 0–16), the respiratory airways 1 (generations 17–21) and the respiratory airways 2 (generations 22–23) are given in Figure 3. In the same figure, for comparison, the concentration of tobramycin in all compartments of the lung and the serum concentrations are combined in one graph.

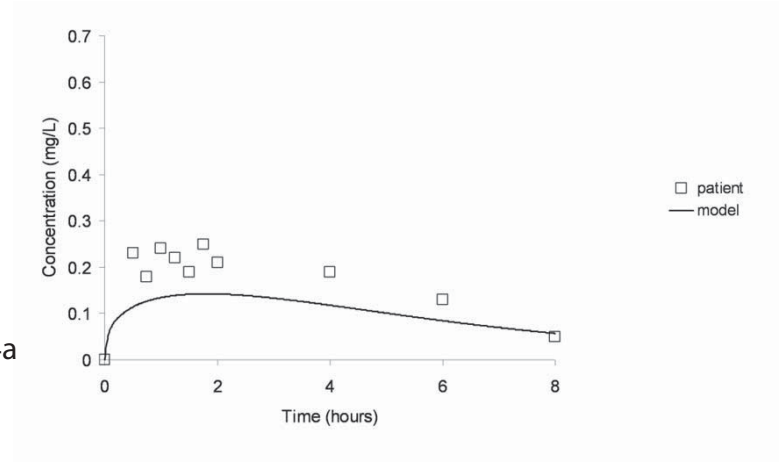
Patient data compared with computer simulation

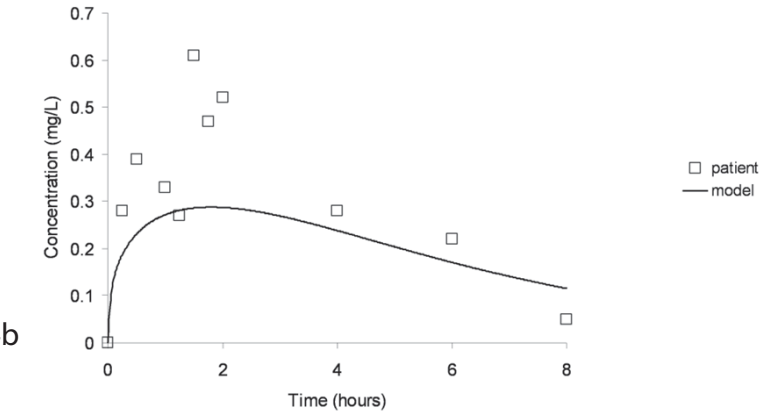
In order to test the model, individual serum concentrations were measured at different time points in one patient after inhaling different doses of dry powder tobramycin (28, 56, and 84 mg). These serum concentrations were compared with the computer simulations based on the patient's characteristics. These comparisons are illustrated in Figure 4. Despite the fact that several serum concentrations were below the lower limit of quantitation of 0.2 mg/L, these values are reported here to allow a rough comparison with the models predicted concentration.

Discussion

The aim of this study was to develop a PK model to predict the time course of the tobramycin concentration in the lungs and serum, taking into account the characteristics of the drug, DPI and patient. The model was tested by performing simulations and by comparison with data from one patient, with good results.

Most parameters incorporated in the model were based on earlier studies (see references in Table 1), except for the elimination from the mouth, throat and anatomical dead volume (gen 0–16) through the GI-tract. An estimate of the elimination from these sites had to be made, knowing that the mucociliary clearance at these sites is a fast process, also because of the cough-reflex [19,34]. Mucociliary clearance by patients with CF or non-CF bronchiectasis is impaired at the sites of the bronchioli (gen 17–21) and alveoli (gen 22–23) [34]. For a better estimation of the elimination of tobramycin from these sites, an analytical experiment should be set up to determine the amount of tobramycin eliminated via the feces after DPI and intravenous administration.





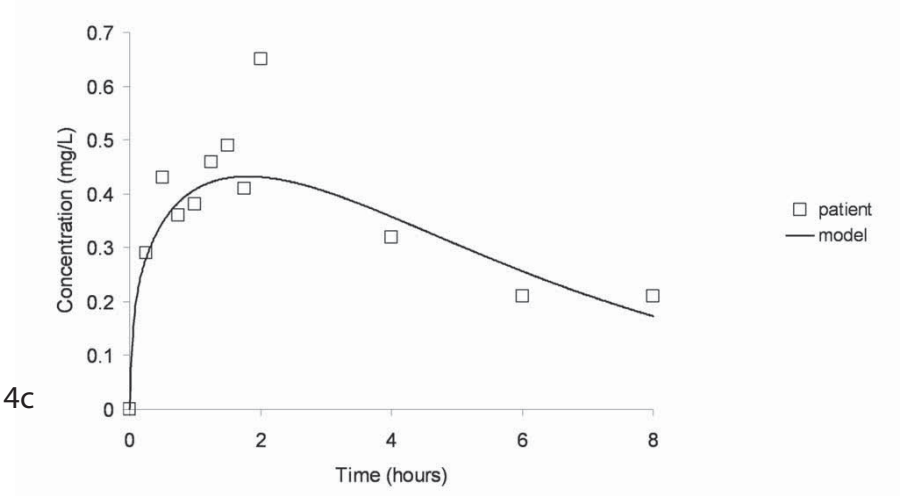


Figure 4 Computer simulation and individual serum concentration versus time after tobramycin inhalation. **4a** 28 mg dose. **4b** 56 mg dose. **4c** 84 mg dose.

It is known that the total capacity of the lungs depends on the height, the age, the sex and the condition of the lungs of the individual [15]. To date, the exact correlation between the total capacity of the lungs and the total surface area is not known. The total surface area may vary up to threefold (i.e. 80–240 m^2) between individuals, so the individualization of the standard surface area of the lungs may improve the model. Using the surface area and the thickness of the ELF in different parts of the lungs, the total volume of ELF in the lungs is 0.205 L (equation 1). Le Brun et al. also determined a pulmonary volume of distribution. Using a completely different method, the volume was calculated to be 0.2 L [10]. The number of cells in ELF and the concentration of ELF were greater in CF patients compared to healthy subjects [35]. As the mucus covering the ELF is more thick and tenacious in CF patients compared to healthy subjects it is probable that the solubility and absorption of ELF in CF patients is different compared to healthy subjects. For patients with non-CF bronchiectasis nothing is known about the composition of ELF or the mucus and its difference with healthy subjects or CF patients. Therefore it is difficult to estimate the influence of the composition of ELF and its role in our model.

To date, in vitro data are the only source to determine the absorption from the lungs. The absorption rate is directly proportional to the surface area of the lungs and the apparent permeability coefficient (Papp). Papp is dependent on the physiological structure of the lung cells. The amount of space in the tight junctions is an important factor that affects the permeability of the cell and thus the Papp [36]. This space is dependent on the state of the lung cells. In damaged lungs, the space of the tight junctions is increased in contrast to healthy lungs due to the leakage of the cells [36,37]. Also, the thickness of the mucus layer has an effect on the permeability of the cell, because the mucus transport depends on the thickness of the mucus layer. The thicker the mucus layer, the lower the mucus transport and thus the higher the permeability of the cell [38]. The Papp also depends on the physicochemical properties of the drug, including lipophilicity and molecular weight. Unfortunately, lung cells are difficult to simulate in vitro, because reconstructed single cell barriers are complex lung tissue, so in vivo data are desirable [39]. In this study we assumed that the apparent permeability is the same for every site of the lungs. In fact, the distance between the alveolar membrane and the plasma membrane is much smaller than in the first generation and so the apparent permeability may be lower in the first generation compared to the last generations. Because the Papp in vivo can be different from the Papp measured in vitro, it is important to know how a change in the Papp will affect the serum concentrations. An increase in the Papp will increase the maximum concentration and slightly decrease the area under the serum concentration – time curve (Figure 2).

The deposition of the fine particle dose in the lungs is mainly dependent on the flow rate, the MMAD and the breath holding time. In our model we assumed that 25% of the amount that reached the respiratory airways reaches the alveoli based on the amount of fresh air that reaches the alveoli during an inhalation (unpublished results). When a smaller amount reaches the alveoli compared to the bronchioles, the time to maximum concentration increases and the maximum concentration decreases (Figure 2), because the absorption occurs more rapidly in the alveoli compared to the bronchioles. Because some patients have a lower forced vital capacity, a smaller volume of inhaled fresh air reaches the alveoli compared to the bronchioles and the time to maximum concentration will increase even more [40]. It is relevant to know the concentrations at different sites of the lungs, because bacterial infections are often located on specific sites and not evenly distributed over the whole lungs. Unfortunately, no reference can be made for these local concentrations, and therefore the predicted values should be interpreted with caution. In the anatomical dead volume (gen 0–16) the initial concentration is 150 mg L^{-1}, in the bronchioles (gen 17–21) 14 mg L^{-1} and in the alveoli (gen 22–23) 108 mg L^{-1} (Figure 3). In serum, the therapeutic serum concentration of tobramycin is between 1–10 mg L^{-1} (12). The concentration of tobramycin in the bronchioles (gen 17–21) is fairly higher than the serum concentration during more than 8 hours after inhalation (Figure 3).

The model was tested with serum concentrations of tobramycin of one patient with non-CF bronchiectasis. Because the lower limit of quantification of the assay was 0.2 mg L^{-1}, the serum concentrations after the lowest dose of 28 mg tobramycin are difficult to compare with the model (Figure 4). After a 56 mg tobramycin dose, the observed serum concentrations are consistently higher than the computer simulation. The profile after the highest dose of 84 mg tobramycin is nearly the same as the simulated profile.

Conclusion

The PK model developed in this study, based on literature data, was able to predict tobramycin concentrations in serum and lung fluids after inhalation by a DPI. More data from inhalation via DPI in healthy volunteers or patients with non-CF bronchiectasis should be obtained to validate the model. Physiological differences between healthy and damaged lungs should then be taken into account in the model so the model can be used for patients with an infection of Pseudomonas aeruginosa. The type of model developed can also be used for other drugs and for other devices, after adapting the drug-specific and device-specific model parameters, to facilitate the design of new studies with inhalation of antibiotics.

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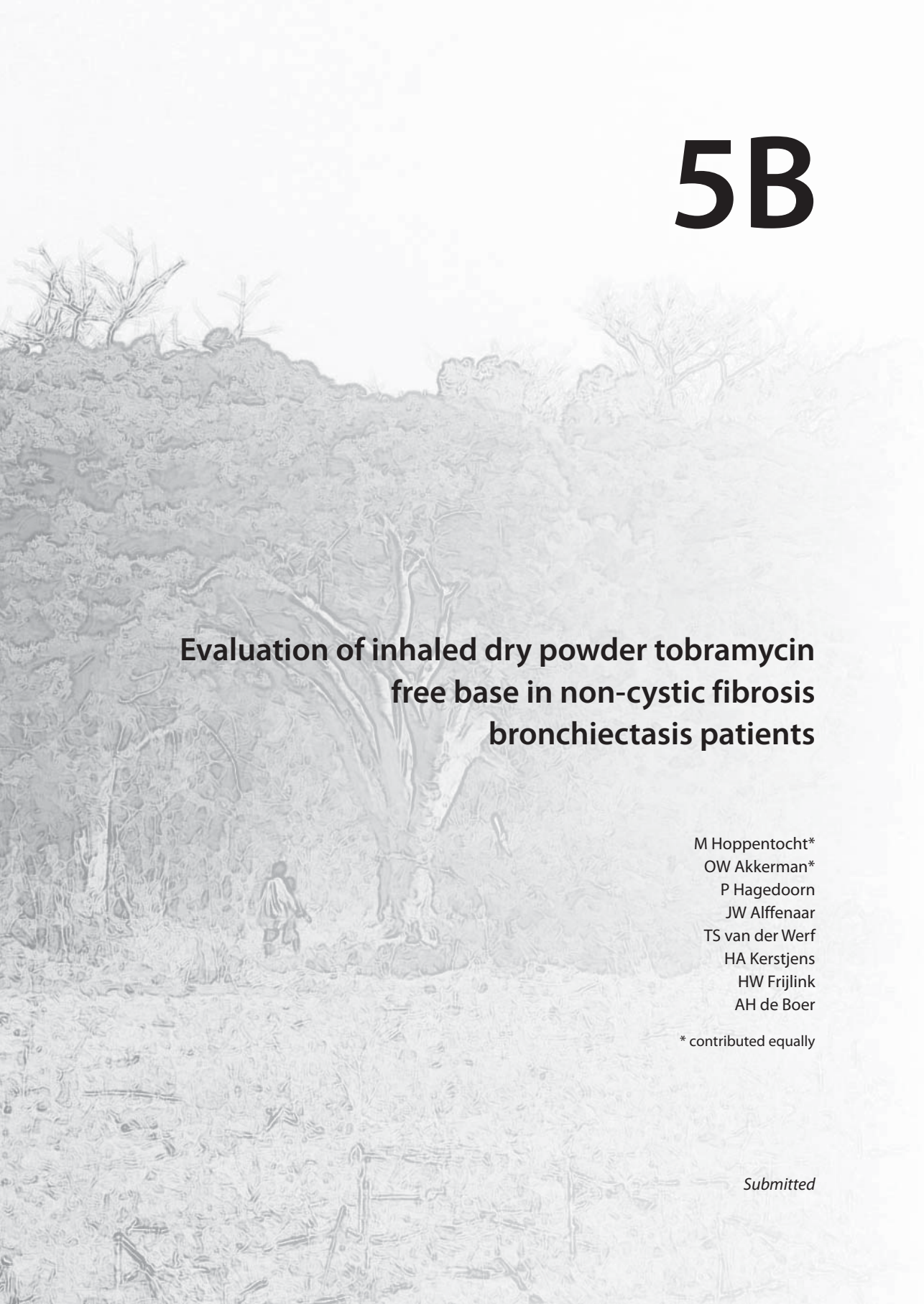

Evaluation of inhaled dry powder tobramycin free base in non-cystic fibrosis bronchiectasis patients

M Hoppentocht*
OW Akkerman*
P Hagedoorn
JW Alffenaar
TS van der Werf
HA Kerstjens
HW Frijlink
AH de Boer

* contributed equally

Submitted

Abstract

Bronchiectasis is a persistent condition characterised by dilated and thick-walled bronchi. The presence of *Pseudomonas aeruginosa* in bronchiectasis is associated with a higher hospitalisation frequency and a reduced quality of life, requiring frequent and adequate treatment with antibiotics.

To assess local tolerability and the pharmacokinetic parameters of inhaled excipient free dry powder tobramycin as free base administered with the Cyclops dry powder inhaler to participants with non-cystic fibrosis bronchiectasis. The free base and absence of excipients reduces the inhaled powder dose.

Eight participants in the study were trained in handling the device and inhaling correctly. During drug administration the inspiratory flow curve was recorded. Local tolerability was assessed by spirometry and recording adverse events. Serum samples were collected before, and 15, 30, 45, 60, 75, 90, 105, 120 min; 4, 8 and 12 h after inhalation.

Dry powder tobramycin base was well tolerated and mild tobramycin-related cough was reported only once. A good drug dose-serum concentration correlation was obtained. Relatively small inhaled volumes were computed from the recorded flow curves, resulting in presumably substantial deposition in the central airways – i.e., at the site of infection.

In this first study of inhaled dry powder tobramycin free base in non-cystic fibrosis bronchiectasis patients, the free base of tobramycin and the administration with the Cyclops dry powder device were well tolerated. Our data support further clinical studies to evaluate safety and efficacy of this compound in this population.

Introduction

Bronchiectasis is a persistent and frequently progressive condition characterised by dilated and thick-walled bronchi. This pathology can result from many underlying conditions, which is divided in cystic fibrosis (CF) and non-cystic fibrosis (non-CF) including post-infectious conditions from, for instance, infection with bacteria (e.g. *Mycobacterium tuberculosis*) or viruses (e.g. measles). The main symptoms of bronchiectasis are cough and chronic sputum production [1]. The infectious burden stimulates neutrophilic and inflammatory mediator responses in the airways [2]. Ongoing structural damage has been referred to as the vicious circle in bronchiectasis [3]. In different studies *Haemophilus influenzae* was isolated in 29–42% and *Pseudomonas aeruginosa* in 13–31% of the patients with stable non-CF bronchiectasis [4,5]. The presence of *P. aeruginosa* in patients with bronchiectasis is associated with increased sputum production, more extensive bronchiectasis on high-resolution computed tomography (HR-CT) of the chest, a higher hospitalisation frequency, and a reduced quality of life [2,6–9].

Current treatment practice for non-CF bronchiectasis patients chronically infected with *P. aeruginosa*, consists of tobramycin (as sulphate) or colistin (as sulfomethate sodium) inhalation in combination with orally administered macrolides [1,10]. The BTS guidelines are still reticent about using macrolides in this population. Both inhaled drugs are most frequently administered by wet nebulisation, although dry powder formulations have recently been introduced. Nebulised tobramycin sulphate is usually administered for 28 days in 2 daily doses of 300 mg each, followed by 28 days without tobramycin therapy to reduce the risk of side effects and antibiotic resistance. This regimen was originally tested in patients with CF [11–13]; trials conducted with inhaled tobramycin in non-CF bronchiectasis patients with chronic *P. aeruginosa* have shown clinical improvement and a reduction in bacterial density too [14]. An alternative to wet nebulisation of tobramycin sulphate is the TOBI® Podhaler™. Tobramycin sulphate inhalation powder (TIP), administered with the Podhaler™ to CF patients that are chronically infected with *P. aeruginosa* appeared to be safe and effective [15]. Pharmacokinetic parameters and efficacy of a 112 mg TIP dose twice daily were similar to 300 mg nebulised tobramycin sulphate solution twice daily [15]. However, the re-usable capsule based dry powder inhaler (DPI) and voluminous powder formulation of the sulphate containing various excipients have some disadvantages, notably, the large number steps to administer one dose [16]. No clinical studies with dry powder tobramycin have been carried out in non-CF bronchiectasis patients to date.

The aim of this study was to assess local tolerability and the pharmacokinetic parameters of increasing doses of dry powder tobramycin free base administered using the Cyclops DPI without excipients to participants with non-CF bronchiectasis.

Methods

Materials

Tobramycin free base was obtained from Spruyt Hillen BV (the Netherlands) and spray dried at the Department of Clinical Pharmacy and Pharmacology of the University Medical Center Groningen (UMCG) following previously described procedures [17]. The free base of tobramycin was chosen instead of the commonly used sulphate salt based on its favourable physico-chemical properties and the sulphate group increases the amount of powder to be inhaled [17]. The Cyclops DPIs used during this study were also described earlier [17].

Participants

Eight participants with non-CF bronchiectasis, confirmed by HR-CT, were recruited in the outpatient department of the Department of Pulmonary Diseases and Tuberculosis of the UMCG. The baseline characteristics of the participants are listed in Table 1. The criteria for exclusion were partly based on the contra-indications and known drug-drug interactions of the TOBI® Podhaler™ [18]. In- and exclusion criteria are listed in Table 2. Written informed consent was obtained from all participants.

Table 1 Participant characteristics

Participant	Sex	Age	FEV_1 /FVC	FEV_1 Predicted (%)	BMI	Asthma
P1	F	60	67	113	43	Yes
P2	F	68	86	74	32	No
P3	F	69	46	31	31	Yes
P4	M	69	65	71	23	No
P5	F	64	71	71	25	No
P6	F	63	77	106	23	No
P7	F	57	71	92	39	Yes
P8	F	73	61	82	29	Yes

Table 2 In- and exclusion criteria

Inclusion criteria:
- Age 18 years or older
- Obtained informed consent
- Patients having bronchiectasis (confirmed with HR-CT of the chest)
Exclusion criteria:
- Patients with cystic fibrosis
- Pregnant or breast feeding
- Subjects with known or suspected renal, auditory, vestibular or neuromuscular dysfunction, or with severe, active haemoptysis
- History of adverse events on previous tobramycin or other aminoglycoside use
- Concurrent use of cyclosporin, cisplatin, amfotericin B, cephalosporins, polymyxins, vancomycin or NSAIDs

Study objectives and design

The primary objectives were to assess both local tolerability and pharmacokinetics of dry powder tobramycin free base administered using the Cyclops in the target population. During four consecutive visits, at least 7 days apart, the participants received a 30, 60, 120 or 240 mg dose of dry powder tobramycin from the Cyclops. Each blister contained 30 mg of tobramycin; the higher doses were administered in multiple successive blisters and inhalers. This study was performed as single centre, dose-escalation study.

Tolerability

Local tolerability was assessed by spirometry, combined with active questioning and passive monitoring by recording remarks about adverse events made by the participants. Spirometry was performed before (S0) inhalation and 20 (S1), 35 (S2) and 95 (S3) minutes after inhalation. A drop in FEV_1 of 10% or more compared to baseline FEV_1 (S0) was considered significant. Active questioning for adverse events was done every time a blood sample was drawn. Furthermore, before inhalation the creatinine level of every participant was measured as baseline to check for decreased kidney function. The creatinine clearance was calculated using the Cockroft Gault formula.

Serum sampling and analysis

Blood samples were collected before pulmonary administration of the study drug (t=0), and 15, 30, 45, 60, 75, 90, 105, 120 min; 4, 8 and 12 hours after inhalation. The samples

were centrifuged for 5 min at 3,000 rpm and subsequently stored at -80°C until analysis. Tobramycin serum concentrations were analysed using a modified immunoassay method Syva® *Emit® 2000* Tobramycin Assay (Siemens Healthcare, Germany) combined with the ARCHITECT c8000 (Abbott Diagnostics, U.S.A.).

Pharmacokinetic analysis

The area under the curve from t=0 to t=12 h (AUC_{0-12}) was calculated using MW/Pharm (Mediware, the Netherlands) [19]. The maximum serum concentration (C_{max}) and time to maximum serum concentration (t_{max}) were derived from the concentration-time curves. The delivered dose was computed from weighed dose and inhaler residue determined by gravimetric analysis for the first two participants and by chemical and gravimetrical analysis for the others. Gravimetrical analysis was performed immediately after inhalation and chemical analysis on the same day of administration. We used a 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) assay to chemically quantify the amount of tobramycin retained in the Cyclops DPIs [17].

Recording of the inspiratory flow curve

Prior to inhalation of the study drug, study participants received inhalation instructions followed by training regarding handling of the device and performing a correct inhalation manoeuvre. Training was done using an empty Cyclops connected to a laptop, with self-written software (LabVIEW, National Instruments, the Netherlands) for recording and processing of flow curves generated through the device. A differential pressure gauge (Sitrans P250, Siemens, Germany) was used to measure the pressure drops generated across the inhaler, after prior pressure drop versus flow rate calibration with a thermal mass flow meter (Brooks Smart Mass Flow Meter 5863S, USA). Inhaler instrumentation was performed without changing the inhaler resistance or interfering with the aerosol delivery [17]. First when a series of consistent flow curves meeting the criteria for good inhaler performance was obtained during training, a similarly instrumented Cyclops with tobramycin was handed to the participant. Also during the drug administration the inspiratory flow-rate was recorded to be able to explain unexpected pharmacokinetic results, and to ascertain that the participants generated a 4 kPa pressure drop – corresponding with the target flow rate of 34 L/min [17].

Ethics

The study protocol was approved by the medical ethical review committee of the UMCG (METC number 2013.024) and was performed according to the Helsinki declaration. The study was registered at www.clinicaltrials.gov (NCT02035488).

Results

Participants

Eight participants were enrolled and all completed the study; see Table 2 for baseline characteristics.

Inhalation manoeuvres

Training of respiratory manoeuvres was successful in all participants. All were also able to hold their breath for 10 sec after inhalation of the drug to facilitate deposition by sedimentation in the airways.

Local tolerability

Administration of dry powder tobramycin free base using the Cyclops was well tolerated. Table 3 shows that four participants showed significant drops in FEV_1 (≥10%) at some time point after dose administration. In total six significant drops were recorded out of 32 measurements (19%), 4 times after a low dose (30–60 mg) and 2 times after a high dose (120–240 mg). The first two participants had slight complaints of a bad taste after inhalation of the first dose (30 mg). For this reason, the participants were advised to rinse their mouth with water after the complete dose was administered. Thereafter, none of the participants reported this adverse event. Two participants reported mild cough – one after a dose of 240 mg, 7 hours after inhalation; the other reported cough after active questioning after a dose of 30 mg, 1 hour after inhalation.

Table 3 Drops in FEV_1 >10% during all four visits. S indicates during which of the 3 spirometry measurements after inhalation the drop occurred

Participant	Visit 1 (30 mg)	Visit 2 (60 mg)	Visit 3 (120 mg)	Visit 4 (240 mg)
P1	No	No	No	No
P2	No	No	Yes (S1: 18%; S2: 11%)	No
P3	Yes (S1: 14%; S2: 10%)	Yes (S3: 10%)	No	No
P4	No	No	No	No
P5	No	No	No	No
P6	No	No	No	No
P7	Yes (S1: 13%; S3: 12%)	No	No	Yes (S1: 10%; S2: 10 %)
P8	Yes (S3: 10%)	No	No	No

Pharmacokinetic analysis

All mean pharmacokinetic parameters investigated are summarised in Table 4. As expected, the mean C_{max} and mean $AUC_{0\text{-}12}$ rose approximately by two-fold after each doubling of the dose. The t_{max} was the same, 1.6 (+ 0.08) h, for all four doses investigated. Figure 1 shows the serum concentration-time curves of the individual participants after 30 (Figure 1a), 60 (Figure 1b), 120 (Figure 1c) and 240 mg (Figure 1d) dry powder tobramycin. Some data points are missing due to failed blood draws. Therefore, the mean $AUC_{0\text{-}12}$ presented in Table 4 was calculated for 6 participants. Apart from inter-individual differences (Figure 1), also large intra-individual differences were observed in some participants. For example, participant 5 showed a C_{max} of 0.57 mg/L after a 120 mg dose (delivered dose 95 mg), but a 240 mg dose (delivered dose 204 mg) resulted in a C_{max} of only 0.58 mg/L. Figure 2 shows the C_{max} per mg delivered dose as function of the inhaled volume; the figure indicates a strong trend for increasing normalised C_{max} with decreasing inhaled volume.

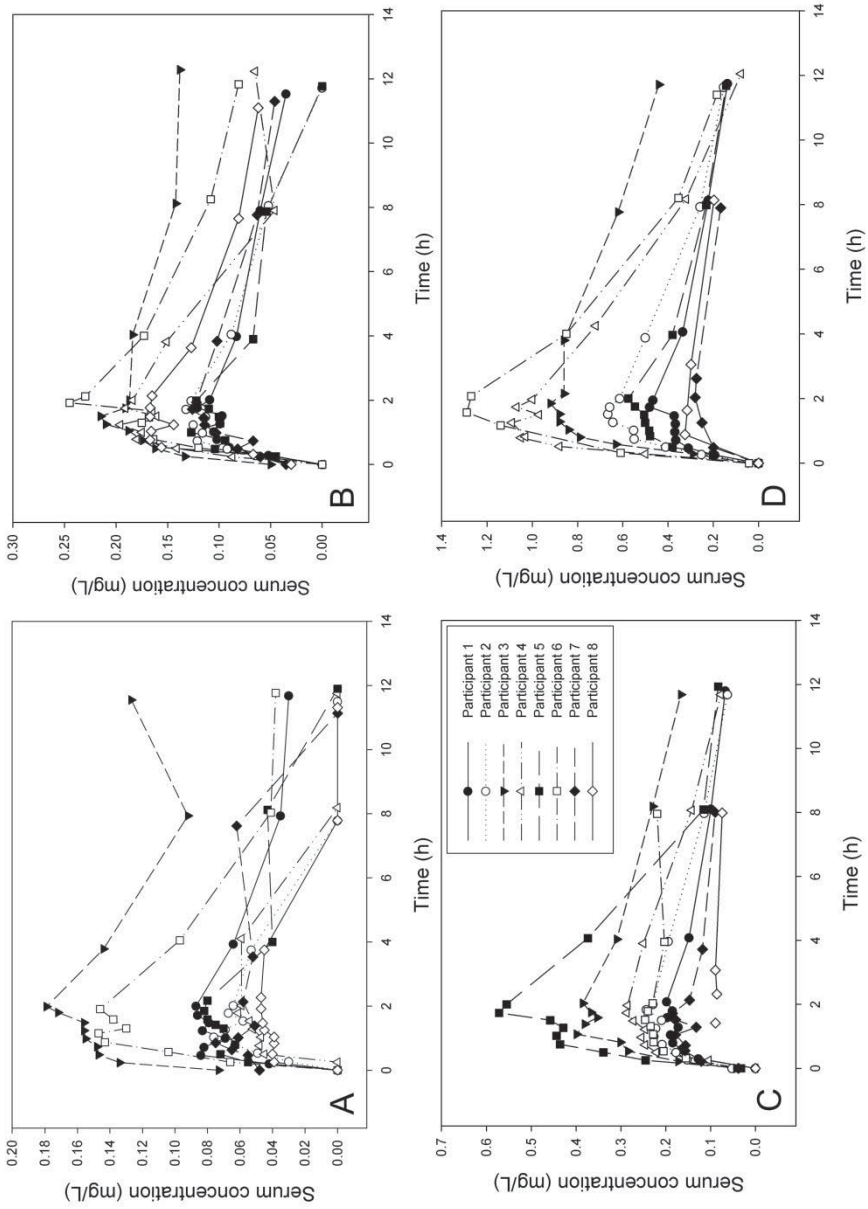

Figure 1 Individual serum concentrations of tobramycin following administration of a 30 (A), 60 (B), 120 (C) or 240 (D) mg dry powder tobramycin dose from the Cyclops.

Table 4 Mean pharmacokinetic parameters and standard deviations. It was particularly difficult to obtain blood from participants 7 and 8 during visit 3 and 4

Parameters	Visit 1 (30 mg)	Visit 2 (60 mg)	Visit 3 (120 mg)	Visit 4 (240 mg)
Delivered dose (mg)	23 ± 4.8	53 ± 2.3	97 ± 9.7	198 ± 11.9
$AUC_{0\text{-}12}$ (h mg/L)	0.40 ± 0.72	1.03 ± 0.56	2.26 ± 0.77	5.36 ± 2.10
C_{max} (µg/L)	105 ± 45	173 ± 48	277 ± 148	703 ± 365
t_{max} (h)	1.57 ± 0.48	1.45 ± 0.41	1.64 ± 0.31	1.60 ± 0.39

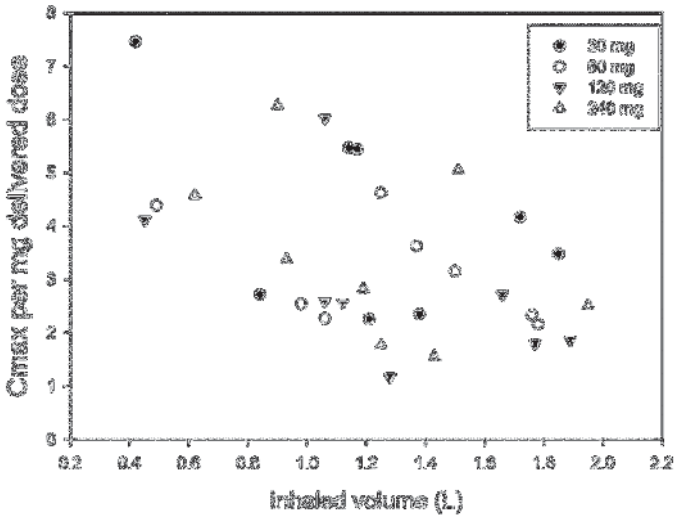

Figure 2 The C_{max} per milligram delivered dose as function of the inhaled volume.

Discussion

In this study we assessed the local tolerability and pharmacokinetic parameters of escalating doses of dry powder tobramycin free base using the Cyclops in participants with non-CF bronchiectasis. We demonstrated that inhalation of dry powder tobramycin base from the Cyclops is well tolerated.

Coughing is often reported as an adverse event immediately after inhalation of tobramycin, either by wet nebulisation or dry powder inhalation, both in CF and non-CF bronchiectasis patients [20–22]. In this study, only two participants started coughing after inhalation, each during only one out of four visits. One participant reported cough 7 hours after inhalation,

making causality of dry powder tobramycin less likely. We believe that the high inhaler resistance to airflow and excellent powder dispersion by the Cyclops may explain the very low frequency of coughing. The tobramycin particles, of which almost 90% is between 1 and 5 μm, enter the respiratory tract at a flow rate of only 34 L/min [17]. This combination of beneficial features prevents the deposition of substantial drug fractions in the oropharynx, which is the common trigger for coughing. Based on the experience with colistin sulfomethate and colistin sulphate [23], where using the sulfomethate salt reduced cough compared to using the sulphate salt, we speculate that the use of tobramycin free base instead of the sulphate salt might also help to reduce cough. In addition, the lower powder dose to be inhaled for the free base (65.6% compared to the sulphate) without excipients may also have contributed to reduced cough. Rinsing the mouth with water after administration of the full dose solved the reported bad taste of the two participants after their first visit. The bad taste is known from nebulised tobramycin sulphate.

In four participants a drop in FEV_1 of 10% or more was observed during one or two visits. These four participants were diagnosed with asthma, all of the drops were without complaints of dyspnoea. In half of the cases the drop in FEV_1 was exactly 10%. No correlation was found between the drops in FEV_1 and the different time points of spirometry nor with the dose administered. All drops in FEV_1 observed during the first two measurements (S1 and S2), were spontaneously reversed without the use of bronchodilators. In a previous study with nebulised tobramycin 3 out of 26 participants showed a drop in FEV_1 >10%, but also 5 out of 27 participants in the placebo group showed a drop in FEV_1 >10%. They considered a drop in FEV_1 of 10% not to be an adverse event to inhaled tobramycin [24]. Others suggest that respiratory adverse events are more common in non-CF bronchiectasis patients than in CF patients. They state that this is probably caused by underlying morbidities like asthma, the greater age of these patients, and a greater history of smoking [14,22,25]. The clinical relevance needs to be determined in larger phase 2 and 3 studies.

The computed normalised C_{max} values (C_{max} per mg delivered dose) in our study are in a wide range between 1.17 and 7.46 μg/L per mg of deliver ed dose, with an overall average of 3.41 μg/L. The delivered doses were derived from the inhaler retentions measured. In a study in healthy volunteers, the Podhaler™ 80 mg dose, after correction for the inhaler losses, the sulphate group and the excipients, yielded a normalised C_{max} value of 9.57 μg/L per mg delivered free base [26]. In CF patients, normalised C_{max} values of approximately 22 μg/L per mg delivered free base could be derived (more or less independent of the dose), assuming that Podhaler™ losses were similar to earlier reports [15]. The wide range of normalised C_{max}

values from the Cyclops in our study and the lower C_{max} value compared to studies in CF patients and healthy volunteers with the Podhaler™ are remarkable. Further investigation is needed to elucidate whether they result from a difference in inhaler performance, or from differences in the study populations – or both.

The Cyclops delivered doses derived from inhaler residues were quite consistent and are on average (all doses, all patients) 82.1% of the doses weighed into the blisters (RSD = 12.2%). Therefore, delivered dose variation does not seem to explain the wide range of normalised C_{max} values in this study. In a previous study good dispersion performance of the Cyclops was already demonstrated [17]; fairly consistent delivered fine particle fractions (FPF < 5 μm) of approximately 75% of the weighed doses were computed. Losses in the oropharynx between the Cyclops and the Podhaler™ may have been different due to a difference in inhaler mouthpiece design, although the exit velocity at 35 L/min from the Cyclops (24.3 m/s in our study) is the same as that from the Podhaler™ at 80 L/min (24.7 m/s) in the studies with this device. Nevertheless, aerosol plume geometry and jet effects resulting in return flows in the oral cavity, may be different and greater for the Cyclops compared to the Podhaler™ in spite of comparable exit velocities. Beyond the oral cavity however, at a distance from the mouthpiece, the more than two times lower flow rate from the Cyclops at the same pressure drop must result in lower inertial deposition in the first bifurcations. Since almost no tobramycin related cough was reported or observed during this study, it is safe to assume that indeed no large losses in the oropharynx from the Cyclops occurred. Therefore, a difference in results from the different studies seems most likely the result of the inhalation manoeuvre or a difference in disease related aspects, between the subject populations. In our study, relatively small inhaled volumes ranging from only 0.42 to 1.95 L were computed from the flow curves recorded during drug administration. They were less than 50% of recorded Vital Capacities, in spite of the instructions given to inhale as deep as possible, and cannot be explained by dyspnoea since all participants were able to comply with the recommended breath-hold pause of at least 10 s after inhalation. These low volumes must have resulted in substantial deposition in the upper and central respiratory tract and only marginal aerosol penetration into the most distal airways, where absorption is supposed to be fastest (resulting in a high C_{max}) [27]. However, the most distal airways may not be the most relevant target area in non-CF bronchiectasis patients, since it is known that bacterial infections in this population are mainly located in the bronchi and less in the bronchioles and alveoli [28]. Surprisingly, a strong trend was found for increasing normalised C_{max} with decreasing inhaled volume (Figure 2). Comparison with the Podhaler™ studies

in this respect is not possible, as flow curves during drug administration were not recorded in the studies performed with this device. With the Podhaler™, almost 40% of the whole lung dose was recovered from the peripheral airways, which suggests that inhaled volumes were considerably higher, presumably causing the higher C_{max} values.

The finding of increased normalised C_{max} with decreasing inhaled volume was surprising, and clearly needs further clinical investigations. In patients with bronchiectasis the bronchial circulation can be increased from 1% to as much as 30% of the cardiac output due to increased inflammation [25,26]. It can be hypothesised that the higher blood circulation increases the absorption rate as drugs like tobramycin and other antibiotics penetrate faster also in opposite direction from the systemic circulation into lung tissue in patients with pulmonary infections like pneumonia [27,28]. Because non-CF bronchiectasis is a progressively deteriorating condition accompanied by increased inflammation, it could be that the C_{max} changes with the degree of inflammation. This could also explain why in previous studies with the TOBI® Podhaler™ normalised C_{max} values were much higher for CF patients compared to healthy volunteers [15,26]. These aspects remain unclear from all deposition studies however, and should be addressed in future clinical investigations with inhaled antibiotics.

Our data are limited to AUC, t_{max} and C_{max} results in serum; the topical tobramycin concentrations in the airways – i.e. at the site of infection – were not measured. A phase 2 study evaluating safety and efficacy in non-CF bronchiectasis patients should be performed next. Based on the current data, we recommend 120 and 240 mg dry powder tobramycin doses by the Cyclops.

Conclusions

This is the first pilot study describing the use of dry powder tobramycin free base in non-CF bronchiectasis patients. The free base was well tolerated and this positive result invites for further clinical studies with the Cyclops dry powder inhaler to evaluate safety and efficacy of this compound in non-CF bronchiectasis patients.

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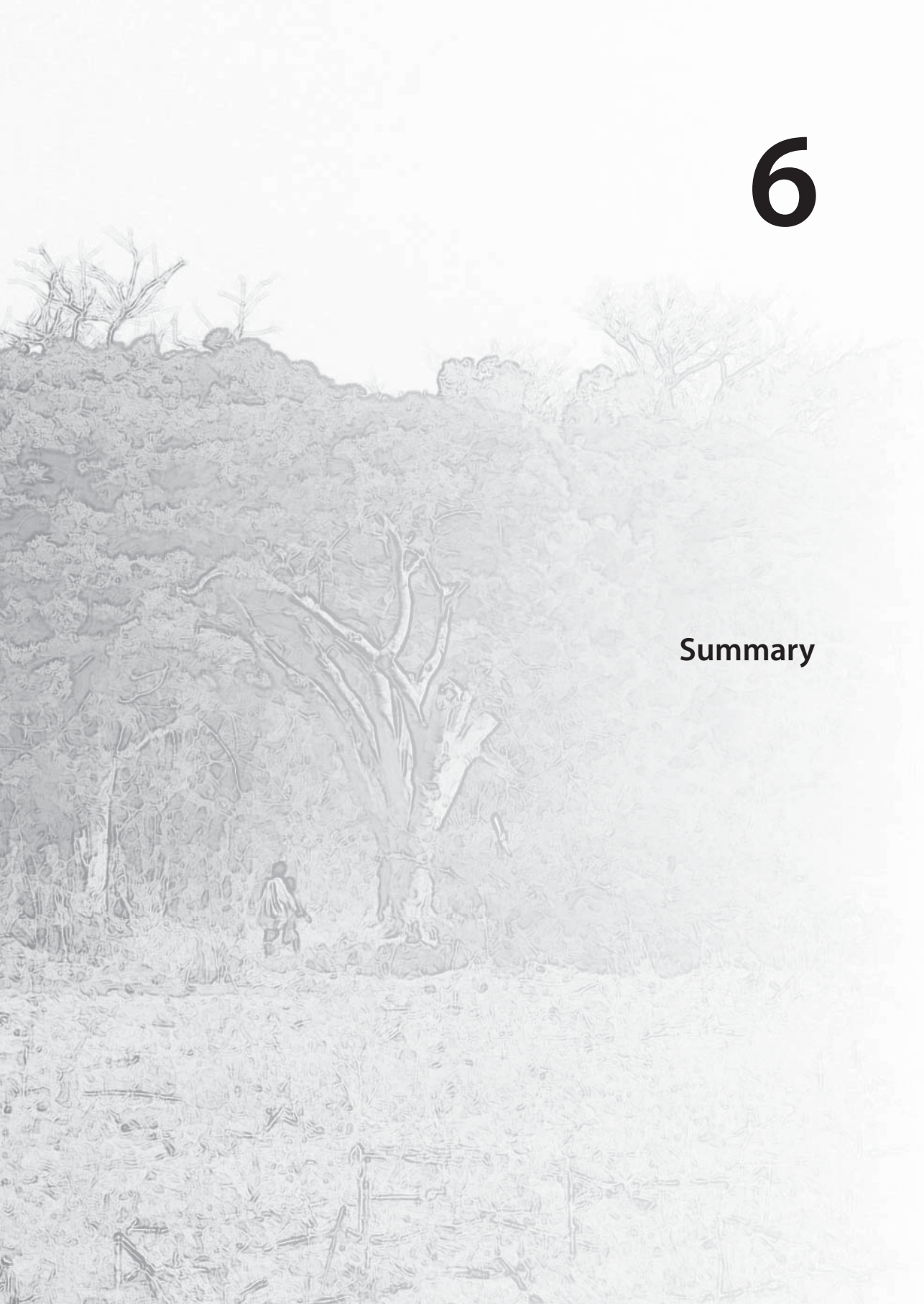

Summary

In this thesis, we touched on molecular diagnostics and epidemiology, as well as treatment aspects of tuberculosis (TB) and a condition that may ensue, once TB – or any other devastating infectious condition of the lungs – is cured: non-CF bronchiectasis, or structural airways disease.

In **chapter 2** we compared the major molecular tests for the diagnosis of TB and we studied an optimized method of fingerprinting – Variable Number of Tandem-Repeats (VNTR) typing generally used for molecular epidemiology of TB.

In **chapter 2a** we focused on the rapid molecular diagnostics of TB and the different in-house and commercially available PCR assays. This was the first study to include comparisons of a large number of diagnostic PCR assays currently available for TB. In general, the bacterial load of the specimen correlates with analytical performance of a test. We mimicked the bacterial load of specimens by dilution steps, with a high dilution mimicking a low bacterial load. Both analytical sensitivities and the detection limits appeared to differ between the PCR tests, with the AmpliSens MTC-FRT PCR kit, the in-house real-time PCR IS*6110*: 10 µl DNA input performing best in both analytical sensitivity and detection limit and with the GeneXpert MTB/RIF assay having the lowest analytical sensitivity.

In **chapter 2b** we evaluated a modified method for VNTR typing. VNTR typing is the current gold standard for performing molecular epidemiological studies with *M. tuberculosis* (MTB) strains. The modification of 7 primers of the original in-house method resulted in a high percentage of complete profiles in the first multiplex PCR run. This reduces workload and laboratory turnaround time and it is much cheaper than the commercially available VNTR method. We tested analytical sensitivity of this optimized VNTR method in standardized broncho-alveolar lavage fluid (BALF) spiked with MTB as we described in the study presented in chapter 2a. This resulted in an analytical sensitivity of 1:10 – or in an almost perfect analytical sensitivity of 1:100. The latter dilution showed a perfect score except for one of the three VNTR analyses with a strain with a single copy of IS*6110* that yielded results for only 22 loci instead of 24. These results show that the assay may provide an asset for clinicians; they may take clinical decisions for empirical treatment based on fast molecular, and standard phenotypic drug sensitivity results if their patient appears infected with an isolate from a known cluster; or, in discriminating between a relapse or a new infection, comparing two different isolates over time in the same patient.

In **chapter 3** we focused on results of epidemiological linking of individual patients. In **chapter 3a** we described an outbreak among non-human primates and a human using results

obtained by another molecular fingerprint method – spoligotyping. With the clinical and radiological data and with the use of this molecular epidemiological technique we described the transmission of TB from great apes to humans. Transmission from animals to humans has rarely been described; our clinical and radiological data appeared highly suggestive that the route of transmission was in fact from non-human primates to humans. Further evidence of the route of transmission was given by spoligotyping resulting in the general conclusion that the animals, and not the human, were the source of the infection.

In **chapter 3b** we described a case study of the results using a blood test, and not only a skin test for TB contact tracing, to detect any transmission from a patient with TB. For the first time, the Quantiferon (QFT) was used next to the tuberculin skin test (TST) for contact tracing around an index case with bovine TB. We showed the discrepant results between the TST and the QFT in three rings of contact tracing. The QFT targets the ESAT-6 and CFP10 gene products coded by the Region of Difference-1 and TB7.7; these genes are almost unique for the *M. tuberculosis* complex. Therefore the results in this study in bovine TB met our expectations, as in human TB the discordant results of both the TST and the QFT in contact tracing have been reported and discussed in the literature [1,2]. The results of the molecular-epidemiological test showed a possible link to another case but thorough investigation by the public health TB department could not relate our patient to this source.

In **chapter 4a** we reported the drug concentrations of second-line anti TB drugs at the site of the infection, in this case, the lung of a patient that needed resection. The hypothesis to be tested by measurements of drug concentrations in the resected lung tissue was that the second-line TB drugs would not reach the severely injured, poorly perfused lung tissue. The hypothesis of insufficient blood supply to the destroyed lung was based on imaging studies of the lungs using technetium-99m-labeled macro-aggregates of albumin, showing that lung perfusion of the injured lung was poor. Surprisingly, even with the caseated tissue of the destroyed lung, tissue drug concentrations were sufficient for antimicrobial killing. The study model presented here is a step forward in gaining more knowledge of the concentrations of these second-line TB drugs at the site of the TB infection. In the letter in **chapter 4b** we commented on a study describing the pharmacokinetics of high dose of rifampicin and moxifloxacin in both serum and cerebrospinal fluid in TB meningitis. We argued that there is a need for studying this devastating type of extra-pulmonary TB that still carries a poor prognosis. Several of the first-line TB drugs have poor penetration into cerebro-spinal fluid; rifampicin has poor penetration, but isoniazid does not. The latter drug should therefore be analyzed for its contribution relative to the other treatment components. Drug exposure

of the different TB drugs is best studied as continuous variable to estimate their relative pharmacokinetic contribution. Studies of the pharmacotherapy and pharmacokinetics of patients with TB meningitis are typically small, and every effort should be made to analyze all available data to help design future studies in this daunting condition.

In **chapter 4c** we commented on a study that related plasma concentrations of isoniazid, rifampicin and pyrazinamide to sputum culture status after 4 and 8 weeks. Only a few studies have related low drug exposures to treatment response. We propose a limited sampling strategy for calculating the pharmacokinetics of the drugs instead of using 2-hour post ingestion as moment to measure levels. For the correct interpretation of the pharmacokinetics, especially the area under the curve, the actual MIC of the isolates is needed. In this way the best predictor of efficacy, the AUC/MIC ratio, can be measured.

In **chapter 5a** we built a model to predict systemic levels of drugs by measuring blood concentrations by absorption in the airways following inhalation. In this model, we describe the different stages of absorption, distribution and clearance of an antimicrobial agent following inhalation. The model includes knowledge about the device used for inhalation and the physico-chemical properties of the antimicrobial product used.

In **chapter 5b** we studied the local tolerability and pharmacokinetics of dry powder tobramycin using a novel device called the Cyclops. We tested four different doses of tobramycin in patients suffering from non-Cystic Fibrosis Bronchiectasis. In general, the tobramycin was well tolerated – in fact much better than currently available methods to deliver tobramycin to the airways of such patients that usually report adverse effects like cough and dyspnea side effects resulting from inadvertent drug resorption from the airways into the blood stream. There were only mild complaints of cough in two out of eight study participants, each at only one of the four visits. Bad taste reported after inhalation of the first dose initially reported was abolished after we introduced rinsing the mouth with water. Pharmacokinetics were calculated and compared to the Tobi Podhaler. The C_{max} in our study was lower compared to the C_{max} values of the Podhaler in studies with healthy volunteers and patients with CF-bronchiectasis.

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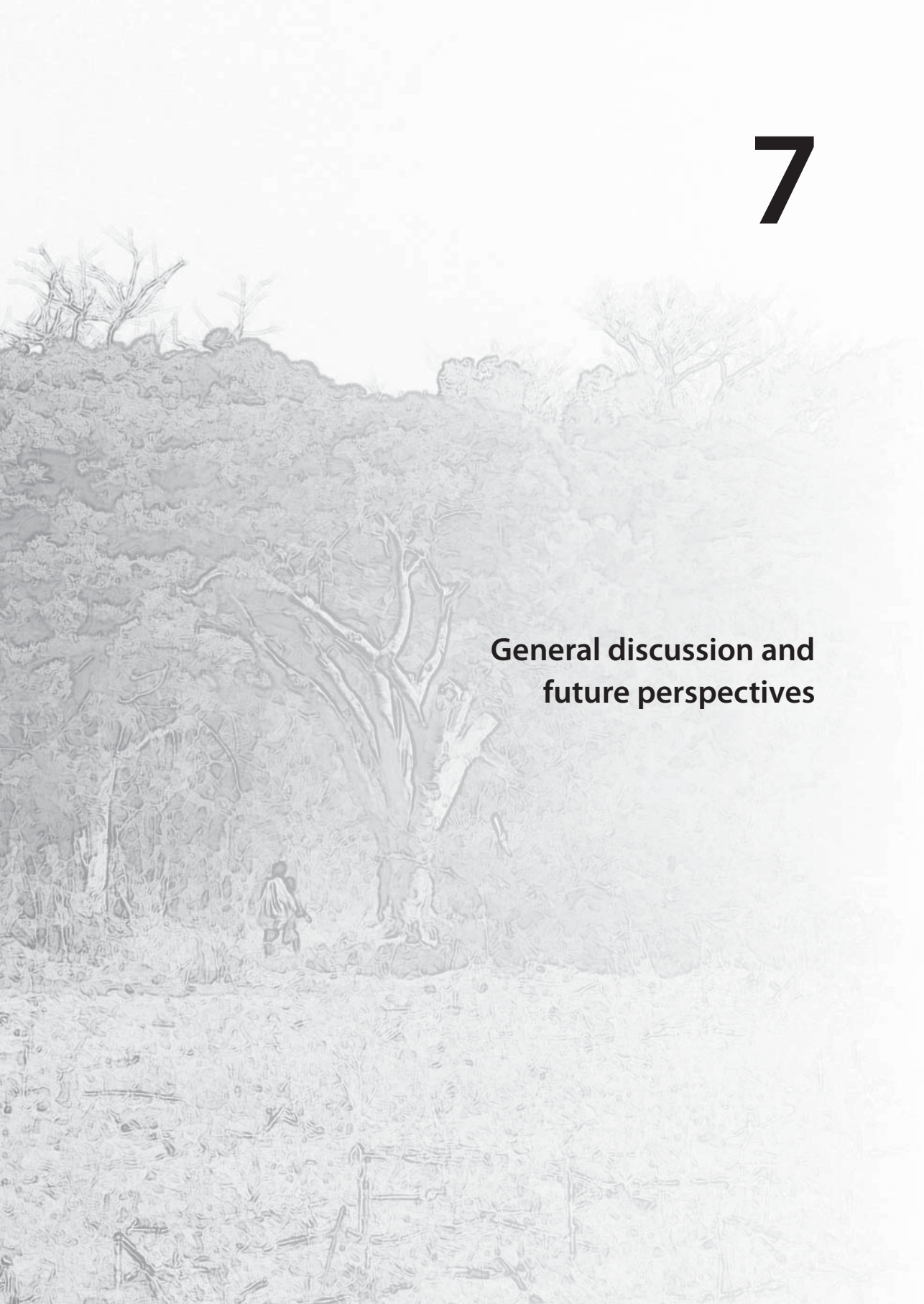

General discussion and future perspectives

TB has caused more deaths than any other infectious disease in the history of mankind. In 2013 alone, there were about 1.5 million deaths worldwide. With 9 million new cases annually, the disease burden of TB is also staggering. The epidemiology of sequelae due to TB has been studied less [1]. To conquer both TB and its sequelae, more knowledge has to be gained about diagnostics, epidemiology and treatment of TB and its sequelae.

Here we discuss these issues in the context of the studies compiled in this thesis, and we provide future perspectives.

Transmission of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb) depends on human hosts for its survival: humans are the dominant reservoir for the organism. Transmission is the critical way for Mtb to survive as a species, as every new human host expands its reservoir. Thus prevention of TB starts by increasing the knowledge about transmission.

Transmission depends on the immunology as well as the behavior of the human host, as we discussed in the introduction. After developing overt pulmonary TB, TB patients start coughing and sneezing, thus producing aerosols containing live Mtb. Once inhaled by a new susceptible host, Mtb may reach the lower airways including the alveolar spaces. Healthy bronchi will clear the Mtb by the ciliary beat driving the tapis roulant; entrapped bacilli are transported to the proximal airways and swallowed or expectorated by coughing. Damaged airways – e.g., as a result of smoking – make the host more susceptible to TB [2–4].

Contact tracing around an index case can tell us about the transmission of TB in a microenvironment [5]. Contact tracing can be done using the tuberculin skin test (TST) or an interferon gamma release assay (IGRA). As latent TB infection (LTBI) is still lacking a clear definition a gold standard is still lacking for the TST and both IGRAs. Before interpreting the results of the TST and the IGRAs the BCG vaccination status, previous contacts, the immune status of the individual tested and the test properties should be known [6–12]. Normally, active case finding is only performed around a case of human TB. However, M. bovis still causes TB – in around 1.0–1.5% of the cases of TB in the Netherlands, the United Kingdom and the United States [13–15]. Our report shows that using an IGRA may help in contact tracing around an index case of bovine TB.

In the macro-environment, the routes of transmission of the strains within and between countries or continents are found using molecular-epidemiological techniques like

fingerprinting techniques. Fingerprinting helps understanding the route of transmission thereby generating knowledge about virulence of certain strains and lineages. Furthermore fingerprinting can provide evidence of unexpected routes of transmission. Moreover, it can provide links that cannot be detected by classical epidemiological techniques. In the past this was done using spoligotyping or IS*6110*-RFLP, but in recent years it has been replaced by VNTR technology [16–18]. Another benefit of fingerprinting is that early knowledge of the results may guide clinicians to embark early on an adequate treatment regimen in a patient with an MDR strain. Early knowledge about the origin of the strain and its susceptibility to SLD's may prevent administering unnecessary drugs or overdosing appropriate drugs, thereby preventing adverse effects of the drugs prescribed.

Therefore the most optimized VNTR technique should be available worldwide with little increase in costs. The combination of the studies comparing 14 different PCR assays and the optimized VNTR technique can guide clinicians in requesting rapid VNTR typing in individual cases.

The knowledge of the routes of transmission and the evolution of Mtb should further increase and results should be known as soon as possible. It can change early knowledge about the strain and thereby not only change management of contact tracing but also treatment for a patient. Whole genome sequencing (WGS) might fill the gap left over by conventional but cheaper techniques like VNTR. So future studies with WGS should make the routes of transmission and evolution of Mtb even clearer and known as soon as possible. For smaller outbreaks, much cheaper optimized epidemiologic techniques should be developed to combat TB worldwide. This means that these techniques should be better affordable without loss of performance using WGS as gold standard.

MDR-TB

In the Netherlands, MDR-TB was steady with around 15–20 patients per year in recent years [13], though in 2014 the number of newly detected MDR-TB cases was slightly lower (i.e., only 6 patients). In 2013, worldwide estimations are that 3.5% of all newly detected cases and 20.5% of the relapses have been reported to be MDR-TB [19]. In Belarus around 75% of the relapses are MDR-TB [20,21]. These facts make MDR-TB one of the biggest infectious threats globally. MDR-TB probably has similar virulence compared to drug-susceptible TB strains. Though this is still debated and therefore controversial, virulence is probably dependent on factors associated with certain MTB lineages with an inherent potential to elicit cytokine

and inflammatory reaction of the host; loss of drug susceptibilities does not appear to result in an equal loss of virulence factors in most studies [22–24].

Two validated, commercialized molecular tests are currently available for testing for possible MDR-TB, the GenoType MTBDR*plus* (Hain Lifescience) and the Xpert MTB/RIF (Cepheid). Molecular testing for sensitivity should be performed as early after diagnosis as possible. In the future the molecular sensitivity testing using WGS should be combined with the viability of bacilli. Knowing the resistance of a strain prevents inadequate treatment with its toxicity. By giving adequate treatment for drug-resistant strains, its transmission can be prevented earlier. Importantly, we show that in-house and commercial assays can successfully be compared in a clinically meaningful way. We have shown for the first time, that most of these assays compare favorably using a platform that mimics real-life. Our experiment indeed mimicked the situation often encountered in low incidence settings like ours, with patients having low bacillary loads. In these sputum smear- and PCR negative patients suspected to have TB, we employ bronchoscopic broncho-alveolar lavage for diagnosis. Broncho-alveolar lavage is a procedure that by definition causes dilution of the alveolar bacillary load by around 1,000 fold. Comparing diagnostic thresholds of these 14 different diagnostic assays is highly relevant in clinical practice. In our study comparing PCR assays, with its limitations, we found the Xpert MTB/RIF to have the lowest analytical sensitivity. In high incidence countries this is of less importance due to a high a priori change of diagnosing TB and finding a higher bacterial load in the population of these countries. In low incidence countries there is a need to further evaluate the performance for detecting resistance of the GenoType MTBDR*plus* and the Xpert MTB/RIF on its own or using both tests together.

In contrast to WHO guidelines, therapeutic drug monitoring (TDM) has indeed been used in the treatment of MDR-TB in the Netherlands, with excellent results [25,26]. By adjusting the dose in case of high serum drug levels possible adverse events of second line anti TB drugs (SLD) can be prevented, while detection of low serum drug levels can be adjusted so as to improve efficacy, and hence, potentially enhance cure rate. Indeed, no treatment failures or early relapses have been detected in the last decades in our center [26,27].

One potential drawback of TDM is, that it assumes a near-perfect correlation of TB drug concentrations in the blood stream and at the site of TB infection. Further knowledge about the correlation between serum levels of TB drugs and the levels of the same drugs at the site of infection would fill important knowledge gaps. Obviously, only occasionally will it

be possible to obtain tissue specimens of infectious TB foci; and only in cases of elective surgery, will such simultaneous measurements in blood and in resected tissue be possible. Nonetheless, it would be a great advantage to obtain more specimens of affected tissues or fluids for measuring levels of drugs with simultaneous plasma drug measurements over time [28]. This also applies to measuring concentrations at target sites that are even more challenging and critical, e.g., cerebro-spinal fluid [29].

The current technology for TDM is burdening to patients and the health care system. One disadvantage of TDM is the large number of serum samples that should be drawn to obtain a reliable pharmacokinetic curve. The reliability and potential benefit of TDM through limited sampling strategy using the dried blood spots (DBS) sent by mail in ambient temperature conditions for processing in a reference lab have to be further examined.

No study has yet compared treatment outcome of MDRTB with individualized treatment using TDM versus standard-dose following the WHO guidelines. Therefore, such a study would be a tremendous asset to evaluate the full potential of TDM using DBS. We hypothesize that this strategy would improve treatment outcome in MDR-TB while also reducing adverse effects and toxicity.

A similar spot card, as used for DBS, might be suitable for detecting molecular genetic mutations in drug targets predicting drug sensitivity in sputum samples, using whole genome sequencing. Both TDM and molecular drug sensitivity might be performed in specimens collected using only one or two spot cards. If this platform provides adequate predictions for outcome, it might be used on a routine basis in national TB programs, and not only for patients that fail on treatment.

Sequelae of TB

Those who survive TB may pay health costs that have received little attention. Side effects – especially, liver test abnormalities – may cause delay in the treatment [30]. Other side effects include vestibular and auditive impairment, especially as a result of group 2 second-line TB drugs – aminoglycosides [31,32].

Sequelae of extra-pulmonary TB have been identified. TB meningitis has the most profound and severe sequelae [33,34]. Indeed, if this condition does not result in death, it may result in severe neurological damage [35–37]; even with anti-inflammatory co-medication, neurological outcome is poor in a large proportion of affected individuals [37–39]. Future studies in this

population are urgently needed to gain more knowledge of the proper treatment and the levels of the drugs in the CSF. In low incidence countries this can be achieved by transferring such patients to a high care TB center where a wide range of facilities are available, and where protocols are in place to achieve the goals of TDM simultaneously in blood- and CSF specimens over time [29].

Sequelae of pulmonary TB were not systematically recorded when the trials on short-course, rifampicin-based therapy were reported [40]. Several authors have published about cavitary lung lesions that were sterilized from TB bacilli following TB treatment but that were subsequently colonized by fungal hyphae, especially, *Aspergillus fumigatus* leading to aspergillomas [1,41–43]. Bronchiectasis after pulmonary TB used to be common although currently, it has become an unusual cause of non-Cystis Fibrosis (CF) bronchiectasis in affluent countries [44–49]. In TB-endemic regions however, this condition is fairly common among the sequelae of TB, and clearly TB is the most common cause of structural lung disease world-wide [49,50].

Unfortunately, also due to a paucity of studies, the data base providing the evidence for the management of non-CF bronchiectasis is still limited [51]. For that reason, treatments are often based on the relatively large studies on which the CF bronchiectasis guidelines are based.

Aggressive treatment for infectious exacerbations improved the survival of patients with CF dramatically in the last 20–30 years. One important aspect of this treatment is antibiotic therapy directed against *Pseudomonas aeruginosa*. The persistent presence of this micro-organism has been shown to be an independent risk factor for more rapid decline in quality of life and pulmonary function [52,53]. Recently a study was performed to test inhalation of tobramycin in patients with non-CF bronchiectasis chronically colonized with *P. aeruginosa* with an acute exacerbation. Oral ciprofloxacin was added for 14 days. This led to a greater reduction in microbial load at day 14 but no significant clinical effect was found [54].

Inhalation of aminoglycoside antibiotics has several potential advantages over intravenous administration. High intravenous dosing of these agents is usually not well tolerated, with considerable renal and audio-vestibular toxicity. Inhaling these drugs may provide high concentration of the drug at the site of infection circumventing the systemic toxicity.

The first generation of aminoglycoside-inhaled therapy was done using a wet nebulizer. In recent years, several devices containing dry powder antibiotics have become available, for example the Podhaler with tobramycin [55–57]. Inhalation of dry powder antibiotics is less

time consuming and easier for patients that prefer a small device to carry on. Indeed low systemic exposure was confirmed in CF patients with the Podhaler [58].

The Cyclops, a device specifically designed for inhalation of dry powder aminoglycosides [59], was tested in patients with non-CF bronchiectasis with excellent tolerability.

Pharmacokinetic results in the population of non-CF BE gained from this study should be compared with results gained from future studies in healthy volunteers and patients with CF-BE. This could help us to understand the mechanism of systemic absorption after inhalation of tobramycin, as there is still a lack of knowledge of what happens after inhalation; no detailed information is available on where particles are deposited, and neither do we know what happens next [60]. Such studies could evaluate and improve our described model as well. Also future studies should evaluate microbiological and clinical outcomes of patients with non-CF BE using the Cyclops compared to the Podhaler, or other devices used for inhaled aminoglycosides.

As the Cyclops was developed for inhalation of dry powder aminoglycosides, kanamycin can be used as well. Future studies should explore the question whether inhalation of kanamycin using the Cyclops – or any other dry powder inhalation device – could possibly replace the injectable therapy of kanamycin. The latter has its inherent problems of injected therapy including long hospital admissions and / or risks incurred by intravenous access, including catheter-related thrombosis and infection.

This thesis might be the start of exploring inhalation therapy of anti TB drugs in dry powder formulation [61]. Perhaps, a reduction of toxicity might be achieved by inhalation therapy. One other theoretical or potential advantage could be that by providing high concentrations into the airways, the time that patients remain infectious might be reduced, thus providing a public health advantage.

Optimization of our mathematical inhalation model can be useful in predicting serum levels at different time-points using different devices for dry powder inhalation.

Concluding remarks

In the Introduction of this Thesis, we started a short history of the long journey of Mtb and humankind. Still, our knowledge about the epidemiology and transmission of TB, and its treatment should be enhanced to effectively fight TB. Not only by working together with

clinicians or molecular biologists but also with molecular epidemiologists, geneticists, immunologists, and people working in the field of inhalation technology, insights can be obtained to improve the fight against TB, and its most threatening variants: MDR- and XDR-TB. Some battles have been won, but winning the war requires a huge consorted effort.

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Samenvatting

Tuberculose

Epidemiologie

Tuberculose is een infectieziekte die de mens al duizenden jaren achtervolgt. Tuberculose wordt veroorzaakt door de tuberkelbacillen, waarvan de officiële benaming *Mycobacterium tuberculosis* is. Wereldwijd ontwikkelen ruim 8 miljoen mensen actieve tuberculose per jaar, terwijl ongeveer een derde van de wereldbevolking de tuberkelbacil met zich meedraagt zonder er ziek van te zijn. Deze mensen hebben een latente tuberculose-infectie. Dit komt er in het kort op neer dat deze mensen besmet zijn met de tuberkelbacil en dat hun afweersysteem de aanwezigheid van de tuberkelbacil in het lichaam herkent bij aanvullend onderzoek. Slechts een kleine minderheid van deze besmette mensen zullen ooit ziek worden en actieve tuberculose krijgen. In 2014 zijn er wereldwijd zo'n 1,3 miljoen mensen overleden aan tuberculose. Degenen die genezen hebben regelmatig een verminderde kwaliteit van leven en soms ook een verminderde levensverwachting door ernstige restschade die is ontstaan door de tuberculose, sequelae genaamd. Helaas weten we nog erg weinig over hoe vaak dit nu precies voorkomt. Omdat tuberculose een gevaar voor de volksgezondheid is, is het in Nederland een aangifteplichtige infectieziekte. Dat wil zeggen dat een patiënt bij de GGD gemeld moet worden. Hierdoor weten we precies hoe vaak tuberculose voorkomt. In Nederland daalt het aantal nieuwe tuberculosegevallen gestaag; in 2014 waren er nog maar 823 nieuwe patiënten.

In de buitenlucht kan de tuberkelbacil hooguit maar een paar uur overleven; de bacterie is gevoelig voor uitdroging en (ultraviolet) licht. De tuberkelbacil overleeft goed bij de mens, dit maakt de mens tot het belangrijkste reservoir voor de tuberkelbacil. De menselijke populatie vormt dan ook de belangrijkste overlevingskans voor de tuberkelbacil en vanuit het menselijke reservoir vindt verspreiding plaats. Misschien is die relatie wederkerig; waarschijnlijk heeft een voorloper van de tuberkelbacil zich samen met de mens in verschillende golven vanuit Afrika over de hele wereld verspreid. Behalve door de tuberkelbacil zelf kunnen mensen ook tuberculose ontwikkelen door aan de tuberkelbacil sterk verwante bacteriën die we gezamenlijk ook wel het *Mycobacterium tuberculosis* complex noemen. De meeste bacillen uit het *Mycobacterium tuberculosis* complex hebben echter niet de mens als reservoir, maar een dier. Een voorbeeld hiervan is *Mycobacterium bovis* welke vooral bij runderen voorkomt. Indien een mens tuberculose ontwikkelt door een bacil uit het *Mycobacterium tuberculosis* complex dan kan die wel andere mensen hiermee besmetten.

Besmettelijkheid

Overdracht van tuberculose gaat bijna altijd via de lucht – door het verspreiden van kleine in de lucht zwevende druppeltjes, zogenaamde aërosolen, die de tuberkelbacil bevatten. Deze aërosolen ontstaan bijvoorbeeld door praten, lachen, hoesten, niezen of zingen. Na inademen van deze aërosolen zorgt het afweersysteem ervoor dat de tuberkelbacil wordt opgeruimd. Indien dit niet gebeurt kan direct actieve tuberculose ontstaan of er ontstaat een zogenaamde slapende of latente tuberculose. Ongeveer 10% van de mensen met latente tuberculose ontwikkelt ooit actieve tuberculose; de meeste mensen hebben van nature een goede afweer en krijgen geen tuberculose. Als de afweer van een persoon verminderd is, dan wordt het risico op het ontwikkelen van actieve tuberculose ineens veel groter – tot bijna 10% per jaar. Voorbeelden van ziekten die de afweer verminderen zijn suikerziekte (diabetes mellitus), maar vooral HIV-infectie. Bij sommige ziekten zoals de ziekte van Crohn en bij reuma (reumatoïde artritis) worden vaak afweer-onderdrukkende medicijnen ingezet, waardoor die personen ook een verhoogde vatbaarheid voor tuberculose hebben.

Voor het vaststellen van latente tuberculose zijn tegenwoordig drie immunologische testen beschikbaar. Deze immunologische testen bevatten deeltjes van de tuberkelbacil, zogenaamde antigene epitopen, die zorgen voor immuun-herkenning van het afweersysteem van de mens als er sprake is van een eerdere besmetting. Ten eerste is er de tuberculine huidtest, vaak gezet volgens de methode van Mantoux. Deze test is al meer dan 100 jaar oud. De antigenen in deze test zijn niet specifiek voor de bacillen van het *Mycobacterium tuberculosis* complex; ze komen ook voor bij aan de tuberkelbacil verwante bacteriën, de zogenaamde non-tuberculose mycobacteriën. Dit betekent dat een positieve uitslag niet altijd wijst op een besmetting met *Mycobacterium tuberculosis*. In het begin van deze eeuw zijn er ook twee bloedtesten ontwikkeld die latente tuberculose kunnen vaststellen. Deze testen bevatten antigenen die specifieker zijn voor het *Mycobacterium tuberculosis* complex, waardoor deze bloedtesten ook specifieker zijn in het opsporen van latente tuberculose ten opzichte van de huidtest. In dit proefschrift, hoofdstuk 3b, hebben we een studie gedaan of er overdracht is geweest tussen een patiënte met tuberculose veroorzaakt door *M. bovis* en anderen. We hebben dit voor het eerst gedaan met behulp van de resultaten van één van die twee bloedtesten, de quantiferontest, die geschikt is voor het ontdekken van latente tuberculose-infectie. Deze resultaten hebben we gebruikt naast de uitslagen van de tuberculine huidtest. De resultaten komen overeen met die van de testen bij humane tuberculose en wat we verwachtten op

basis van de eerdere genoemde aangrijpingspunten van deze testen. Dit betekent dat deze test in de toekomst ook gebruikt kan worden bij contactonderzoeken rondom patiënten met tuberculose veroorzaakt door *M. bovis*.

De tuberkelbacil reist niet alleen met de mens mee over de wereld maar kan ook binnen de mens reizen, zowel via de lymfebanen als via het bloed. Tuberculose kan zich dan ook bijna in het hele lichaam uiten. Meestal presenteert tuberculose zich in de longen, maar ook vaak in de lymfeklieren. Als de longen zijn aangedaan noemen we dit pulmonale tuberculose. Een infectie elders in het lichaam wordt extrapulmonale tuberculose genoemd. Indien er zowel een infectie in de longen is als elders in het lichaam dan spreken we van pulmonale en extrapulmonale tuberculose. De wervelkolom of het centraal zenuwstelsel zijn minder vaak aangedaan door tuberculose, echter bij deze vormen komen vaker ernstige sequelae voor.

Om verdere verspreiding van tuberculose onder controle te krijgen en te stoppen en tuberculose zelfs helemaal uit te roeien is het erg belangrijk om te weten hoe de verspreiding precies verloopt. Hiervoor is het belangrijk om zowel de route als het tijdspad van verspreiding te kennen. Kleine veranderingen in de basevolgorde in het DNA van *Mycobacterium tuberculosis* maken dat er kleine geleidelijke genetische veranderingen optreden in de loop van de tijd, gedurende de verspreiding. Genetisch variante organismen hebben de zelfde genetische vingerafdruk en behoren daarmee tot hetzelfde cluster; de technieken om vast te stellen of geïsoleerde bacteriën tot hetzelfde cluster behoren worden fingerprinting genoemd. De breed beschikbare en relatief goed betaalbare technieken zijn spoligotypering, *IS*6110 Restriction Fragment Length Polymorphism en Variable Number of Tandem Repeats (VNTR). Al deze technieken vermenigvuldigen bepaalde stukjes van de genetische code – het DNA – van de bacterie. Nauwkeuriger is het om niet een deel maar het gehele genoom van de bacterie in kaart te brengen om alle mogelijke overeenkomsten en verschillen te bestuderen. Met 'whole genome sequencing' kan men beter beoordelen of er kleine veranderingen in het genoom zijn opgetreden. Deze techniek is nog niet wijdverbreid beschikbaar en vooralsnog ook erg kostbaar. Op dit moment wordt de VNTR-typering het meest gebruikt voor het doen van moleculair-epidemiologische studies met *Mycobacterium tuberculosis* stammen. In hoofdstuk 2b hebben we onderzocht hoe we de VNTR-methode kunnen optimaliseren. In dit onderzoek hebben we de originele zelfgemaakte (in-house) methode aangepast en dit heeft geleid tot een verhoogde gevoeligheid van de VNTR. Dit resulteert in een verlaagde werkbelasting en zorgt er ook voor dat er minder tijd nodig is voor deze methode, die ook nog eens veel goedkoper is dan de commercieel beschikbare VNTR-methode. Deze aangepaste VNTR-methode hebben we ook getest op de gevoeligheid volgens de methode die we in

hoofdstuk 2a hebben beschreven; verderop komen we hierop terug. Deze VNTR-methode bleek nog een redelijke gevoeligheid te hebben waardoor het ook voor klinisch werkzame dokters een interessante methode is geworden. Doordat we sneller meer weten over de verspreiding van de tuberculose, weten we ook of het een gevoelige of resistente vorm van tuberculose kan zijn. En als bij de bron resistente tuberkelbacillen waren ontdekt kan de nieuwe patiënt direct beter behandeld worden op basis van de kennis over de gevoeligheid voor medicijnen bij de bronpatiënt.

Een andere fingerprinting techniek, namelijk spoligotypering, hebben we gebruikt om een uitbraak van tuberculose te beschrijven tussen mensapen en de mens in hoofdstuk 3a. Deze techniek hebben we gebruikt naast de gegevens die waren verzameld van de patiënt en de radiologiebeelden van de patiënten. De overdracht van tuberculose van dieren naar mensen wordt maar zelden beschreven en onderkend. Onze resultaten wijzen er op dat de mensapen mensen hebben besmet.

Fingerprinting hebben we ook gebruikt in het onderzoek rond de patiënte met tuberculose door *M. bovis* die we hier (en in hoofdstuk 3b) al eerder hebben beschreven. We hebben dit gebruikt om een mogelijke verklaring te vinden voor de besmetting met *M. bovis* die uiteindelijk tuberculose bij deze patiënte had veroorzaakt. De fingerprint liet een sterke overeenkomst met die van een andere patiënt zien; de medewerkers van de afdeling tuberculosebestrijding van de GGD hebben nagegaan of die twee patiënten ooit contact hadden gehad maar zij konden geen verbinding leggen tussen die twee patiënten.

Diagnostiek van tuberculose

Het diagnosticeren van tuberculose begint bij de klinische en radiologische verdenking hierop; het aantonen met een microbiologische test is nodig om het bewijs rond te krijgen. Een goed monster van het aangedane deel van het lichaam is nodig om de diagnose zo goed mogelijk te stellen. Meerdere microbiologische technieken zijn beschikbaar voor de diagnostiek naar *Mycobacterium tuberculosis*. Dit kan door gebruik te maken van een microscoop waarbij verschillende kleuringstechnieken worden gebruikt. De gouden standaard voor het aantonen van bacteriën was vanouds de kweekproef; specifieke (vaste) voedingsbodems werden gebruikt, waarbij remmende stoffen werden toegevoegd om sneller delende bacteriën en schimmels te onderdrukken. De remmende stoffen veroorzaken ook enige groeiremming van de tuberkelbacillen, die op zich al vrij langzaam delen. Kweekproeven, hoewel heel goed, zijn niet erg gevoelig, en vooral ook erg traag. Met de

invoering van vloeibare kweekmedia werd de kweekmethode veel sneller en gevoeliger, maar nog steeds moesten artsen weken wachten op de resultaten. In de jaren '80 van de vorige eeuw werd een moleculaire techniek, polymerase kettingreactie (polymerase chain reaction – PCR) genaamd, ontwikkeld. Deze test kwam begin jaren negentig beschikbaar voor de diagnostiek naar tuberculose. PCRs zijn gevoelig, specifiek en vooral heel snel: in enkele uren in plaats van weken kunnen testresultaten beschikbaar komen.

Bij deze kettingreactie wordt heel snel een kopie van de basepaarvolgorde van het in het monster aanwezige DNA van de bacterie gemaakt. Dit moet op een voor die bacterie specifiek DNA-deel aangrijpen en starten. De meeste PCRs voor tuberculose maken gebruik van het feit dat er in de meeste tuberkelbacteriën een stukje DNA-volgorde in vele kopieën voorkomt, op verschillende plaatsen in het DNA; die ingesloten DNA-eilandjes heten het insertiesegment 6110 (IS*6110*). Nu verschillen tuberkelbacillen onderling in het aantal van deze IS*6110*; er zijn zelfs ook tuberkelbacillen zonder dat insertiesegment. Tegenwoordig zijn er zowel commercieel beschikbare als in-house testen beschikbaar. De PCR-techniek is gevoeliger dan de microscopie. Het doel van het onderzoek naar de diverse PCR-testen in dit proefschrift, hoofdstuk 2a, was om de meest gevoelige test te vinden voor een situatie waarin weinig tuberkelbacillen aanwezig zijn in het te onderzoeken monster, zoals bij sommige vormen van tuberculose. We hebben in totaal 14 verschillende PCR-tests gebruikt, zowel de zelfgemaakte als verschillende commercieel verkrijgbare. Onze studie was de eerste die zoveel verschillende PCR-testen vergeleek. De gevoeligheid van de test hangt vooral ook af van de hoeveelheid bacteriën (bacterieload) in een monster. Wij hebben deze variabele bacterieload nagebootst door de monsters steeds verder te verdunnen, waarbij een hoge verdunning een lage bacterieload nabootst. Uit onze studie kwamen twee PCR-testen als meest gevoelige uit de proeven, te weten de AmpliSens MTC-FRT PCR kit en de in-house real-time PCR IS*6110* met10 µl DNA. Zeker omdat tuberculose weinig voorkomt in Nederland willen we de meest gevoelige test hebben om tuberculose aan te tonen. Door dit onderzoek kunnen de laboratoria in Nederland dit meenemen in hun beslissing om te kiezen voor een bepaalde PCR-test, en weten artsen die deze tests aanvragen beter hoe ze uitslagen moeten interpreteren.

Helaas zijn er nog geen technieken operationeel die levende van dode bacillen kunnen onderscheiden. Interpretatie van de uitslagen van de microscopie en de PCR kan dan ook alleen als ook de klinische en de radiologische gegevens van de patiënt hierbij worden meegenomen.

Verder dient er ook altijd gekweekt te worden. Als de kweek de bacterie heeft aangetoond, kan er nadien getest worden voor welke medicijnen tegen de tuberculose de gekweekte tuberkelbacil gevoelig is. Tegenwoordig zijn ook al PCR-testen of andere testen van DNA beschikbaar, waaronder de Xpert MTB/RIF, om te kijken of er sprake kan zijn van resistentie voor sommige antibiotica.

Behandeling van tuberculose

Normaal gevoelige tuberculose houdt in dat de tuberculose gevoelig is voor de beste anti-tuberculose medicijnen, de zogenaamde eerstelijns anti-tuberculose medicijnen. Indien tuberculose resistent is tegen de twee meest werkzame van deze medicijnen, namelijk isoniazide en rifampicine, dan spreken we van multidrug resistente tuberculose.

De behandeling van multidrug resistente tuberculose is veel lastiger en duurt ook veel langer en geeft ook vaak meer bijwerkingen. In hoofdstuk 4a beschrijven we een studie die we gedaan hebben bij een patiënte met multidrug resistente tuberculose. Bij deze patiënte dachten we dat de long zodanig aangetast was en hierdoor slecht doorbloed dat de medicatie dit deel van de longen niet kon bereiken en hierdoor lage concentraties van de medicijnen zou geven. We hebben de geneesmiddelconcentraties vastgesteld ter plaatse van de infectie in de long, waar de tuberculose zat, Verrassend genoeg waren de spiegels van de medicijnen in de bloedbaan vergelijkbaar met die in het aangedane deel waar veel weefselversterf ('verkazing') was opgetreden. De resultaten van dit onderzoek zijn een eerste stap om meer kennis te verkrijgen over de spiegels van tweedelijns anti-tuberculose medicijnen op de plaats van de ziektehaard.

In Nederland behandelen we patiënten met multidrug resistente tuberculose op basis van de farmacokinetiek en farmacodynamiek van de medicijnen en de tuberculosebacil. De farmacodynamiek is de wetenschap die bestudeert hoe het geneesmiddel aangrijpt op het ziekteproces, vrij vertaald wil dat bij tuberculose zeggen hoe gevoelig de bacterie is voor het betreffende medicijn. Het mycobacteriologisch laboratorium van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) is een door de Wereldgezondheidsorganisatie erkend referentielaboratorium; daar wordt voor patiënten in Nederland de gevoeligheid van de verschillende medicijnen voor de tuberkelbacil vastgesteld.

De farmacokinetiek is de wetenschappelijke bestudering van het lot van medicijnen in het lichaam; farmacokinetiek bestudeert wat het lichaam doet met het medicijn. Dit betreft

de opname van het middel vanuit het maagdarmkanaal, maar ook de verdeling in het lichaam via het bloed, de omzetting van medicijnen – vaak in de lever – en de uitscheiding uit het lichaam – meestal via de gal en de ontlasting of via de nieren, in de urine. De meest gebruikte methode voor de bestudering van de farmacokinetiek is het meten van de geneesmiddelconcentraties van de medicijnen in het bloed, op verschillende momenten na het innemen van die middelen. Op basis van deze gemeten concentraties – bloedspiegels – en de bepaling van de gevoeligheid voor het medicijn van de in het laboratorium gekweekte tuberkelbacil kunnen we de behandeling optimaliseren. Een te hoge concentratie van het medicijn ten opzichte van de gevoeligheid kan zorgen voor onnodige en vermijdbare bijwerkingen; en te lage concentraties ten opzichte van de gevoeligheid kunnen er voor zorgen dat het middel niet werkzaam is en dat er resistentievorming optreedt.

In hoofdstuk 4c van dit proefschrift hebben we commentaar gegeven op een onderzoek dat de plasmaconcentraties van isoniazide, rifampicine en pyrazinamide relateert aan de uitslagen van de sputumkweken na een behandelduur van 4 en 8 weken. In plaats van gebruik te maken van de medicijnconcentratie in het bloed 2 uur na inname kan beter gekozen worden voor het verzamelen van meerdere bloedmonsters om de farmacokinetiek van de verschillende medicijnen te bestuderen. Verder is ook de gevoeligheid van de tuberculosestam, de minimaal remmende concentratie (MIC), van belang om in combinatie met de farmacokinetiek – met name het oppervlak onder de concentratie-tijd curve (AUC) – te bepalen of de dosering afdoende is. De verhouding van die twee: de ratio van AUC/MIC, moet berekend worden om te bepalen of de medicatie goed gedoseerd is.

In hoofdstuk 4b van dit proefschrift hebben we commentaar gegeven op een studie bij patiënten met tuberculose van het centraal zenuwstelsel en dan met name van de hersenvliezen. Deze studie beschrijft de farmacokinetiek bij deze patiënten van hoge doseringen rifampicine en moxifloxacin in zowel het serum als in het hersenvocht. Net als die onderzoekers pleiten wij voor meer onderzoek omdat deze vorm van tuberculose een slechte overlevingskans heeft, maar ook veel restverschijnselen bij degenen die de ziekte overleven. Rifampicine dringt heel slecht, maar isoniazide juist heel goed, door in het hersenvocht. Wij vinden dan ook dat de bijdrage van isoniazide in de behandeling van tuberculeuze hersenvliesontsteking beter onderzocht moet worden. We beargumenteren dat de blootstelling aan diverse anti-tuberculosemedicijnen namelijk het best wordt onderzocht als continue variabele. Verder menen wij dat bij de onderzoeken naar patiënten met tuberculeuze hersenvliesontsteking alle beschikbare gegevens geanalyseerd moeten worden voor een bijdrage aan toekomstige onderzoeksrichtingen bij deze vorm van tuberculose.

Bronchiëctasieën

Bronchiëctasieën zijn lokale uitstulpingen of verwijdingen van de luchtwegen, waarvoor veel oorzaken zijn. Wereldwijd is dit heel vaak het gevolg van restverschijnselen of sequelae na genezing van pulmonale tuberculose. Bij jonge mensen in Nederland is de meest voorkomende oorzaak van bronchiëctasieën 'taaislijmziekte' of cystic fibrosis (CF).

Bronchiëctasieën worden gekenmerkt door recidiverende infecties van de luchtwegen door vele verschillende bacteriën. Een bepaalde bacterie, genaamd *Pseudomonas aeruginosa*, zorgt ervoor dat er vaker opvlammingen van ziekteactiviteit zijn van de bronchiëctasieën met hierdoor verdere toename van de bronchiëctasieën als ook verslechtering van de longfunctie en afname van de kwaliteit van leven.

Het voorkómen van deze exacerbaties gebeurt tegenwoordig door het inhaleren van antibiotica. Bij bronchiëctasieën door CF is de effectiviteit en veiligheid van zo'n behandeling al bewezen maar voor bronchiëctasieën door andere oorzaken – bijvoorbeeld, door tuberculose – is er nog geen voldoende wetenschappelijk bewijs. De overleving van patiënten met CF is sinds de jaren '80 van de vorige eeuw duidelijk toegenomen, en de gedachte is dat ook bij 'non-CF bronchiëctasieën' veel winst te behalen valt.

Inhalatie van antibiotica

Inhalatie van medicatie is een bekende manier van inname van medicatie – het wordt al meer dan 4000 jaar toegepast. Het inhaleren van een medicijn tegen tuberculose werd voor het eerst in 1950 beschreven, hoewel het toen als een niet succesvol experiment werd beschouwd.

Tegenwoordig is het inhaleren van antibiotica vooral gebruikelijk onder patiënten met CF. Toch worden ook patiënten met non-CF bronchiëctasieën tegenwoordig steeds vaker behandeld met inhalatie van antibiotica. Inhaleren van antibiotica kan door een vernevelaar waarbij de medicijnen in een waterige oplossing zijn gebracht; maar ook door het inhaleren van het medicijn in de vorm van een droogpoeder. Dit laatste wordt de laatste 10 jaar steeds meer onderzocht en er zijn nu ook droogpoeder antibiotica op de markt. Voordelen van droogpoederinhalatie zijn de korter durende inhalatietijd, wellicht minder kans op besmetting met schimmels en bacteriën van het toedieningssysteem; en misschien ook wel een grotere efficiëntie, door betere afzetting van de actieve stof in de luchtwegen. Bijwerkingen kunnen lokaal zijn, zoals optreden van hoestklachten of verergering van bestaande hoestklachten; of een vieze smaak in de mond. Met het inademen – inhaleren

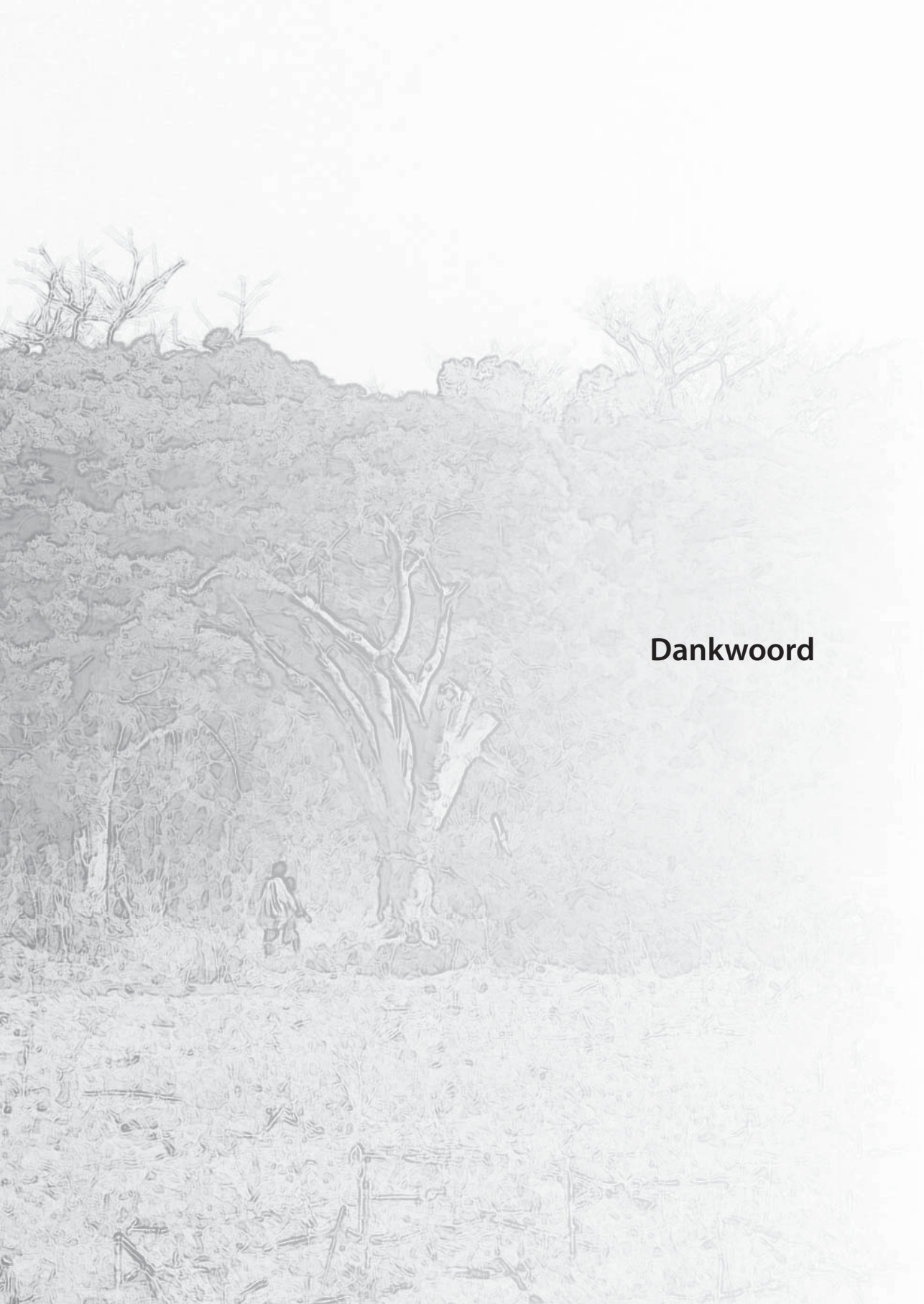
– van antibiotica kan het medicijn ook in het bloed komen. Doordat de medicijnen in het bloed komen kunnen de bijwerkingen van de antibiotica ook van algemene aard zijn of elders in het lichaam optreden. De bijwerkingen kunnen soms al bij lage dosering en lage bloedconcentraties ('lage blootstelling') optreden; soms zijn bijwerkingen afhankelijk van de dosering en zullen alleen bij hogere blootstelling optreden.

Aminoglycoside antibiotica worden niet in het bloed opgenomen als ze als tablet worden doorgeslikt: deze medicijnen geven we doorgaans als injectie, bijvoorbeeld via het infuus. Kanamycine en amikacine zijn aminoglycisides die bij geneesmiddel-resistente tuberculose worden ingezet. Tobramycine is een aminoglycoside dat veel wordt ingezet bij personen met bronchiëctasieën. Onze onderzoeksgroepen zijn geïnteresseerd in het idee om ooit kanamycine per inhalatie in plaats van per infuus te gaan testen. We weten nu nog niet of dat gaat lukken want we willen dan graag dat het middel goed wordt opgenomen in het bloed, via de kleine luchtwegen en de longblaasjes.

Bij bronchiëctasieën zijn we juist geïnteresseerd in de vraag of we de antibiotica in de ontstoken luchtwegen kunnen laten komen, in de haard van de infectie, zonder te grote opname in het bloed. Zoals eerder gezegd kunnen juist deze bloedconcentraties bijwerkingen van het medicijn geven. Het kan dus heel belangrijk zijn om voorafgaande aan nieuwe studies te kunnen voorspellen wat de concentraties zijn in het bloed van verschillende doseringen antibiotica. In het algemeen zijn de voor- en nadelen echter lastig te voorspellen. Het lijkt er wel op dat mensen (patiënten, proefpersonen) een vast inhalatiepatroon aanleren waardoor de voor- en nadelen voor die persoon redelijk voorspelbaar zijn. In hoofdstuk 5a van dit proefschrift hebben we een model gemaakt zodat we bloedspiegels van medicijnen kunnen voorspellen na inhalatie van een bepaald medicijn. In dit model beschrijven we de verschillende stappen na inhalatie van een antibioticum. Het model maakt gebruik van de kennis van het inhalatieapparaat en van eigenschappen van het gebruikte antibioticum. Hiervan zou bij nieuwe onderzoeken gebruik gemaakt kunnen worden doordat we nu weten bij welke dosering de spiegels te hoog worden.

Bij tobramycine (en andere 'aminoglycosides') weten we dat algemene bijwerkingen (nierfunctiestoornis, schade aan het gehoor en evenwichtsorgaan) vooral samenhangen met de hoogte van de blootstelling. In hoofdstuk 5b van dit proefschrift hebben we onderzoek gedaan naar de inhalatie van tobramycine in droogpoedervorm met een nieuw inhalatieapparaat, genaamd de Cyclops. We hebben door acht proefpersonen, allen patiënten met non-CF bronchiëctasieën, tobramycine laten inhaleren via de Cyclops. We hebben in totaal vier

verschillende doseringen onderzocht. Hierbij hebben we gekeken of ze dit goed konden verdragen en verder hebben we ook bloed afgenomen op verschillende tijdstippen zodat we bloedspiegels konden meten van de tobramycine. Het blijkt dat de tobramycine ook in de hoogste dosering goed wordt verdragen. Onze proefpersonen hadden opvallend weinig klachten van hoesten en kortademigheid na het inhaleren van tobramycine. De bloedspiegels die wij maten hebben we vergeleken met die concentraties die gevonden worden bij het apparaat dat nu wordt gebruikt voor inhalatie van tobramycine, de zogenaamde Tobi Podhaler. Ook deze waren lager ten opzichte van de onderzoeken van de Tobi Podhaler, ook al waren die onderzoeken uitgevoerd bij gezonde vrijwilligers en patiënten met CF. Samenvattend denken we dat de Cyclops een apparaat is dat verder getest moet worden bij grotere groepen patiënten met non-CF bronchiëctasieën, en daarbij vergeleken moet worden op belangrijke uitkomstmaten voor patiënten: effectiviteit, veiligheid, gebruiksgemak en bijwerkingen.



Dankwoord

Het doen van onderzoek kan niet zonder hulp van anderen. Mijn proefschrift was dan ook niet tot stand gekomen zonder hulp van al die anderen, waarvan ik helaas velen tekort doe als ik er toch enkele noem.

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Mijn co-promotor dr. A.G.M. van der Zanden, beste Adri, wat is jouw hulp en enthousiasme belangrijk geweest voor mijn promotieonderzoek. De avonden waarop we via facetime met elkaar gesproken hebben over de interpretatie van resultaten leidde tot nieuwe ideeën en hebben mij als clinicus zeker anders naar uitslagen leren kijken. Jouw adagium dat een clinicus ook kennis moet hebben van, en moet kunnen meepraten over onderwerpen vanuit het laboratorium neem ik nog dagelijks ter harte.

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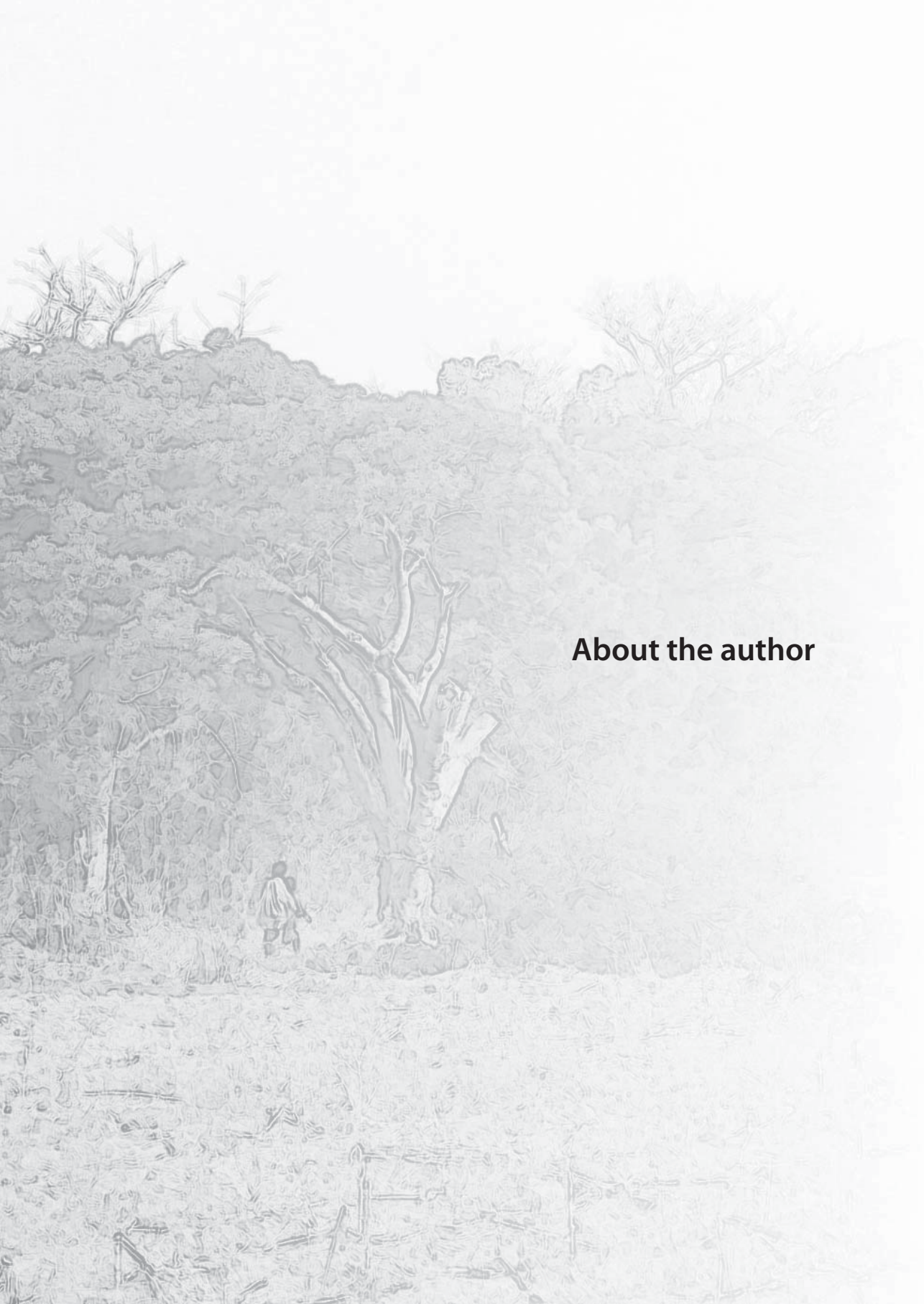

About the author

Curriculum vitae

Onno Willem Akkerman was born in Sneek, the Netherlands. In 1995, he graduated from the rijksscholengemeenschap Magister Alvinus (VWO) and started to study Medicine at the Erasmus University Rotterdam.

From 1999 until 2000, before his internships, he spent valuable time in hospital Manoel da Silva Almeida, Recife, Brasil.

In 2002, he graduated as a medical docter. After graduation, he started working at the Zuiderziekenhuis (Rotterdam, the Netherlands). From 2003 till 2004, he worked at Saint Francis Hospital (Katete, Zambia). In December 2004, he started his specialization to become a chest physician at Medical Spectrum Twente (Enschede, the Netherlands), under supervision of Dr Jaap Klein, Dr Hugo Schouwink and Dr Paul van der Valk. In December 2010, he was registered as a chest physician and he started working at the Röpcke Zweers ziekenhuis until 2011.

Since 2011, Onno has worked as a chest physician at the department of Pulmonary Diseases and Tuberculosis of the UMCG where he is responsible for the medical care of the Tuberculosis Center Beatrixoord (Haren, the Netherlands); in January 2015 he joined the Cystic Fibrosis team for adults in the UMCG.

Onno lives with his wife Anne Akkerman-Nijland and their cat, Tommie (2003). He enjoys travelling and swimming.

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