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## Non-Tuberculous Mycobacterial infections of veterinary relevance

Franck Biet, Maria-Laura Boschioli

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# European Symposium on Non-tuberculous Mycobacteria

24th - 27th June 2015

Manor House, Research Center Borstel



## Keynote Speakers

Michel Arthur (Paris, France) · Kenneth Bruce (London, UK) · Andres Floto (Cambridge, UK)  
Jean-Louis Herrmann (Paris, France) · Jakko van Ingen (Nijmegen, The Netherlands)  
Laurent Kremer (Montpellier, France) · Kenneth Olivier (Bethesda, MD, USA)  
Diane Ordway (Fort Collins, CO, USA) · Richard Wallace (Tyler, TX, USA)

## Scientific Advisory Board

Andres Floto (Cambridge, UK) · Jean-Louis Herrmann (Paris, France)  
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Ulrich Schaible (Borstel, Germany) · Dirk Wagner (Freiburg, Germany)

## Local Organizing Committee:

Christian Herzmann · Sven Müller-Loennies · Jutta Passarger · Bianca Schneider  
Nasstaja Wassilew · Otto Holst (Chair)



# European Symposium on Non-Tuberculous Mycobacteria



June 24<sup>th</sup> - 27<sup>th</sup> 2015

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## Financially Supported By



# Program

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## June 24<sup>th</sup>, 2015

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16:00 – 18:00 Registration

18:00 – 21:30 Get together

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## June 25<sup>th</sup>, 2015

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08:50 **Welcome Address**

*Chair* **Patrick Brennan, Fort Collins, USA**

09:00 – 09:45 **Kenneth Olivier, Bethesda, MD, USA**

Evolving Geographic and Ecologic Patterns of NTM

09:45 – 10:05 **Torsten Eckstein, Fort Collins, CO, USA**

Phylogenetic characterization of the five subspecies of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*

10:05 – 10:25 **Walter Oelemann, Rio de Janeiro, Brazil**

Comparison of lipid profiles from *Mycobacterium paratuberculosis* (MAP) isolated from the infected host and after subculture *in vitro*

**10:25 – 10:55 Coffee Break**

*Chair* **Dirk Wagner, Freiburg i. Br., Germany**

10:55 – 11:40 **Diane Ordway, Fort Collins, CO, USA**

Understanding non-tuberculous mycobacteria: pathogenesis and treatment

11:40 – 12:00 **Dariimaa Ganbat, Borstel, Germany**

Functional studies of the initial phase of mycobacterial infection in human lung tissues

12:00 – 12:20 **Andrea Sanchini, Berlin, Germany**

*Mycobacterium avium* subsp. *hominissuis* isolated in Germany: characterization of a new genomic island

**12:30 – 14:00 Lunch**

*Chair*            **Christoph Lange, Borstel, Germany**

14:00 – 14:45 **Richard Wallace, Tyler, TX, USA**

*Mycobacterium avium* lung disease and the modern day broad street pump: a tale of two cities

14:45 – 15:05 **Franck Biet, Tours, France**

Feature of adhesions expressed in *Mycobacterium intracellulare*

15:05 – 15:25 **Fabienne Misguich, Montigny-le-Bretooneux, France**

Type VII secretion pathway involved in *Mycobacterium abscessus* intracellular survival

**15:25 – 15:55 Coffee**

*Chair*            **Ulrich Schaible, Borstel, Germany**

16:00 – 16:45 **Laurent Kremer, Montpellier, France**

Deciphering and imaging the physiopathological events of *Mycobacterium abscessus* infection in zebrafish embryos

16:45 – 17:30 **Jean-Louis Herrmann,**

*Mycobacterium abscessus*: a peculiar behavior for a rapid growing mycobacterium inside cells

**18:00 Dinner**

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## June 26<sup>th</sup>, 2015

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*Chair*            **Faustine Dubar, Lille, France**

09:00 – 09:45 **Jakko van Ingen, Nijmegen, Netherlands**

Assessing drug susceptibility of non-tuberculous mycobacteria in the clinical and pre-clinical setting

09:45 – 10:05 **Sanne Zweijpfenning, Nijmegen, The Netherlands**

Characteristics and treatment outcomes of non-tuberculous mycobacterial pulmonary disease in mycobacterial disease reference clinic

10:05 – 10:25 **Caroline Demangel, Paris, France**

Systemic immune suppression imposed by mycolactone: implications for the treatment of Buruli ulcer disease

**10:25 – 10:55 Coffee Break**

*Chair*            ***Delphi Chatterjee, Fort Collins, USA***

10:55 – 11:40 **Michel Arthur, Paris, France**

Mycobacteria and betalactams: mode of action and therapeutic perspectives

11:40 – 12:00 **Heike Köhler, Jena, Germany**

Comparative analysis of systemic and local host responses against MAP and MAH in an experimental goat model

12:00 – 12:20 **Kris Huygen, Brussels, Belgium**

Analysis of the virulence and immunogenicity of porcine and human *M. avium* ssp. hominissuis isolates in an experimental mouse model

**12:30 – 14:00 Lunch**

*Chair*            ***Brendan Loftus, Dublin, Ireland***

14:00 – 14:45 **Andres Floto, Cambridge, UK**

Using whole genome sequencing to uncover the biology of NTM infection

14:45 – 15:30 **Kenneth Bruce, London, UK**

Cystic fibrosis airways microbiota – from ecology to treatment

15:30 – 18:00 **Coffee and Poster**

**18:00 Dinner**

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**June 27<sup>th</sup>, 2015**

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**Departure**

# Abstracts

**Keynotes and Oral Presentations**



## **Evolving geographic and gologic patterns of NTM**

Kenneth N Olivier, MD, MPH

National Heart, Lung, and Blood Institute/NIH  
Bethesda, MD, USA

Reports from around the world note a similar awareness and often increasing prevalence of nontuberculous mycobacteria (NTM), particularly in association with lung disease. Population based studies have been limited by lack of reporting requirements for NTM, overlap of signs and symptoms of disease with other causes especially in TB endemic areas, the need for appropriate radiologic and culture based methods for detection, and a lack of access to complete information for proper disease characterization. In studies that have looked at serial changes in prevalence, there appear to be a consistent increase across disparate geographical areas. Quite a bit of variability has been noted with regard to relative prevalence of individual species of NTM. In general, many studies note a predominance of species within the *M. avium* complex, with the rapid grower mycobacteria such as *M. abscessus* being next most common followed by *M. xenopi*.

Historically, NTM have been associated with neoplasms and structural lung disease such as bronchiectasis and chronic obstructive pulmonary disease. Recent case control studies have supported these associations as well as factors such as thoracic skeletal abnormalities and low body mass index likely reflective of the “Lady Windermere” population of predominantly women without identified predisposing disease risks. The use of immunomodulatory drugs including steroids or autoimmune conditions such as rheumatoid arthritis have also been associated with NTM pulmonary disease. Regional differences in host characteristics have been described with nonsmoking women with idiopathic bronchiectasis predominating in the US and smoking men with COPD/emphysema more common in Europe.

In addition to host factor associations, there have been a number of recent reports of potential geographic and environmental risks with marked regional variations in prevalence for countries like the US, Japan, and Australia which may relate to differences in atmospheric, soil, or water conditions.

In summary, recent reports indicate an increasing prevalence of NTM pulmonary disease. It is likely a combination of host characteristics and environmental factors which influence disease occurrence. Marked regional variation is noted with regarding to both host characteristics and predominant species of NTM associated with disease.

# Phylogenetic characterization of the five subspecies of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*

Torsten M. Eckstein and Julia M. Inamine

Colorado State University, Department of Microbiology, Immunology, and Pathology, Fort Collins, CO 80523, USA

For a long time the *M. avium* complex consisted of three bacterial species: *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. All three mycobacterial species are known for their highly immunogenic surface glycopeptidolipids generating 28 different serovars for *M. avium/intracellulare* and one for *M. scrofulaceum*. Twenty-five years ago, *M. paratuberculosis* and *M. silvaticum* were reassigned as subspecies of *M. avium* resulting in three subspecies: subsp. *avium*, subsp. *paratuberculosis*, and subsp. *silvaticum*. Recently, it was demonstrated that the subsp. *avium* should be divided into two subspecies: one for bird isolates (subsp. *avium*) and one for non-bird isolates (subsp. *hominissuis*).

With the large amount of variation within this complex and new species identified that might be closely related to the *M. avium* complex it is necessary to assign the various serovars and subspecies to its correct bacterial species. Here we present our 16S rRNA gene sequence analyses to assign the various serovars to its original bacterial species and demonstrating that all subspecies recently designed belong to *M. avium*. We could show that all subspecies do far declared are subspecies of *M. avium*. We also could demonstrate that there are clusters for *M. avium*, *M. intracellulare*, and *M. scrofulaceum*. In addition, we identified another cluster outside the three clusters. Serovars 1 – 6, 9 – 11, and 21 are *M. avium*. Serovars 12 – 17, 19, 20, and 25 are *M. intracellulare*. Serovar 7 is split between *M. intracellulare* (2 strains) and *M. scrofulaceum* (2 strains). Serovar 8 can be *M. avium* or *M. intracellulare*. Serovar 18 was found in the cluster of *M. intracellulare* and in the cluster of *M. scrofulaceum*. Serovar 22 was seen within the *M. intracellulare* cluster and in the outside cluster. Further studies on the actual carbohydrate composition and reassurance of the serovar determination of the serovars with split assignments are needed.

## Comparison of lipid profiles from *Mycobacterium paratuberculosis* (MAP) isolated from the infected host and after subculture *in vitro*

Walter M. R. Oelemann<sup>1</sup>, Matthias Stehr<sup>2</sup>, Regina Engel<sup>3</sup>, Katarzyna A. Duda<sup>3</sup>  
and Ralph Goethe<sup>2</sup>

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<sup>2</sup>Department of Infectious Diseases, Institute for Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany

<sup>3</sup>Division of Structural Biochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany

*Mycobacterium avium* ssp. *paratuberculosis* (MAP) is the etiological agent of Johne's disease or paratuberculosis. MAP is able to infect monogastric animal species and humans. Similar to other pathogenic mycobacteria, the highly complex lipid repertoire of the cell wall contributes to host-pathogen interaction and bacterial survival in the host. In the host, mycobacteria modify significantly their lipid metabolism, and during the course of the resulting long-term infection, mycobacterial lipids seem to constantly influence the immune response of the host. Some reports suggest that the lipid pattern changes from more polar molecules in the beginning of an infection to more apolar compounds during later stages. In the present work we compare the lipid profiles of MAP isolated by sucrose gradient centrifugation from the intestinal mucosa of an infected cow and the same isolate harvested after *in vitro* surface culture on Middlebrook medium supplemented with mycobactin. Both bacterial preparations were lyophilized and non-covalently bound lipids were extracted by thorough stirring in successive mixtures of chloroform/methanol in the proportions of 2:1, 1:1 and 1:2. The resulting crude lipids were fractionated on silica gel columns using a polarity gradient for elution. Lipid fractions were analyzed by one-dimensional and two-dimensional thin layer chromatography (TLC), using solvent systems which are suited for the different mycobacterial lipid classes (modified from Pirson et al. *Vet. Res.* **43**:54 (2012)). These experiments indicate quantitative and qualitative differences in the lipid repertoire of MAP recovered from the gut and from the respective *in vitro* culture. The differences are predominantly observed in the fractions containing apolar lipids, as well as the polar fractions that contain phosphatidylinositol mannosides (PIMs). The PIM-containing fraction of MAP isolated from the gut appeared more complex than that of the corresponding isolate cultured *in vitro*. In contrast, MAP from culture appeared to synthesize more apolar lipids. Presently, the fractions of interest are being separated/purified utilizing HPLC with reverse phase column. The obtained fractions will be chemically characterized by GC/MS and ESI MS.

## Understanding non-tuberculosis mycobacteria: pathogenesis and treatment

Diane Ordway<sup>1</sup>, Kimberly Arnett<sup>1</sup>, Deepshikha Verma<sup>1</sup>, Megan Blackledge<sup>2</sup>, Christian Melander<sup>2</sup>, Anne Lenaerts<sup>1</sup>

<sup>1</sup>Colorado State University, D.Ordway@colostate.edu

<sup>2</sup>North Carolina State University

Over the past decade there has been a significant increase in cases of drug resistance associated with nontuberculous mycobacterial (NTM) species. Little is known about the nature of the pathogenesis of NTM infections and how these organisms overcome host immunity and persist *in vivo*. This explains the current lack of development of more efficacious and novel targeted therapies. The cure rate for chronic lung and disseminated infections due to NTM is dismal despite prolonged treatment with various multi-drug regimens. *Mycobacterium abscessus* outcomes are much worse than other rapidly growing mycobacteria (RGM), in fact this organism seems to be essentially untreatable, with only ~50% sputum conversion seen in multiple chemotherapy studies. Incorrect drug susceptibility predictions—combined with minimal pharmacokinetics and pharmacodynamics information for current treatment regimens—has led to these pathogens being associated with alarming high fatality rates. Our preliminary results with animal models of NTM infection support the hypothesis that these NTMs are capable of using different routes of infection resulting in biofilm formation and phenotypic antimicrobial resistance. Our studies provide strong evidence that through inhibition of biofilm formation with small molecule inhibitors during *in vitro/in vivo* infection with *M. abscessus* increased bactericidal activity is present when combined with the standard anti-NTM treatment. Moreover, additional approaches using adjuvant inhibitors combined with immunomodulatory agents show promise towards NTM prevention.

## Functional studies on the initial phase of mycobacterial infection in human lung tissues

Dariimaa Ganbat<sup>1</sup>, Elvira Richter<sup>3</sup>, Sebastian Marwitz<sup>2</sup>, Ekkehard Vollmer<sup>2</sup>, Christian Kugler<sup>4</sup>,  
Torsten Goldmann<sup>2</sup>

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<sup>2</sup>Clinical and Experimental Pathology, Research Center Borstel, Borstel, Germany

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<sup>4</sup>Hospital Großhansdorf, Thoracic surgery, Germany

Mycobacterial infections are still significant causes of morbidity and mortality. There are still surprising gaps in our knowledge about these infectious diseases because of limitations of available model systems. Particularly the early phase of mycobacterial infection remains unclear, whereas the late and active stages have been characterized by histological and clinical investigations. There are several lines of epidemiological evidences supporting that innate immunity might prevent or even eliminate mycobacterial infection. This attractive hypothesis and the limitations of human complex models for mycobacterial infection, the primary goal of the study was to establish a novel *ex vivo* STST (**Short-Term Stimulation of Tissue**) model for the initial phase of mycobacterial infection also allowing further functional studies.

From surgical material of 65 patients, 0.5-1 cm<sup>3</sup> sizes of the human vital lung specimens were obtained and incubated with one of three different mycobacterial species (*M. tuberculosis*, *M. avium*, *M. abscessus*). The establishment of the *ex vivo* STST model was assessed by two main criteria: 1) viability of tissues, including histopathology, MTT and TUNEL assay; 2) infection of tissues, including histo and molecular pathological methods.

The morphologic comparisons between *ex vivo* incubated and non-incubated lung specimens did not show any noticeable differences. The MTT assay showed that the optical measurements of the *ex vivo* incubated samples ( $2.34 \pm 0.12$ ) intervened between positive ( $3.52 \pm 0.06$ ) and negative controls ( $0.22 \pm 0.01$ ). By a TUNEL assay we determined that  $92.85 \pm 6.99\%$  of cells in the *ex vivo* stimulated tissues were alive. Serial sections of a whole block verified that diffusion of infectious agents deep into tissues was sufficient. Genetic material of mycobacteria could be detected in the stimulated tissues. We observed that different cell types were infected, including macrophages, neutrophils, monocytes and pneumocytes-II. A total of 900 infected cells were counted and macrophages and monocytes predominated (76.58% and 3.03%) regardless of the mycobacterial strain ( $p=0.539$  and  $p=0.761$ ). Overall incidences of infected neutrophils was 7.46%, infection of pneumocytes-II exhibited 12.93%. Moreover, neutrophils were predominantly infected by *M. abscessus*, whereas infected pneumocytes-II were more commonly found after infection within *M. tuberculosis*. Infected cells also responded with cellular changes, including karyopyknosis, karyrrhexis, and karyolysis depending on the mycobacterial species. Transcriptome analysis showed that in sets of *M. abscessus* 840, *M. tuberculosis* 1018, *M. avium* 5944 genes were up-regulated upon stimulation compared to medium control.

Tissue viabilities were acceptable and infections of the tissues were successful. All collected patients' lung specimens were infected and showed that the initial host defense mechanism is actively working via phagocytosis without any additional stimulation, unlike *in vitro* experiments. The combination of short-term stimulated vital lung specimens and HOPE fixation technology allow the extraction of high quality mRNA and a genome-wide expression profile upon mycobacterial infection can be investigated on mRNA level.

## ***Mycobacterium avium* subsp. *hominissuis* isolated in Germany: characterization of a new Genomic island**

Andrea Sanchini<sup>1</sup>, Annesha Lahiri<sup>1</sup>, Torsten Semmler<sup>2</sup>, Inga Eichhorn<sup>2</sup>, Niyaz Ahmed<sup>3</sup>, Narender Kumar<sup>3</sup>, Kshitij Tandon<sup>3</sup>, Astrid Lewin<sup>1</sup>

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<sup>3</sup> Pathogen Biology Laboratory, Department of Biotechnology and Bioinformatics, University of Hyderabad, India

Incidences of infections caused by Non-tuberculous mycobacteria (NTM) are increasing worldwide. The specie *Mycobacterium avium* is a one of the most relevant among the group of NTM. Within the *M. avium* specie, the subspecies *M. avium* subsp. *hominissuis* (MAH) is an opportunistic human pathogen causing lymphadenitis, lung, skin and disseminated infections. It is also widespread in the environment, especially dust, soil and water. Genomic Islands (GI)s are mobile genetic elements that can be involved in pathogenesis, fitness, metabolism, or drug-resistance. Previous work identified a new GI in 4 MAH genomes available in GenBank. Here we identify such GI in environmental and clinical strains of MAH isolated in Germany and we characterize the GI gene composition.

A total of 50 MAH strains were analyzed. Genomic DNA has been extracted by Phenol/Chloroform extraction. Whole genome sequencing has been performed using Illumina MiSeq Desktop Sequencer. Reads were assembled using CLC Genomics Workbench in form of contigs. Contigs were annotated using RAST. The conserved flanking genes present on either sides of the GI were used for the identification of the GIs in the contigs. All genes of the GIs have been extracted using Geneious. The 28 most frequent genes of the GIs which have hypothetical annotation have been analyzed by I-Tasser, SWISS modeling and DALI server in order to putatively assign protein function.

In 18/50 strains (36%) a complete GI has been identified. In 2/50 strains (4%) no GI has been identified. In 30/50 strains (60%) the GI is present but it is not fully covered by the assembled sequences, suggesting sequence variability and the needs of further assembly methods. The most common GI is of 16.4 kb. The size of the GI (from 13,4 to 84,9 kb) and the gene composition varies across the strains. The GI of some strains carries a homolog of the *mmp10* gene (involved in other mycobacteria in lipids transport and cell wall biosynthesis). Computational biology of genes annotated as suggests that it may be involved in drug-resistance. Computational biology of genes with hypothetical protein annotation revealed that 8/17 (47%) proteins may have a function in transcription regulator. 7/17 (41%) proteins may have enzymatic activity 2/17 (12%) proteins may act as fibrinogen-binding protein.

The recently identified GI is present also in a German collection of MAH strains isolated from various sources. Such GI is a region of high diversity. The GI may carry genes potentially interesting for drug-resistance (e.g. *mmp10*). Generation of knock-out mutants, gene up/down regulation and functional studies will assess if the GI play a role in drug-resistance or host-pathogen interaction.

## ***Mycobacterium avium* lung disease and the modern day broad street pump: a tale of two cities**

Richard J. Wallace, Jr., Elena Iakhiaeva, Barbara A. Brown- Elliott, Julie V. Philley, Jeana L. Benwill, Rebecca R. Wilson, Sruthi Vasireddy, Joseph Falkinham III

Departments of Microbiology and Medicine, the University of Texas Health Center at Tyler, Tyler, TX, U.S.A.; Department of Biological Sciences, Virginia Tech, Blacksburg, VA, U.S.A.  
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Little is known of the long term consequences of high risk patients to exposure to municipal water supplies containing multiple genotypes of *Mycobacterium avium*.

We identified 17 patients with NTM lung disease from two adjacent small southern U.S. towns (designated A and B) followed with serial sputum AFB cultures for 4-10 years. Patients were older, non-smoking white females and 17/18 had nodular bronchiectatic MAC lung disease. Town A received its municipal water from a single purification plant processing surface water, while Town B received its water from an aquifer. MAC species were identified by sequencing and strain comparison by VNTR.

Approximately 400 sputum cultures (mean 45.3 per patient) were obtained from nine patients from Town A over a mean of 6 years. Of six sputum genotypes of *M. avium* identified, two were recovered from multiple patients (VNTR types 23 and 38). Multiple patients also had *M. yongonense*. 8/9 patients had *M. avium* type 38 over the mean 6 year period and 3/9 patients had all three genotypes at different times. Biofilms from household water systems in Town A at the end of the study period revealed the same three epidemic MAC species/genotypes. Town B was less than one mile from Town A but as noted had a different water supply. Approximately 220 sputum cultures (mean 27.6 per patient) were obtained from eight patients in Town B over a mean of 5 years follow up. Only 2/8 patients had *M. avium* identified in their sputum, and none of the six *M. avium* genotypes or *M. yongonense* found in Town A were found in the sputum or household water of patients in Town B. Equivalent numbers of patients in both towns were infected with *M. intracellulare*, *M. abscessus*, and *M. chimaera*, but none of these species were recovered from household water.

This study implicates municipal water systems as the major source of *M. avium* nodular lung disease, and that the predominant *M. avium* biofilm genotypes can be acquired over many years from the same water source (hence the historical reference to the contaminated Broad Street pump described by Edward Snow as a source of cholera in London in the 19<sup>th</sup> century). Thus patients with multiple genotypes of *M. avium* in their municipal water supply may in part explain the frequent recurrence of new genotypes of MAC in patients with nodular disease. This is the first study to evaluate longitudinal acquisition of MAC from individual communities and their relationship to household (municipal water systems).

## Feature of adhesins expressed in *Mycobacterium intracellulare*

Lefrancois<sup>1</sup>, L. H., M. Branger<sup>1</sup>, T. Cochard<sup>1</sup>, O. Peuchant<sup>2</sup>, D. Raze<sup>3</sup>, C. Locht<sup>3</sup>, P. Lanotte<sup>1</sup>, and F. Biet<sup>1</sup>

1 INRA-Centre de Tours, UR1282, Infectiologie et Santé Publique (ISP-311), F-37380 Nouzilly, France. 2 Laboratoire de Bactériologie, Hôpital Haut-Lévêque-CHU de Bordeaux, Avenue Magellan, F-33604 Pessac France., 3 Inserm U1019, Lille France, CNRS UMR 8204, Lille, France, Institut Pasteur de Lille. Center for Infection and Immunity of Lille Lille, France, University Lille Nord de France, Lille, France, 4 Service Bactériologie-Virologie, Hôpital Bretonneau-CHRU de Tours, 2 boulevard Tonnellé, F-37044 Tours cedex France

<sup>1</sup>Franck.Biet@tours.inra.fr

The *Mycobacterium avium* MAC complex includes two closely related species *Mycobacterium avium* and *Mycobacterium intracellulare* (Mi). These non-tuberculous mycobacteria are opportunistic pathogens in humans and responsible of severe diseases in a wide variety of animals. These bacteria are also isolated from various environmental sources. In humans Mi is the second cause of illness after the strains of *Mycobacterium tuberculosis* complex. The genomic data available for the reference strain ATCC 13950 have enabled the development of genotyping but few studies concern identification of factors involved in the pathogenicity of this mycobacteria.

The objective of this study was to identify, isolate and characterize the adhesins belonging to the family of HBHA and LBP from the reference strain and from a panel of clinical isolates from patients with different pathologies associated with Mi infection.

Based on the genomic sequence of Mi we investigated whether the genes of HBHA and LBP were present and conserved between the different strains of the panel. The expression of adhesin HBHA and LBP in Mi was investigated by Western blot from lysates of human isolates Mi. An approach using affinity chromatography on heparin Sepharose was used to isolate and purify native adhesins from Mi.

Our results allow for the first time the characterization of the expression of adhesins HBHA and LBP in Mi. This preliminary work opens numerous perspectives on the role of these molecules in human pathologies but also in diagnostic approaches of NTM infections in human.



## **Type VII secretion pathway involved in *Mycobacterium abscessus* intracellular survival**

Laencina Laura, Herrmann Jean-Louis, Misquich Fabienne

EA3647 EPIM Laboratory of Diagnostic and Physiopathology of Microbial Infection, U1173 INSERM Infection - Inflammation, UFR des Sciences de la Santé Simone Veil, Université de Versailles Saint-Quentin-en-Yvelines, Montigny-le-Bretonneux, France, fabienne.girardmisquich@uvsq.fr

The *Mycobacterium* genus brings together species ranging from harmless saprophytic organisms to major human pathogens. The well-known pathogenic species such as *Mycobacterium tuberculosis*, *Mycobacterium marinum* and *Mycobacterium ulcerans* belong to the subgroup of slowly growing mycobacteria (SGM). By contrast, rapidly growing mycobacteria (RGM) form a group of over 150 species essentially composed of non-pathogen saprophytic organisms. However, *Mycobacterium abscessus*, an RGM, was recently considered as an opportunistic human pathogen, responsible for a wide spectrum of infections, from pulmonary to skin and soft tissues infections. *M. abscessus* is considered, with *Mycobacterium avium*, as the major mycobacterial pathogen in patients with cystic fibrosis.

*Mycobacterium abscessus* has been shown to behave as an intracellular pathogen, able to survive to the bactericidal response of macrophages and fibroblasts in lungs and skin, survival usually not observed for RGM. Sequence analysis of *M. abscessus* genome showed that this bacterium is endowed with the metabolic pathways typically found in environmental micro-organisms with contact to soil, plants, and aquatic environments, where free-living amoeba are frequently present. Very little is known about the genetic requirements that *M. abscessus* has developed or acquired to survive in an intracellular environment. We aimed to find if *M. abscessus* possess genomics advantages to survive in antigen presenting cells such as macrophages or in environmental protozoa such as amoeba.

A transposon mutant library of a clinical strain of *M. abscessus* subspecies *massiliense* was screened by its ability to persist in amoeba and macrophages. Several mutants were identified to have a reduced ability to survive in those intracellular environments. Interestingly, twelve on 6,000 mutants screened appeared to be muted in genes homologs to the type VII secretion pathway of *M. tuberculosis* also present in *M. abscessus*. These twelve mutants were all in the ESX-4 locus. This evolutionary system of secretion could manipulate the host cell biology to make macrophages and amoeba attractive hosts for *M. abscessus*. We showed here for the first time, genes allowing *M. abscessus* to survive inside phagocytic cells present in the environment and in the human host.

# Deciphering and imaging the physiopathological events of *Mycobacterium abscessus* infection in zebrafish embryos

Laurent Kremer

Centre d'étude des Pathogènes pour la Biotechnologie et la santé (CPBS), CNRS FR3689, University of Montpellier, 1919 route de Mende, 34 293 Montpellier, France  
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The emerging pathogen *Mycobacterium abscessus* is a rapidly-growing mycobacterium causing severe lung infections, particularly in cystic fibrosis patients. The Smooth (S) morphotype expresses glycopeptidolipids (GPL) at the bacterial surface while the Rough (R) morphotype is characterized by the loss of surface GPL. Previous studies suggested that the R variant is involved in more severe clinical forms, associated with a hyper-proinflammatory response. However, the molecular mechanisms responsible for the virulence associated to the R variant remain elusive. The zebrafish embryo offers many advantages that motivated its use for a better understanding of mycobacterial infections.

We have developed a zebrafish model of infection to investigate and compare the pathogenesis of R and S variants. This model allows to describing and imaging the chronology of the infection by videomicroscopy. Both the antisense morpholino technology and zebrafish reporter lines were used to evaluate the contribution of the innate immune system. In contrast to the S variant, the R morphotype induced a more robust and lethal infection in embryos, characterized by the rapid development of bacterial foci within the Central Nervous System. The use of a *mpeg1:mCherry* transgenic zebrafish line, harbouring red fluorescent macrophages, demonstrated the presence of isolated or small aggregated bacilli within macrophages during early infection. Later stages were characterized by the presence of extracellularly-replicating mycobacteria, leading to rapid tissue damage (abscess) and to larval death. The high propensity of the R variant to form cords *in vivo* prevented the bacilli from being phagocytosed by macrophages or granulocytes, representing a mechanism of immune evasion. We also demonstrated the critical role of TNF signaling in controlling the infection of S and R variants. TNF receptor morphants were characterized by increased macrophage death, promoting uncontrolled extracellular growth of mycobacteria, and resulting in hyper-cording of the R variant and enhanced larval death.

In addition, we propose to use this model to monitor, at spatiotemporal level, the *in vivo* therapeutic effects of molecules active against *M. abscessus*, notorious for being one of the most antibiotic-resistant mycobacterial species.

# ***Mycobacterium abscessus*: a peculiar behavior for a rapid growing mycobacterium inside cells**

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*Mycobacterium abscessus* is considered as an emerging pathogen in cystic fibrosis patients, with recent studies showing its genomic and phenotypic heterogeneity. Our main objective was to understand the particular sensitivity of cystic fibrosis patients, and more specifically of the CFTR<sup>-/-</sup> macrophages and dendritic cells towards *M. abscessus*. We studied *M. abscessus* pathogenicity *in vivo* or *ex vivo* in mice or within macrophages and dendritic cells wild type, DF508, and *cftr*<sup>-/-</sup> respectively. By using *in vivo/ex vivo* animal and cellular models already developed in our laboratory, we were able to show the capacity of *M. abscessus* to resist to the intracellular bactericidal activity of macrophages, independently of the presence of a CFTR defect.

# Assessing drug susceptibility of non-tuberculous mycobacteria in the clinical and pre-clinical setting

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Treatment of non-tuberculous mycobacterial (NTM) disease has developed along 2 lines; first, for slow growers such as *Mycobacterium avium*, drug regimens for tuberculosis have been modified to exclude pyrazinamide and include macrolides after their in vitro activity was shown. Yet, the use of rifampicin, ethambutol and streptomycin cannot be supported on basis on in vitro activity since these drugs have very little activity in vitro, both alone and in combinations. For the rapid growers, treatment regimens have been based on the species-specific in vitro susceptibilities. Yet, for some key drugs, such as aminoglycosides, the breakpoints to define susceptibility have largely been based on wild type minimum inhibitory concentration (MIC) distributions, even if these are concentrations impossible to achieve in serum or at the site of infection owing to toxicity. The recent insights in pharmacokinetics and pharmacodynamics of antimycobacterial drugs need to be used to redesign treatment regimens. The poor outcomes of currently recommended treatment regimens are testimony to the fact that these regimens need to be rebuilt from ground level.

In this presentation, correlations between in vitro susceptibility and in vivo treatment outcomes of major NTM diseases in trials and case series will be discussed. Then, an up-to-date overview of results from recent time-kill kinetic assays performed for aminoglycosides, macrolides, linezolid, moxifloxacin and clofazimine against *M. abscessus* subsp *abscessus*, *M. fortuitum*, *M. avium* subsp *hominissuis*, *M. xenopi* and *M. simiae* will be presented. Also, the first results of hollow fiber model experiments to determine the optimal dose and dosing strategy for amikacin against *M. abscessus* subsp *abscessus* will be presented.

# Characteristics and treatment outcomes of nontuberculous mycobacterial pulmonary disease in a mycobacterial disease reference clinic

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The incidence of nontuberculous mycobacterial pulmonary disease (NTM-PD) is increasing in the last few years in the Netherlands. Evidence concerning treatment is poor. In this study we give an overview of clinical aspects, treatment and outcome of all patients with suspicion of clinical relevant NTM-PD referred to the center of expertise for mycobacterial infections (University lung center Dekkerswald, Nijmegen, The Netherlands).

We conducted a retrospective study of all patients seen between 2008 and 2013 with at least one NTM isolate and diagnosed using the American Thoracic Society (ATS) diagnostic criteria. Patients with non classical radiographic features that were not described in the ATS-criteria were included as well.

334 patients were reviewed. Sixty-four patients with NTM-PD were included. Half of them were female and mean age was 64,2±12,3 years. *M. avium* complex (MAC) was the predominant species (n=38, 59,3%); *M. malmoense* and *M. kansasii* were seen in 7 and 6 patients. *M. abscessus* was seen in 4 patients, *M. chimaera* in 1 patient, *M. simiae* in 3 patients, *M. xenopi* in 2 patients and 3 patients had combined infections. The most predominant radiologic feature were nodules and consolidations (60,9%). Most patients had underlying COPD (73,4%), bronchiectasis was present in 54,7%, 40,6% smoked at time of diagnoses and 32,8% had a history of smoking. 87,8% of patients treated for MAC-PD were given a rifamycin-ethambutol-macrolide regimen. Five patients with MAC were not treated. From the patients who received rifamycin-ethambutol-macrolide regimen five patients additionally received clofazimine and 7 patients received amikacine and clofazimine. All six patients with pulmonary *M. kansasii* disease were treated with rifampicin and ethambutol; four also received isoniazid. The overall cure rate was 62,5%; 66% for MAC, 66,7% for *M. kansasii* and 57,1% for *M. malmoense*. All-cause mortality during treatment was 20,3%; ten patients experienced treatment failure (=15,6%), and 7 patients, 10,9% relapsed.

In our setting, MAC was the predominant NTM and treatment largely followed ATS guidelines. Treatment success rates are comparable with data from the literature. The poor outcomes and toxicity underline the need for clinical trials addressing NTM-PD treatment.

## **Systemic immune suppression imposed by mycolactone: implications for the treatment of Buruli ulcer disease**

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The release of mycolactone by *Mycobacterium ulcerans* in host skin causes a combination of ulcerative, analgesic and anti-inflammatory effects. While ulcer formation is caused by the pro-apoptotic activity of mycolactone on skin cells *via* hyper-activation of Wiskott-Aldrich Syndrome proteins, analgesia results from neuronal hyper-polarization *via* signaling through angiotensin II type 2 receptors. Mycolactone also blunts the capacity of immune cells to produce inflammatory mediators, by an independent mechanism of protein synthesis blockade. In an attempt to isolate the structural determinants of mycolactone's immunosuppressive activity, we screened a library of synthetic subunits of mycolactone for inhibition of cytokine production by activated T cells. The minimal structure retaining activity was a truncated version of mycolactone (hereafter called mini-mycolactone), missing one of its two polyketide chains. This compound inhibited the inflammatory cytokine responses of human primary cells at non-cytotoxic doses, and bound to angiotensin II type 2 receptors comparably to mycolactone *in vitro*. Intraperitoneally-delivered mini-mycolactone diffused rapidly into mouse peripheral blood cells. Moreover, like full mycolactone, it conferred systemic protection against chronic skin inflammation and inflammatory pain. In addition to identifying the structural basis of mycolactone bioactivity, our results establish its systemic effects against inflammation and pain. They highlight the need for continuous monitoring of mycolactone levels during the course of antibiotic therapy, in order to anticipate paradoxical reactions delaying the healing of Buruli ulcers.

## **Mycobacteria and beta-lactams: mode of action and therapeutic perspectives**

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The essential targets of  $\beta$ -lactams in virtually all bacteria are the high-molecular weight penicillin-binding proteins (PBPs) that catalyze the last cross-linking step of peptidoglycan polymerization. These enzymes are active-site serine D,D-transpeptidases that generate 4 $\rightarrow$ 3 cross-links connecting glycan strands. Mycobacteria contain additional targets for  $\beta$ -lactams since their peptidoglycan mainly (75-80%) contains 3 $\rightarrow$ 3 cross-links formed by L,D-transpeptidases. These enzymes are structurally unrelated to PBPs, contain an active-site cysteine residue, and use an acyl donor containing a stem tetrapeptide, in contrast to D,D-transpeptidases, which use a pentapeptide. The essential tetrapeptide-containing substrate of the L,D-transpeptidases is formed by D,D-carboxypeptidases that cleave the D-Ala<sup>4</sup>-D-Ala<sup>5</sup> bond of pentapeptide stems assembled in the cytoplasm. The latter enzymes represent a third class of targets for  $\beta$ -lactams since they belong to the active-serine PBP family. PBPs are potentially inactivated by all classes of  $\beta$ -lactams whereas L,D-transpeptidases are only inactivated by carbapenems. Other  $\beta$ -lactams only partially inactivate L,D-transpeptidases since formation of covalent adducts and their hydrolysis occur at similar slow velocities. However, this is not sufficient for resistance since inhibition of the D,D-carboxypeptidases indirectly inhibits the L,D-transpeptidases by blocking the supply of the tetrapeptide substrate. Intrinsic resistance to  $\beta$ -lactams in mycobacteria does not appear to result from inefficient inactivation of the targets, the PBPs and the L,D-transpeptidases, by these drugs. Instead, the mycomembrane limits access of  $\beta$ -lactams to the periplasm. The activity of the drugs is further limited by broad-spectrum class C  $\beta$ -lactamases. Imipenem (a carbapenem) and cefoxitin (a cephamycin) are part of the reference treatment of pulmonary infections due to *Mycobacterium abscessus* since these drugs are only slowly hydrolyzed by the  $\beta$ -lactamase Bla<sub>Mab</sub>. Among approved drugs, avibactam is the only  $\beta$ -lactamase inhibitor that efficiently inactivates Bla<sub>Mab</sub> *in vitro*. Avibactam also inactivates Bla<sub>Mab</sub> in human macrophages and in zebrafish embryos infected by *M. abscessus*. This inhibitor may potentially increase the panel of  $\beta$ -lactams useful to treat infections due to this bacterium, raising the possibility to use better tolerated and more efficient drugs in cystic fibrosis patients.

# Comparative analysis of systemic and local host-responses against MAP and MAH in an experimental goat model

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Domestic ruminants are natural hosts for *Mycobacterium (M.) avium*-complex (MAC) infections. Paratuberculosis caused by *M. avium* subsp. *paratuberculosis* (MAP) is prevalent in cattle and goats, while *M. avium* subsp. *hominissuis* (MAH) infections are only incidentally observed. Experimental ruminant models of both infections allow insights into differences in host-pathogen interaction. This study aimed at examining the kinetics and intensity of systemic and local host responses of goats to MAP and MAH.

21 goat kids each were orally inoculated 10 times every 2-4 days with MAP or MAH, receiving total doses of  $6,3 \times 10^8$  or  $2,1 \times 10^{10}$  cfu per animal, respectively. Clinical symptoms, faecal shedding of the organisms, course of specific interferon-gamma (IFN- $\gamma$ ) and antibody responses as well as composition and specific activation of T cell subsets in blood were monitored regularly up to 13 months post inoculation (mpi) in inoculated animals and 10 age-matched controls. At necropsy, which occurred at different time points pi, bacterial burden was culturally determined in intestinal tissues, intestinal lymph nodes (ILN) and in peripheral organs. Gene expression of immune modulatory cytokines (IFN- $\gamma$ , IL-1 $\beta$ , IL-10, IL-12p40, IL-17, IL-18, IL-23, TGF- $\beta$ , TNF- $\alpha$ ) in organized gut associated lymphatic tissue (oGALT) and ILN was examined.

MAP-inoculated and control goats were clinically healthy throughout the experiment. In contrast, 9 of the MAH-inoculated goats developed progressive disease (fever, depression, weight loss) and died or were euthanized 2-3 mpi. The other goats had mild transient symptoms and were in good general condition when necropsied at 13 mpi. Already 7-10 weeks (w) pi, a strong antigen-induced IFN- $\gamma$  response was noted in MAP- and MAH-inoculated animals. While the initial response was very high in the MAH-inoculated goats, it only gradually increased up to 4 mpi in the MAP-inoculated animals. The proportion of CD4<sup>+</sup> T cells was up-regulated and that of  $\gamma\delta$  TcR<sup>+</sup> T cells down-regulated in MAH-goats up to 6 mpi. Activation of CD8<sup>+</sup> T cells in blood was temporarily observed in both MAP and MAH inoculated groups. MAP-specific antibody titers started to increase about 3 mpi and reached peak levels at 7 mpi. MAH-specific antibody titers were already measurable 2 mpi but S/P% values were considerably lower than in MAP-inoculated goats.

High to very high amounts of MAP were culturally isolated from intestinal mucosa, oGALT and ILN in animals necropsied 4 and 7 mpi, while low to moderate amounts were recovered at 13 mpi. Extra-intestinally, MAP was most often recovered from hepatic lymph nodes. Moderate to high amounts of MAH were recovered from intestinal mucosa, oGALT and ILN of the animals at 2-3 mpi. Massive dissemination to extra-intestinal tissues including liver, lung and spleen was also noted. At 13 mpi, MAH was almost eliminated and could only be isolated in low amounts from a few tissues of 7/10 animals.

A differential regulation of the gene expression of IFN- $\gamma$ , IL-1 $\beta$ , IL-10, IL-12p40, IL-17, IL-18, IL-23, TGF- $\beta$  and TNF- $\alpha$  was noted in the different tissues depending on mycobacterial species and time pi. The up-regulation of IL-17 in oGALT of MAH- and MAP-inoculated animals necropsied 2-3, 4 and 7 mpi in comparison to 13 mpi was most prominent. At 13 mpi, IL-23 expression was increased in oGALT and ILN of MAP-inoculated goats compared to MAH- and control animals.

In conclusion, MAP and MAH interact differently with their natural host, the goat. Comparative studies will foster insights into protective mechanisms against MAC members.



# Analysis of the virulence and immunogenicity of porcine and human *M. avium* ssp. *hominissuis* isolates in an experimental mouse model

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Nontuberculous mycobacteria of the *Mycobacterium avium* complex (MAC) are opportunistic pathogens of increasing concern for public health, due to their ability to cause lymphadenitis in children and systemic infections in immunocompromised patients. In pigs these bacteria can cause granulomatous lymphadenitis. Detailed comparison of human and swine MAC genotypes may shed more light on their zoonotic potential. Of ninety-nine human MAC isolates more than 95% were identified as *M. avium* ssp. *hominissuis* (*Mah*), and thirty-eight different genotypes were identified using multispacer sequence typing (MST). In 5 % of suspected cases of submandibular granulomatous lymphadenitis in swine, found in Belgian slaughterhouses, ten different MST types of *Mah* were detected.

Using an experimental intranasal infection in BALB/c mice, we compared the virulence and immunogenicity of porcine and human isolates with shared genotype (MST **12**, **22** and **91**) or with specific human (MST **15**) and porcine genotype (MST **103**).

Isolates varied in virulence, with human/porcine genotype **22** and porcine genotype **12** showing higher bacterial numbers in lungs and more dissemination to spleen and liver than human/porcine genotype **91** and genotype **15**. Human genotype **12** and genotype **103** showed an intermediate virulence. Mycobacteria specific cellular and humoral immune responses against a series of 37 recombinant *M. avium* proteins also showed pronounced variations.

In conclusion, intranasal infection of BALB/c mice may be a valuable experimental model for the development of tools for the immunosurveillance of *Mah* infection and the elucidation of its zoonotic potential.

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# Using whole genome sequencing to uncover the biology of NTM infection

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*Mycobacterium abscessus* is a rapidly emerging respiratory pathogen affecting ~5-15% of individuals with Cystic Fibrosis around the world. It is inherently multi-drug resistant and challenging to treat, leads to accelerated decline in lung function and progressive lung damage, and is a contra-indication to lung transplantation in many centres. Prior to our work, infection with *Mycobacterium abscessus* was thought to be acquired by susceptible individuals independently from the environment. However, given the rise in infection rates worldwide, we examined whether person-to-person spread could be occurring.

Using whole genome sequencing and detailed epidemiological analysis of a cohort of patients attending the CF centre at Papworth Hospital, we found strong evidence for transmission from person to person. Our subsequent multi-national study has demonstrated the presence of a number of dominant global clones of *M. abscessus* with frequent person-to-person transmission events occurring in CF centres around the world.

The presence of dominant circulating clones combined with evidence for transmission between patients suggests that *Mycobacterium abscessus* may be adapting to the lung environment. Deep sequencing of longitudinal isolates has allowed us to examine the changes in genetic diversity within a single individual over the course of chronic infection. We found that within-host variation correlates with bacterial burden and that both of these are reduced by effective antibiotic treatment. We observed phenotypic heterogeneity in antibiotic resistance and colony morphology which suggests that the development of within-patient variation can lead to clinically relevant changes. In addition to this we found evidence of convergent evolution in genes linked to virulence and of mutations conferring a hypermutator phenotype.

Our data provides the first evidence that *Mycobacterium abscessus* is frequently able to transmit from person-to-person and offers novel insights into population dynamics and within-patient evolution of this organism.

# Cystic fibrosis airways microbiota – from ecology to treatment

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Despite important therapeutic advances, mortality for the majority of individuals with cystic fibrosis is still due to respiratory failure resulting from damage triggered by airways infection. As such, it is important to detail and describe the roles of the microbes driving these infections. Over the past decade, culture-independent techniques have started to define the microbes – collectively the microbiota – in the lower airways of individuals with CF. For adults with cystic fibrosis, the airways microbiota has been shown typically to be highly complex with a phylogenetically-diverse range of bacterial species present. The species detected in microbiota studies include those such as *Pseudomonas aeruginosa* that have been regarded conventionally as pathogens in this context. Microbiota studies have also however detailed many species seldom if ever previously reported in the CF airways. In addition to anaerobes from the oral cavity, there is now increasing interest in understanding the role that NTM play in relation to the pathophysiology of CF airways disease progression. The focus of this presentation therefore is to contextualise NTM within the wider bacterial community of the CF airways microbiota. The impact of antibiotics on the CF airways microbiota will be discussed and future directions for research towards gaining therapeutic benefits for individuals with CF also identified.

# Poster

## Detection and immune characterization of *Mycobacterium abscessus* infection in cystic fibrosis patients

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Cystic fibrosis (CF) is a genetic disease caused by mutations of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. CF patients suffer from multiple infections with different pathogens, especially non-tuberculous mycobacteria [e.g. *Mycobacterium (M.) abscessus complex* (MABSC)]. Specific immunological or PCR-based tests for MABSC infection are not available. The aim of this study was to establish specific tests for rapid detection of MABSC infections and to characterize T-cell immunity against MABSC.

Whole blood collected from CF patients (n = 35) was re-stimulated *in vitro* using purified protein derivatives (PPD) of different mycobacteria (MABSC, *M. tuberculosis*, *M. avium*) and intracellular cytokine analysis was performed. Subpopulation marker (i.e. CD4, CCR7, CD45RA) as well as cytokine expression profiles (i.e. TNF $\alpha$ , IFN $\gamma$ , CD40L, IL-2) of mycobacteria specific T cells were compared between CF patients with and without mycobacterial infections. For a subgroup of patients (n = 21), expectorated sputum was analyzed by mycobacterial culture and direct PCR based DNA strip test concomitantly.

Four patients with acute MABSC infections were positively tested by sputum culture and DNA strip test. These results were confirmed by MABSC PPD-induced T-cell immunity that also suggested previous mycobacterial infections in MABSC negative CF patients, as well as in a subgroup of CF patients not able to expectorate sputum. Quantification of CD40L/IL-2 double-positive T cells was optimal for detection of MABSC specific immunity and CD40L positive but IL-2 negative T cells qualified as candidate markers for acute MABSC infection in CF patients.

In conclusion, *in vitro* T-cell cytokine analysis in combination with PCR DNA strip tests enabled rapid diagnosis and characterization of MABSC infection, and may contribute to diagnosis especially where sputum samples are not available.

# Unusual outcome of an experimental infection of goat kids with *Mycobacterium avium* subsp. *hominissuis*

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*Mycobacterium avium* subsp. *hominissuis* (MAH) is one of 4 subspecies of *M. avium*. It is an opportunistic pathogen with a wide host spectrum including humans. Granulomatous lesions of the intestinal tract, especially intestinal lymph nodes and liver, but also systemic infections may occur. In the following, the findings after an experimental infection of goats are described.

Eighteen goat kids were orally inoculated (10 times, every 2-4 days) with a total dose of  $10^{10}$  cfu MAH strain 09MA1289. Unexpectedly, 9 goats developed progressive disease (fever, depression, weight loss) and died or were euthanized in extremis 2-3 months post inoculation (mpi). The other goats had mild transient symptoms and were in good general condition when necropsied at 13 mpi. At necropsy, tissues were collected for histology and cultural isolation of mycobacteria. Lesions were characterized in HE-stained paraffin sections. MAH was labeled by immunohistochemistry (IHC).

MAH strain 09MA1289 used for inoculation was a porcine isolate with a multibanded IS1245 RFLP pattern and the MIRU-VNTR genotype INMV 46 unique for Germany until now but found one time before for a French isolate originating also from pig (Randomski et al., 2010). Phenotypic and other genotypic characteristics of this strain (presence of ISMpa1 and a selection of specific GPL genes) were commonly found in other MAH strains in Germany. Goats necropsied with severe clinical signs were in bad nutritional condition. There was extensive ulceration of organized gut associated lymphatic tissue (oGALT) and necrosis of intestinal lymph nodes (ILN). Ulcerations were associated with granulomatous infiltrates composed of many lymphocytes, small groups of epitheloid cells and variable numbers of neutrophils. The confluent granulomas in ILN were delineated by granulomatous infiltrates. Granulomatous infiltrates were also detected in multiple other sites. MAH and mycobacterial material was detected in variable amounts and MAH was cultured from oGALT, ILN and numerous other sites of the 9 goats.

All of the apparently healthy goats necropsied at 13 mpi had severely enlarged ILN with large granulomas and several had multiple small granulomas in oGALT. Mycobacterial material was regularly detected in ILN and occasionally in ileal Peyer's patches. MAH was cultured from ILN of 4 goats.

In conclusion, goats have to be included in the list of species susceptible to MAH infection. After oral exposure, lesions dominate in oGALT and ILN, but there is also systemic spread. Even in apparently healthy animals, lesions were regularly found and may contain viable MAH. The different outcome of the MAH infection in a homogenous group of animals under the same experimental conditions and the severity of lesions in young animals are unusual.

# **Morphological characterization of granulomas in goats experimentally infected with *Mycobacterium avium* subsp. *hominissuis***

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*Mycobacterium avium* subsp. *hominissuis* (MAH), a non-tuberculous mycobacterium (NTM), causes chronic granulomatous diseases in humans and animals. This study describes the morphology of granulomas induced by MAH and compares them with those seen in *M. tuberculosis* complex (MTC) infection.

Granulomas were detected in organized gut associated lymphatic tissues (oGALT) and intestinal lymph nodes (ILN) of goats experimentally infected with MAH when they were necropsied 2-3 months post inoculation (mpi) and 13 mpi. According to a modified scheme from Wangoo et al. (2005) granulomas were characterized in HE-stained paraffin sections as (1) stage 1: initial infiltrate (2) stage 2: small, solid with minimal necrosis, (3) stage 3: monocentric with central necrosis and mineralization (4) stage 4: multicentric with central necrosis and mineralization and (5) stage 5: predominantly fibrotic organization. Macrophages, T-cell subsets, B-lymphocytes, plasma cells and MHC-II positive cells were labeled in frozen sections by immunohistochemistry (IHC) to determine the cellular composition of granulomas. MAH was stained in frozen sections with the Kinyoun method.

At 2-3 mpi, granulomas of stage 1, 2, 3 and 4 were seen in ILN, but not in oGALT. Granulomas of stages 3 and 4 predominated. They were characterized by extensive caseous necrosis with minimal mineralization surrounded by moderate numbers of epitheloid cells, CD4+ cells, few macrophages, single CD8+ cells and scattered  $\gamma\delta$  T-cells. Single MAH were seen in few epitheloid cells in all granuloma stages and in variable quantities in areas of necrosis in type 3 and 4 granulomas. Many MAH were seen in epitheloid cells and necrosis of type 3 and 4 granulomas in one goat.

At 13 mpi, granulomas were found both in oGALT and ILN. In addition to granulomas of stage 1 to 4, stage 5 granulomas were present in ILN. Stage 3 granulomas where the central necrosis and mineralization was surrounded by many epitheloid cells, few multinucleated giant cells and CD4+ T-cells followed by a layer of tertiary lymphoid tissue dominated in oGALT. Large multicentric granulomas with marked fibrotic organization and little cellular infiltrate (stage 4 and 5 granulomas) were frequent in ILN. Single MAH were seen in scattered epitheloid cells and areas of necrosis in few granulomas only.

In conclusion, MAH elicits granulomas highly similar to those in MTC infections and might serve as large animal model for the pathogenesis of these granulomas.

# Distribution of specific glycopeptidolipids (GPLs) within subspecies of *Mycobacterium avium*

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Members of *Mycobacterium avium* (*M. avium*) belong to the direct and indirect human environment; they have been found in soils and waters worldwide. *M. avium* subsp. *hominissuis* (MAH) is an opportunistic environmental pathogen responsible for infections in humans, swine and other mammals. *M. avium* subsp. *avium* (MAA) and *M. avium* subsp. *silvaticum* (MAS) are the causative agents of avian tuberculosis; *M. avium* subsp. *paratuberculosis* (MAP) causes Johne's disease in ruminants. The interaction of *M. avium* members with their environment is crucial in the determination of their pathogenicity. Cell wall glycolipids (GPLs) are discussed to contribute to the virulence of *M. avium*. Different genes involved in the synthesis of GPLs would be expected to modify therefore indirectly the interaction of the bacteria with their hosts.

The aim of this study was to get deeper insight into the distribution of genes involved in the synthesis of GPLs among subspecies of *M. avium* and among strains of subsp. *paratuberculosis*. Therefore, *M. avium* isolates of different origin were screened for the presence of six selected genes of the GPL cluster.

Altogether 76 *M. avium* isolates belonging to MAA (n=10), MAH (n=25), and MAP (n=40) originating from different hosts and some from the environment were investigated including the reference strains ATCC 25291 (MAA), ATCC 19698 (MAP), DSM 44175 (MAS), and additionally the ATCC 13950 (*M. intracellulare*). The isolates were tested for the presence of selected nonspecific (ns)GPL genes *gtfA*, *rtfA*, and *mtfC* as well as serotype-specific (ss)GPL genes: *mdhA*, *merA*, *mtfF* by PCR using primers from Johansen et al. (2009). All *M. avium* isolates had been characterized before by MIRU-VNTR, RFLP and MLST genotyping.

The selected ssGPL genes *mdhA*, *merA*, and *mtfF* were detected in most examined strains with the exception of two MAH isolates and *M. intracellulare*. All MAH and MAA strains, the reference strains of MAS and *M. intracellulare*, and 16 MAP strains (comprising 15 MAP-C strains and one MAP-S strain) revealed the three nsGPL genes *gtfA*, *rtfA*, and *mtfC*. In 24 MAP isolates no nsGPL gene sequences could be found. One bovine MAP strain lacked only the *gtfA* gene sequence. Furthermore, there was no association between presence or absence of the three nsGPL genes in MAP and a specific MAP genotype. The presence of *gtfA*, *rtfA*, and *mtfC* in more than one third of the studied MAP strains and in the MAS reference strain is in contrast to previous results of Eckstein *et al.* (2003). The importance of this division of MAP strains into two groups with or without selected nsGPL genes regarding specific membrane characteristics and different interactions with their hosts has to be clarified in further investigations.

## References:

Eckstein et al. (2003) Microbiology 149, 2797-2807;

Johansen et al. (2009) BMC Microbiology 9:159 doi:10.1186/1471-2180-9-159.



# MLSA-Typing of non-tuberculous mycobacteria isolated from bovine lymph nodes in Switzerland

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With more and more studies revealing that non-tuberculous Mycobacteria (NTM) cause severe disease in humans and animals, NTM finally start to emerge from the enormous shadow of *M. tuberculosis*. However, despite their increased recognition as causative agents of numerous skin and soft tissue infections, lung diseases and visceral or disseminated diseases, still surprisingly little is known about NTM prevalence in Switzerland.

In order to evaluate the spectrum of NTM species infecting Swiss cattle, we investigated 63 *M. tuberculosis* complex (MTBC) negative *Mycobacteria* strains isolated from bovine lymph nodes. By multi locus sequence analysis (MLSA) based on 16S rRNA, rpoB, hsp65 and 16S-23S internal transcribed spacer (ITS), the species of 53 strains could be identified successfully. While the predominant species was *M. avium* subsp. *hominissuis* (29 %), multiple strains were also isolated of *M. nonchromogenicum* (11 %), *M. neoaurum* (8 %), *M. kansasii* (6 %), *M. monacense* (5 %), *M. xenopi* (5 %) and *M. vaccae* (3 %). Additionally eleven NTM species were isolated only once from the investigated lymph nodes. Of those particularly interesting are *M. abscessus* and *M. engbaekii* which to our knowledge both have not been detected in cattle before.

While some studies identified NTM as important pathogens, others revealed that certain NTM can further interfere with tuberculosis (TB) diagnosis. The most commonly used tools for TB testing, tuberculin skin tests and Interferon-gamma release assays (IGRA), both measure reactivity of the immune system against the *M. tuberculosis* antigens ESAT-6 and CFP-10 whose genes are located within the so called region of difference 1 (RD1). Since RD1 was also found in the genome of several NTM including *M. marinum*, *M. szulgai* and *M. kansasii*, an infection with the respective agent can result in false-positive TB-diagnosis. In this context our identification of four *M. kansasii* strains is of utmost interest. With this finding the paradigm that *M. kansasii* infections do not exist in Swiss cattle could finally be disproved. In order to investigate if additional NTM strains do also possess the RD1 region, *esat-6* and *cfp-10* specific PCRs were performed. While most of the other strains were RD1 negative, our study revealed that *M. gordonae* does possess the *cfp-10* gene.

Summarized our results not only show for the first time that Swiss cattle are infected by various NTM species, they also indicate that some of these infections interfere with tuberculosis diagnosis. Therefore further investigation of NTM prevalence in Swiss cattle is crucial, also regarding an efficient TB control.

## **Non-tuberculous mycobacterial infections of veterinary relevance**

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Mycobacteria play an important role in human and animal health fields. Here we examined the place of NonTuberculous Mycobacteria (NTM) infections in the veterinary context. In this review relevant aspects regarding a reference laboratory experience and the literature review are presented. Importance is given as regards productivity loss per se but also to economic losses due to misdiagnosis with bovine tuberculosis and paratuberculosis. The importance of the role played by NTM is variable depending on the part of the world, ecology, husbandry, extent of surveillance programs and bovine tuberculosis and paratuberculosis prevalence. The role in animal disease of the most relevant NTM are summarized with a focus on *Mycobacterium avium* subsp. paratuberculosis which occupies a particularly important position, being the causative agent of paratuberculosis, a disease responsible of high economic consequences for ruminant livestock.

## Comparison in the mouse model of several potential vaccine targets against *Mycobacterium abscessus* in order to obtain protection in the context of cystic fibrosis

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In the fight against multi-resistant bacteria to antibiotics, vaccinal strategies represent a therapeutic possibility. *Mycobacterium abscessus*, a currently emerging pathogen belonging to the group of rapidly growing mycobacteria (RGM), is one of the most resistant bacteria responsible for respiratory and cutaneomucous infections. Cystic fibrosis patients represent one of the most susceptible population of patients. In order to obtain protection against this bacterium, various antigens were tested using different immunization routes. Virulence factors such as PLC (phospholipase C) or MgtC have been tested, an enzyme from the family of Nitrilases (MAB\_2545c) and a more complex fraction from surface extract selected specifically for its ability to activate the response innate via TLR2. We studied the immune response and protection in mice infected intravenously (IV) or by aerosol route with *M. abscessus* after DNA or protein immunization of these *M. abscessus* antigens. The mice were either wild-type mice or mice with the most common mutation observed in the context of cystic fibrosis ( $\Delta F508$ ). The three monovalent antigens show all, to varying degrees (depending on the infectious mode, the immunization method chosen and the type of mouse studied), a significant protective effect in infected mice. Generally speaking, the elimination of bacteria is faster when the infection occurs by the aerosol route, and it is more efficient for  $\Delta F508$  mice as for their wild-type littermates (FVB). DNA vaccination is similar to the protein vaccination for PLC antigen but does not work for the Nitrilase antigen while the latter immunization as purified protein is very efficient. Regarding the TLR2-activating complex fraction, there is no protection after infection by the aerosol route but it is effective after IV infection, curiously only on certain organs tested (liver and lungs).

In conclusion, several proteic antigens, particularly one of them, the Nitrilase MAB\_2545c, are good vaccine candidates against *M. abscessus* in the context of cystic fibrosis.

## **Detection and immune characterization of *Mycobacterium abscessus* infection in Cystic Fibrosis patients**

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Cystic fibrosis (CF) is a genetic disease caused by mutations of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. CF patients suffer from multiple infections with different pathogens, especially non-tuberculous mycobacteria [e.g. *Mycobacterium (M.) abscessus complex* (MABSC)]. Specific immunological or PCR-based tests for MABSC infection are not available. The aim of this study was to establish specific tests for rapid detection of MABSC infections and to characterize T-cell immunity against MABSC.

Whole blood collected from CF patients (n = 35) was re-stimulated *in vitro* using purified protein derivatives (PPD) of different mycobacteria (MABSC, *M. tuberculosis*, *M. avium*) and intracellular cytokine analysis was performed. Subpopulation marker (i.e. CD4, CCR7, CD45RA) as well as cytokine expression profiles (i.e. TNF $\alpha$ , IFN $\gamma$ , CD40L, IL-2) of mycobacteria specific T cells were compared between CF patients with and without mycobacterial infections. For a subgroup of patients (n = 21), expectorated sputum was analyzed by mycobacterial culture and direct PCR based DNA strip test concomitantly.

Four patients with acute MABSC infections were positively tested by sputum culture and DNA strip test. These results were confirmed by MABSC PPD-induced T-cell immunity that also suggested previous mycobacterial infections in MABSC negative CF patients, as well as in a subgroup of CF patients not able to expectorate sputum. Quantification of CD40L/IL-2 double-positive T cells was optimal for detection of MABSC specific immunity and CD40L positive but IL-2 negative T cells qualified as candidate markers for acute MABSC infection in CF patients.

In conclusion, *in vitro* T-cell cytokine analysis in combination with PCR DNA strip tests enabled rapid diagnosis and characterization of MABSC infection, and may contribute to diagnosis especially where sputum samples are not available.

## Development of new anti-mycobacterial agents against *Mycobacterium abscessus* infections

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*Mycobacterium abscessus* is an emerging human pathogen responsible of broncho-pulmonary infections in patients with cystic fibrosis (CF). It is considered as the most virulent and drug-resistant rapid-growing mycobacterial species. In CF patients, the presence of these mycobacteria is a contraindication to lung transplantation. This pathogen has two morphotypes: a smooth (S) avirulent variant characterized by the presence of glycopeptidolipids (GPL) and a rough (R) virulent variant, devoid of GPL surface expression. Recently, we developed a zebrafish model to study the chronology and physiopathology of *M. abscessus* infections thanks to the optical transparency of the embryos which allowed monitoring the evolution of the infectious process in real time. This model was also used to evaluate the therapeutic activity of antibiotics in an *in vivo* context.

With the aim to identify new compounds active against *M. abscessus*, we screened a chemical library against whole bacteria. *In vitro* drug assessments were performed following the recommendations of the Clinical and Laboratory Standards Institute and activity was further confirmed in both liquid and solid media against a panel of *M. abscessus* clinical isolates from CF and non-CF patients. A route of chemical synthesis was also established to produce large amounts of material required to evaluate its therapeutic activity in pre-clinical studies in the zebrafish model of infection.

One compound, FMD-5 was identified as being very active against the *M. abscessus* reference strain with an MIC ≤ 0.20 µM. Similar values were obtained against different clinical strains (S and R) exhibiting different profiles of susceptibility to imipenem or amikacin. Importantly, our preliminary results suggest that FMD-5 has potent *in vivo* activity in infected zebrafish. Exposure to compound was associated to i) a decrease of the pathophysiological symptoms and particularly the infection foci within the central nervous system, and ii) an increase survival of the infected embryos.

We identified a scaffold for a new anti-infective agent against *M. abscessus*. With the aim to better define the pharmacophore, a structure-activity relationships study has been initiated. Further studies will also be dedicated to address the antimycobacterial activity of the compounds in *cftr*-morphants, used to mimic a CF-like microenvironment.

# LOOXSTER<sup>®</sup> technology: overcoming obstacles in enrichment of mycobacteria from whole blood

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Disseminated mycobacterial infections are serious diseases with a fatal outcome unless detected and treated fast. Compared to pulmonary manifestations they do occur only in immunocompromised patients, e.g. in patients with HIV/AIDS. Thus the detection of mycobacteria in immunocompromised patients often requires more laborious diagnostic methods than smear microscopy or cultivation of respiratory samples on standard media. Mycobacterial blood culture systems, such as MYCO/F LYTIC or BacT/ALERT MB, are the usual methods for the detection of mycobacteria in whole blood. Culturing techniques require several weeks to identify mycobacterial growth. Moreover, non-growing mycobacteria like *M. genavense* or *M. tilburgii*, which are mainly detected in immunocompromised patients, are not readily detectable with these methods. A rapid and culture independent identification technology, e.g. DNA enrichment procedure and a subsequent nucleic acid amplification test (NAAT), is needed urgently. New pre-analytic approaches aim to increase the sensitivity of NAATs through enrichment of microbial DNA. Cytosine methylation and frequency of non-methylated CpG dinucleotides are genomic properties varying greatly between mammals and bacteria. Analytik Jena's LOOXSTER<sup>®</sup> uses the specific affinity of the CXXC zinc finger 1 domain to non-methylated CpG dinucleotides for powerful bacterial and fungal nucleic acid enrichment. Due to the removal of more than 95% of eukaryotic background DNA large sample sizes become accessible to analytical and preparative downstream applications, that is even more important when detecting very low microbial counts.

Within this study a manual workflow consisting of the PureProve<sup>®</sup> Blood & Tissue DNA Maxi Kit for total DNA extraction followed by the enabling technology of the LOOXSTER<sup>®</sup> Enrichment Kit was used to extract and enrich *M. fortuitum* DNA from spiked blood samples. An in-house Real-time PCR method targeting an Internal Transcribed Spacer fragment and subsequent Sanger Sequencing was performed to verify the origin of the enriched DNA. The blood was spiked with different cfu/ml of the bacterium; PCR was performed before and after the enrichment.

DNA of *M. fortuitum* could be detected in all samples independent on the cfu/ml after the enrichment with the LOOXSTER<sup>®</sup> Enrichment Kit. In contrast, the DNA could only be detected in the DNA extracts that have not been enriched by the technology when the blood sample was spiked with high amounts of bacteria. The results show that the workflow consisting of the PureProve<sup>®</sup> Blood & Tissue DNA Maxi Kit for total DNA extraction followed by the enabling technology of the LOOXSTER<sup>®</sup> Enrichment Kit is suitable for the detection of mycobacterial DNA in spiked blood samples.

# Multi-tiered map containing both the coding and regulatory architecture of the *Mycobacterium abscessus* genome

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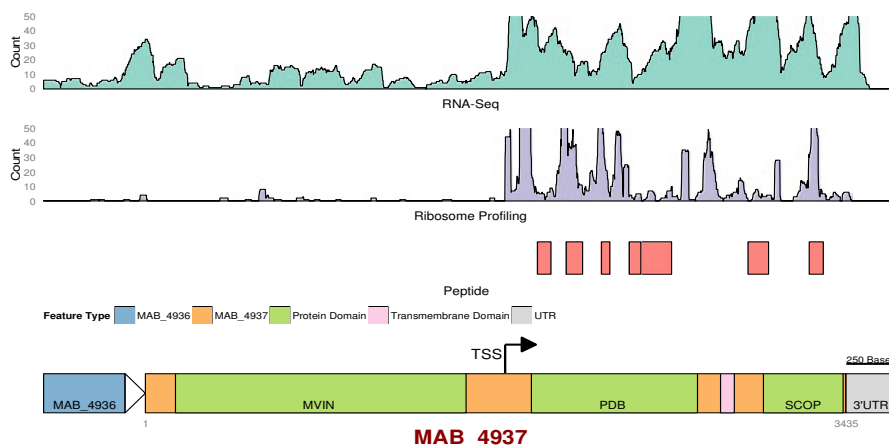
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*Mycobacterium abscessus* is a rapidly growing mycobacterium (RGM) that is both intrinsically highly antibiotic resistant and an important CF pathogen. The *M. abscessus* genome shares notable features with with slow growing mycobacteria including important virulence related genes while harboring notable differences at the genic level as a result of lateral gene transfer. Evolution of regulatory elements occurs at a more rapid rate however and a genome wide identification of regulatory elements that control the transcriptome and proteome has yet to be carried out in *M. abscessus*. Understanding the extent to which these regulatory features are unique to *M. abscessus* or are shared across mycobacteria will shed important light on the biology of *M. abscessus* and its survival strategies within the host. Therefore we generated a multi-tiered omics map of the *M. abscessus* genome encompassing a transcriptional start site (TSS) map, combined with RNA-seq to identify genome wide RNA elements within the genome. In addition we have generated ribosome profiling and LC-MS proteomics data to identify which elements are coding (Fig 1). Together these provide a valuable genomic resource to understand the biology of *M. abscessus*.

Our analysis has identified 48 small un-annotated genes, many of which are highly expressed and a number of which are secreted. We identify numerous genes regulated by 5'UTR regulatory elements including leader peptides and putative riboswitches. We identify a small set of regulatory RNAs conserved across mycobacteria including those essential for growth and predict their mechanism of action. Finally we identify genes that appear to be partially translated offering a mechanism by which they can have independently both essential and non-essential domains for growth (Fig. 1).



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