IntechOpen

Basic Biology and Applications of Actinobacteria

Edited by Shymaa Enany

BASIC BIOLOGY AND APPLICATIONS OF ACTINOBACTERIA

Edited by **Shymaa Enany**

Basic Biology and Applications of Actinobacteria
http://dx.doi.org/10.5772/intechopen.72033
Edited by Shymaa Enany

Contributors

Thet Tun Aung, Roger Beuerman, Oleg Reva, Karen Van Niekerk, Rian Pierneef, Ilya Korostetskiy, Alexander Ilin, Gulshara Akhmetova, Sandeep Chaudhari, Athumani Msalale Lupindu, Erasto Mbugi, Abubakar Hoza, Jahash Nzalawahe, Adriana Ribeiro Carneiro Folador, Artur Silva, Vasco Azevedo, Carlos Leonardo De Aragão Araújo, Patricia Nascimento Da Silva, Jorianne Thyeska Castro Alves, Larissa Maranhão Dias, Joana Montezano Marques, Alyne Cristina Lima, Mohamed Harir

First published in London, United Kingdom, 2018 by IntechOpen
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard copies can be obtained from orders@intechopen.com

Basic Biology and Applications of Actinobacteria, Edited by Shymaa Enany
p. cm.
Print ISBN 978-1-78984-614-0
Online ISBN 978-1-78984-615-7

Meet the editor

Dr. Shymaa Enany is an Associate Professor of microbiology and immunology at the Suez Canal University, Egypt. She received her PhD from the School of Medical and Dental Sciences, Niigata University, Japan, and completed her postdoctoral work in collaboration with many laboratories in San Diego, California, USA and in Niigata, Japan. She is an editorial board member and reviewer of many journals and scientific associations and has many publications in eminent journals as well as books. She has excellent experience in bacterial genomics and proteomics.

Contents

Preface VII

Chapter 1 **Paralleling of Diagnostic Endeavor for Control of Mycobacterial Infections and Tuberculosis 1**
Athumani Msalale Lupindu, Erasto Vitus Mbugi, Jahash Nzalawahe and Abubakar Shaaban Hoza

Chapter 2 **Zoonotic Tuberculosis: A Concern and Strategies to Combat 23**
Ravi N. Teppawar, Sandeep P. Chaudhari, Shilpa L. Moon, Shilpshri V. Shinde, Wiqar A. Khan and Archana R. Patil

Chapter 3 **Nontuberculous Mycobacterial Infections: Negligent and Emerging Pathogens 39**
Thet Tun Aung and Roger W. Beuerman

Chapter 4 **The Genus *Corynebacterium* in the Genomic Era 59**
Carlos Leonardo Araújo, Jorianne Alves, Alyne Lima, Larissa Dias, Patricia Silva, Joana Marques, Vasco Azevedo, Artur Silva and Adriana Folador

Chapter 5 **Clade-Specific Distribution of Antibiotic Resistance Mutations in the Population of *Mycobacterium tuberculosis* - Prospects for Drug Resistance Reversion 79**
Karen van Niekerk, Rian Pierneef, Oleg N. Reva, Ilya S. Korostetskiy, Aleksandr I. Ilin and Gulshara K. Akhmetova

Chapter 6 ***Streptomyces* Secondary Metabolites 99**
Mohammed Harir, Hamdi Bendif, Miloud Bellahcene, Zohra Fortas and Rebecca Pogni

Preface

Actinobacteria constitute one of the largest phyla. They are a group of Gram-positive aerobic bacteria that are distributed in aquatic and terrestrial ecosystems. Members of Actinobacteria have adopted different lifestyles, and can be pathogens such as *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Tropheryma*, and *Propionibacterium*, soil inhabitants such as *Streptomyces*, plant commensals such as *Leifsonia*, or gastrointestinal commensals such as *Bifidobacterium*. They have an extensive bioactive secondary metabolism and produce a huge amount of the naturally derived antibiotics in current clinical use, as well as many anticancer, anthelmintic, and antifungal compounds. Consequently, these bacteria are of major importance for biotechnology, medicine, and agriculture. The first antibiotics discovered in Actinobacteria were actinomycin from a culture of *Streptomyces antibioticus* in 1940, which was then followed by many antibiotic production discoveries, including major classes of clinical antibiotics such as aminoglycosides, β-lactams, macrolides, oxazolidinones, tetracyclines, chloramphenicol, streptogramins, glycopeptides, and β-lactamase inhibitors like clavulanic acid. Moreover, some antitumor agents such as daunorubicin, landomycin, and moromycin were also produced from Actinobacteria.

We decided to write this book to discuss the different types of Actinobacteria and applications of their members in medicine, agriculture, and industry as presented by international leaders in their respective fields.

This book consists of several review chapters. Each chapter starts with a brief introduction, including its aim, and then goes on to provide detailed information about current research relevant to the field. The authors give an overview of the Actinobacteria that they used in their research as important microorganisms that exert beneficial effects on humans and/or the environment in a simple way that allows the reader to form a complete picture of these beneficial microorganisms and their suitability as therapeutic and biomaterial agents. Through the chapters, the authors explore Actinobacteria and how these microorganisms could be of great importance to our life. They examine the beneficial effects of Actinobacteria and the different usages of their secondary metabolites. Future aspects in the development of technologies for the production of improved products are also reviewed here.

We believe that our book is ideal for scientists, especially those who are interested in Actinobacteria. We hope you enjoy reading it. Finally, we would like to thank all the contributing authors without whose dedication and brilliant research this project would not have been accomplished.

Dr. Shymaa Enany
Department of Microbiology and Immunology
Faculty of Pharmacy
Suez Canal University
Ismailia, Egypt

Chapter 1

Paralleling of Diagnostic Endeavor for Control of Mycobacterial Infections and Tuberculosis

Athumani Msalale Lupindu, Erasto Vitus Mbugi,
Jahash Nzalawahe and Abubakar Shaaban Hoza

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77316

Abstract

Mycobacterial infections and tuberculosis pose global public health threats. High tuberculosis morbidities and mortalities are due to the diagnosis problems among other causes. This chapter describes and compares diverse mycobacterial infections and tuberculosis diagnostic efforts and point-out the direction so as to inform areas of and motivate research toward early, rapid, and accurate diagnosis for effective TB control. We have grouped diagnostic approaches according to the type of sample taken for or organ targeted during diagnosis. The sputum-based methods include smear microscopy, culture, and rat sniffing. Interferon-γ (INF-γ) release assays, transcriptional blood signatures, and proteomic profiling use blood samples while colorimetric sensor array (CSA) and mass spectrometry use urine samples. Patho-physiological methods include tuberculin skin tests (TSTs) and radiography. Chromatography and acoustic wave detection can also be used to diagnose TB from breath. Comparative description of these methods is based on a time frame to diagnosis, accuracy, cost, and convenience. The trend shows that there is a move from time-consuming, slow and narrow-spectrum to quick and broad-spectrum TB diagnostic procedures. The sputum-based and patho-physiological approaches remain conformist while blood-based procedures lead research developments. Absence of single best approach calls for synergistic research combinations that form accurate, rapid, cheap, and convenient package at point-of-care centers.

Keywords: tuberculosis, zoonosis, Cricetomys gambianus, tuberculin, interferon, latent TB infection

1. Introduction

Tuberculosis caused by *M. tuberculosis* is the leading cause of human deaths from single infectious agent and fall in the top 10 causes of deaths worldwide. More than 10 million people suffered from TB in 2016 and the global case fatality and incident rates were 16% and 140/100,000 [1].

Inhalation of *M. tuberculosis* stimulates host cellular immune response with a consequence of either clearance of the organism or infection. Infection may result into latent mycobacteria infection (LTBI) (no clinical evidence) [2] or active tuberculosis (pulmonary or extra-pulmonary) [1].

The global LTBI prevalence is 23%, which is approximately 1.7 billion of the population [3]. About 5–15% of LTBI may progress to active TB [4]. LTBI is currently diagnosed by tuberculin skin test (TST) or gamma-interferon release assays (IGRA) [5]. On average the prevalence of active TB globally is 0.14% and the figures may vary according to regions [1].

The zoonotic potential of *M. bovis* constitutes a public health concern. Ingestion of *M. bovis* contaminated material is reported to be a primary cause of infection in humans and the resulting TB may take both pulmonary and extra-pulmonary form [6]. The burden of tuberculosis in humans due to *M. bovis* is variable in different countries, ranging from 5 to 28% with mortalities of 8.7% [6]. Therefore, this pathogen should be given due weight in TB diagnosis and consequently control plan.

According to WHO report, 53 million deaths have been averted from 2000 to 2016 following diagnosis and treatment. There is a big gap between diagnosis and treatment otherwise the number of deaths prevented could be even higher [1]. Every year the health systems fail to capture 3 million TB patients [7] some of them may be due to the nature of mycobacteria and diagnostic setup [6].

When prevention of pathogen-host contact is inadequate, early, rapid, and discriminative diagnosis become the primary factor for treatment success and reduction of further transmissions. Inappropriate diagnosis of symptomatic patients with active TB, LTBI, or other confounding diseases, for example, sarcoidosis, usually lead to delayed or wrong administration of full TB treatment or prophylactic dose [8]. This chapter, therefore, focuses on mycobacterial infections and tuberculosis diagnosis by assessing and comparing different procedures so as to suggest a best diagnostic method(s) and research direction.

The assessment and comparison of diagnostic procedures in this chapter are based on either single or combination of features such as the type of sample tested, pathological, or morphological changes or immunological reactions. Some of the known mycobacterial infections and tuberculosis diagnostic sample sources include sputum for smear, sniffing, and culture, blood for biomarkers such as interferon, inflammatory cells, proteins and transcriptomes, urine, and breath for volatile organic compounds (VOCs). Moreover, anatomical and physiological changes in tissues can allow diagnosis of tuberculosis through imaging techniques and skin reaction. Among these procedures, IGRA, TST and transcriptomic studies can detect mycobacterial infections while smear microscopy, culture and sniffing results of sputum,

http://dx.doi.org/10.5772/intechopen.77316

radiography, and volatile organic compound determination can detect active tuberculosis. The latter group of procedures is assisted by the appearance of clinical symptoms. As the form of mycobacterial detection differs among these methods, sensitivity, and time frame to diagnosis vary as well. Nevertheless, the goal remains to end TB by 2035 [7].

2. Blood tests in TB diagnosis

Whole blood sample can be used to diagnose both mycobacterial infections and tuberculosis based on the host immune response. Characteristics of blood parameters such as interferon-induced genes, myeloid genes, inflammatory genes and B and T-cells regulation genes, and proteomic profiles are used as markers in the detection of mycobacterial infections and tuberculosis [9].

2.1. Interferon-γ release assays (IGRAs)

IGRAs are immune cell-mediated in vitro blood tests that measure mononuclear cell (lymphocytes: T and B cells) release of interferon-γ (INF-γ) after stimulation by antigens specific for Mycobacterium. Following infection, the body immune response is triggered and immune cells such as macrophages, T-cells, B-cells, and natural killer cell are involved. Macrophages, which form the first defense line, engulf, kill, and eliminate *Mycobacteria tuberculosis*. Some *M. tuberculosis* escapes the immune mechanism and survives and replicate in macrophages. T-cells, especially CD4 and CD8 T-cells, produce cytokine—interferon-gamma, which in turn activate infected macrophages to produce reactive nitric oxide and related reactive nitrogen intermediates to kill the *M. tuberculosis* and eliminate it through the actions of phagosomes and lysosomes [10]. The lymphocytes can produce interferon-gamma in vitro whenever stimulated by *M. tuberculosis* antigen. This feature is used to assist in the diagnosis of *Mycobacteria tuberculosis* infection. Principally, the lymphocytes of a person infected with *M. tuberculosis* release interferon-gamma when mixed with *M. tuberculosis* derived antigens. There are two enzyme-linked assays; enzyme-linked immunosorbent assay (ELISA), and enzyme-linked immunosorbent spot (ELISPOT) assay. The assays detect INF-γ release by lymphocytes of sample donor following exposure to antigens found on the *M. tuberculosis* complex (MTBC).

In ELISA based IGRA, fresh whole test blood sample is mixed with *M. tuberculosis* antigens and control. The antigen is made up of 6kDaA early secreted antigen target (ESAT-6), 10-kDa culture filtrate protein (CFP-10), and TB7.7 [11]. Normal saline is used as a control. The assay gives the concentration of INF-γ in international units per milliliter (IU/ml). The test result is considered positive when the difference in INF-γ response to the TB antigen between the test antigen value and the control value is greater than the cut-off of 0.35 IU [12].

Principally, the ELISPOT assay works on peripheral blood mononuclear cells (PBMCs) from a whole blood sample. The PBMCs are mixed with synthetic peptide antigens (ESAT-6 and CFP-10) and the control and incubated to stimulate interferon-γ secretion by the cells. Secreted interferon-γ is captured by specific antibodies and accumulated as spots. Thus, ELISPOT assay gives the number of interferon-γ producing cells (spots). The test result is

considered positive when the difference in a number of spots between the test sample and the negative control is greater than eight (8) [13].

Both IGRAs have the ability to diagnose latent mycobacterial infection in 24 h following the one-time patient visit and submission of the blood sample to a health center [11]. The sensitivity of the IGRAs in adults is 80–90% when mycobacterium standard culture is used as gold [13]. In contrast to tuberculin skin test, IGRAs do not cross-react with Bacille Calmette-Guérin (BCG) antigen hence prior BCG vaccination does not cause false positives [11]. IGRAs cannot discriminate active tuberculosis from LTBI and its sensitivity is low in immunocompromised patients and children under the age of 5 years [13]. The cost of mycobacterium infection diagnosis by interferon- γ release assays is more than 40 USD per sample, which is higher compared to 10.56–25.97 USD for tuberculin skin test [14, 15]. This factor may influence the availability of and accessibility to this diagnostic service, especially in low-income countries.

2.2. Mycobacterium global gene signature/transcriptional blood signatures

Whole blood transcriptional signature can assist in distinguishing latent from active tuberculosis by showing features, which are not present in asymptomatic patients. Moreover, the transition from latent infection to active TB can be predicted by assessing the blood parameter [9]. This approach is important because of the reported 10–20% of detectable tuberculosis in asymptomatic individuals diagnosed with latent *M. tuberculosis* infection [16].

TB risk signature or global gene expression based on whole blood RNA genotyping polymerase chain reaction (PCR and sequencing) can be used to predict the chance of progression from latent to active TB. It has been reported that the expression of signature genes (that is quantities of functional RNA) increases toward active TB development in contrast to non-TB progressor (latent mycobacterium infection). For instance, Zak and colleagues [17] have reported a 16-gene signature with a sensitivity of 71.2% and specificity of 80% (95% CI: 66·6–75·2) at a 6 month time before tuberculosis diagnosis. This approach shows promising future of TB diagnosis as early as possible when samples are submitted since PCR and sequencing can give diagnostic results within 24 h.

Transcriptional blood signatures studies have been conducted in many parts of the world including high-income countries (US, UK, and German), middle-income countries (Brazil, South Africa, and Indonesia) and low-income countries (Kenya, Gambia, and Malawi) [9]. However, the procedure is under research and there is hope that it will become a handy tool in TB diagnosis and treatment decision.

2.3. Blood proteomic profiling/fingerprinting

Proteome, the entire set of proteins produced or modified by a cell, system or organism, vary with time, requirement, stress or disease state that cell, and system or organism experiences. Studies on types and levels of plasma or serum proteins show variations between individuals with LTBI, active TB and those under different stages of treatment. These features can be used to differentiate LTBI from active TB and other perplexing diseases. Plasma or serum protein peaks are identified using high-performance liquid chromatography (HPLC)-tandem matrix-assisted laser desorption/ionization-TOF-MS (MALDI-TOF-MS) and the results are

statistically analyzed to define mass spectral patterns. Results show unique abundant spectra (up-regulation) in plasma from subjects with active TB, which are distinct from those of subjects with LTBI or controls.

Proteomic fingerprinting of plasma by surface-enhanced laser desorption/ionization—time of flight (SELDI-TOF) demonstrates that active TB can be discriminated from LTBI and other confounding diseases. Sandhu and colleagues [8] have reported that plasma from active TB patients had more distinct spectral peaks (at 5.8–11.5 kDa) than symptomatic LTBI and non-LTBI controls. The procedure discriminates the active TB patients from the undifferentiated controls with an accuracy of 87% (sensitivity 84% specificity 90%). Moreover, active TB can be differentiated from symptomatic controls with LTBI at 87% accuracy (sensitivity 89%, specificity 82%) and from symptomatic controls without LTBI at 90% accuracy (sensitivity 90%, specificity 92%). In this study Serum Amyloid A and transthyretin were reported as potential protein biomarkers for TB diagnosis. Similar procedure was also used by Agranoff and colleagues [18] on a serum to distinguish active TB patients from symptomatic controls, with an accuracy of 94% (sensitivity 93·5%, specificity 94·9%). Again, Serum Amyloid A and transthyretin were identified as potential biomarkers for TB diagnosis.

Liu and colleagues have also used SELDI-TOF MS to screen serum samples from TB patients and controls (lung cancer, pneumonia, chronic obstructive pulmonary disease, and healthy volunteers). Among different TB related peaks that were detected, fibrinogen with mass to charge ratio of 2554.6 Da was up-regulated in TB patients compared to controls. This discrimination of TB patients from controls had an accuracy of 83.8% (sensitivity of 83.3% and specificity of 84.2%) and suggests that fibrinogen could be a potential TB biomarker [19]. A similar procedure was performed by Zhang and colleagues [20], whereby TB patients were differentiated from controls (non-TB controls such as pulmonary cancer, pneumonia, chronic bronchitis, emphysema, asthma and flu, and healthy subjects) with a sensitivity of 96.9% and specificity of 97.8% (accuracy up to 97.3%). The most distinct protein peak at mass/charge ratio of 5643 Da was up-regulated in TB patients and identified as orosomucoid protein.

In most proteomic finger printings for TB diagnosis, comparison of active TB patients, LTBI, and controls (healthy subjects or patients with other non-TB diseases) has been done. The peaks of identified protein biomarkers in controls have served as references. However, the identified protein biomarkers are not TB specific.

According to expert opinions, blood-based mycobacterial diagnostic procedures for example, IGRAs can give supporting information in diagnosis in some situations like extra-pulmonary TB, testing of negative acid-fast bacilli (AFB) in sputum and/or culture negative for *M. tuberculosis*, TB diagnosis in children, or in the differential diagnosis of infection with non-tuberculous mycobacteria (NTM) [21]. IGRA can also support the diagnosis of *M. bovis* infection when single intradermal comparative tuberculin test (SICTT) is negative [22].

3. Sputum tests in TB diagnosis

Sputum of patients with pulmonary TB contains mycobacterium pathogens, which serves a role in transmission. The presence of the pathogens and their features such as viability,

staining, or release of odor compounds are useful in diagnosis. Staining and culture are two common procedures, which use sputum sample to diagnose tuberculosis, especially active form. An emerging third method, which also utilizes sputum, is sniffing by trained African giant pouched rats, the *Cricetomys gambianus*.

3.1. Sputum sample collection

In TB diagnosis, a collection of right samples is necessary. First morning samples are preferred as the overnight accumulation of secretions give better results. Sputum samples are normally collected from self-reporting presumptive TB cases at TB clinic. For samples collected far away from the diagnostic center, the samples are collected in transport medium, cetyl-pyridinium chloride (CPC) [23] for best subsequent results.

3.2. Ziehl Neelsen and fluorescent staining

A smear is made by even spreading of sputum over the glass slide to make a 20 mm by 10 mm eclipse shape. It may be dried and fixed by heat.

Smears from sputum samples are commonly stained using Ziehl Neelsen (ZN) or by fluorescence staining technique and examined microscopically for the presence of AFB. AFB testing is done as a screening procedure at the point of sample collection and on arrival at TB reference laboratory before culture and molecular confirmation. The waxy mycobacterial cell wall of mycobacterium is resistant to stain by conventional dyes and in one way the phenol-carbolfuchsin stain is forced to penetrate the cell wall by gentle heating underneath the slide flooded with carbolfuchsin. Then acid decolorizing solution is applied to remove the dye in cells, tissue, and any organisms in the smear except mycobacteria, which retain the dye. After decolorization, the smear is counterstained with either malachite green or methylene blue, which stains the background material green or blue, respectively. The contrasting background color enables the red Acid-Fast Bacilli to be visualized under a light microscope using the 100x oil immersion objective.

On the other hand, sputum smear can be stained with Auramine O or Auramine rhodamine dye and decolorized by a decolorizing agent (containing ethanol and hydrochloric acid). Counterstaining of the background by potassium permanganate enables visualization of orange-yellow acid-fast mycobacteria against a black back ground under a fluorescence microscope.

On comparison, fluorescent microscopy is faster in giving results (1 min) compared to ZN stain (4 min) and more sensitive (up to 97%) than ZN staining (up to 94%) and generally the accuracy of fluorescent microscopy is higher (Area Under the curve (AUC) = 96) compared to ZN staining (AUC = 94). However, due to resources constraint, ZN staining is more popular in low-income TB endemic areas [24].

3.3. Mycobacterial culture and molecular characterization

Culture isolation of mycobacteria is the gold standard method for TB diagnosis. Sputum smear stain positive samples are a good candidate for culture and molecular characterization.

However, manipulation of suspected mycobacterial samples should be handled in a containment facility to minimize the danger of exposure to personnel and the surrounding environment. If samples are collected far from the culture laboratory, and to prevent contamination, transport in CPC is recommended. Culturing of such samples should be within 7–8 days of collection and storage has given accurate results compared to culture from non-CPC stored samples [25]. Recent reports have shown good yield from CPC stored sputum samples for up to 21 days [26]. This is relevant in low-income countries where focal sample processing and culture centers are far from collection sites. Transport and storage times are worth noting as positivity from culture has been reported to be significantly affected beyond 2 weeks [27].

3.3.1. Sputum sample processing, culture, and identification

During sample processing, the sputum-CPC mixture is normally concentrated by centrifuging at 4000 rpm for 15 min, supernatants poured off into a splash proof container. The sediments can then be mixed with 20 ml of sterile distilled water, suspended by inverting the tubes several times, and then centrifuged at 3500 rpm for 15 min. The supernatant is then removed with the remaining pellets re-suspended in water for the inoculums ready for culture. Two Löwenstein-Jensen slants, one containing 0.75% glycerol and the other 0.6% pyruvate are used for inoculation of the sediments and incubated at 37°C. Growth of Mycobacterial inoculum is examined weekly for 8 weeks; at this juncture cultures with no growth after 8 weeks should be considered negative.

3.3.2. DNA extraction procedures

Several methods are available for extraction of mycobacterial DNA and follow pre-existing protocols depending on the purpose. However, boiling a loop full of bacteria in 100 μL H_2O at 80°C for 60 min can suffice to provide sufficient DNA for subsequent analysis. The crude DNA extracted as per protocol is ready for subsequent analyses or storage at −20°C until typing is done. The most used genotyping methods for mycobacterium DNA are both polymerase chain reaction (PCR)-based spoligotyping; and mycobacterial interspaced repetitive units-variable number tandem repeat (MIRU-VNTR) typing. These techniques are useful and can characterize mycobacterial DNA to strain level and later differentiate into sub-lineages that despite enabling the establishment of transmission chains [28] can assist the mapping of TB strains across regions. This can be done by matching strains in question to existing mycobacterium database at *Mtb* complex (http://www.MIRU-VNTRPlus.org/). Whole genome sequencing can be done to further characterize mycobacteria pathogens. Clinically, whole genome sequencing for *M. tuberculosis* has an advantage in the sense that it provides a more rapid and comprehensive view of the genotype of the pathogen, and with the timely and reliable prediction of drug susceptibility including detection of resistance mutations in clinical samples [29]. Despite the robust software and database tools, Whole genome sequencing needs to be developed for its full potential; it provides the highest resolution when investigating transmission events in possible outbreak scenarios [30]. All these methods are geared at improving treatment outcomes when integrated into routine diagnostic workflows, early reporting of drug resistance rather than dependence on phenotypic drug-susceptibility tests

[31]. In our context, we will discuss other characterization techniques, particularly spoligotyping, and MIRU-VNTR genotyping methods as our main focus rather than mycobacterium whole genome sequencing.

3.3.3. Spacer oligonucleotide typing (Spoligotyping)

In humans, TB infection is mostly due to MTBC, which comprises of a group of seven genetically similar species [32] namely *M. tuberculosis*, *M. bovis*, M. africanum, *M. microti*, *M. canettii*, *M. pinnipedii*, and *M. caprae*. To differentiate such genetically closely related species, special molecular techniques have been in place for quite some time now. Fortunately, despite the high genetic homogeneity among the members, they display divergent phenotypes, eliciting different pathologies, and while some show a degree of host specificities [33]. These qualities have made it possible to differentiate mycobacteria species to strain level and epidemiologically, determining their transmission chain using molecular techniques. Spoligotyping is among PCR-based technique that is used in combination with other molecular methods to establish strain variability in a given population. Using commercially available kits, spoligotyping can be done with reference to existing protocols described previously by Kamerbeek et al. [34]. This PCR-based fingerprinting method detects the presence or absence of 43 variable spacer sequences situated between short direct repeat (DR) sequences in the *M. tuberculosis* genome. The DNA from reference *M. tuberculosis* H37Rv and *M. bovis* BCG clones are commonly used as positive controls while autoclaved ultrapure water is used as a negative control. Visualization of presence (black squares) or absence (blank squares) of variable spacer sequences on film is achieved after incubation with streptavidin-peroxidase and detection of hybridized DNA using chemiluminescent detection liquid followed by exposure to radiography uses electromagnetic radiations (x-ray) film. Resulting spoligotypes are normally reported in octal and binary formats and compared to existing patterns in an international spoligotyping database profiles (SpolDB4.0) [35] available at http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/). Spoligotype patterns can then be grouped as spoligotype international types (SITs) if they share identical spoligotype patterns with patterns present in the existing database. Such strain comparisons using SITs are used to identify TB strains circulating in the population as well as new strains and assign names for new strains, which cannot be found in previous studies where possible [35, 36]. By using spoligotyping, it is possible to map TB strains with their diversity within and between regions [37], not only revealing differences in circulating *M. tuberculosis* strains [38] but also identification of new strains [39] even within the country in endemic areas. Further, molecular characterization has yielded molecular type patterns suggestive of similar strains in humans and in wild ungulates [40].

3.3.4. Mycobacterial interspaced repetitive units-variable number tandem repeat (MIRU-VNTR) typing

This is a PCR-based method that exploits the presence of interspaced repetitive DNA in mycobacterium and other genomes to characterize mycobacteria strains and within strains circulating in a given population. In combination with spoligotyping, the method forms a large-scale, high-throughput genotyping of *M. tuberculosis* [28]. The method is based on the presence of more extensive loci that contain variable number tandem repeat (VNTR) of genetic elements named mycobacterial interspersed repetitive units (MIRUs) located

mainly in intergenic regions dispersed throughout the *M. tuberculosis* genome [28, 41]. The method combines the analysis of multiplex PCRs for the target loci on a fluorescence-based DNA analyzer with computerized automation of the genotyping [42]. Initially, the genotyping system used only 12 loci for epidemiological molecular studies in combination with spoligotyping, but now a more discriminatory 24 loci MIRU-VNTR is in place. This current genotyping system in combination with spoligotyping has been found to be 40% superior in a number of types among isolates from cosmopolitan origins, compared to those obtained with the original set of 12 loci [28]. Nevertheless, researchers still continue to optimize the 24-loci MIRU-VNTR genotyping in an attempt to reduce the turnaround time of typing and financial burden [33] although the MIRU-VNTR typing manual [43] remains primary and key guide to all MIRU-VNTR typing of MTBC strains.

The standardized 24 loci MIRU-VNTR typing protocol by Supply et al. [42] is performed using primers that amplify 24 polymorphic loci on the mycobacterial genome per DNA isolate. The number of tandem repeat units present at each locus is then calculated from the size of DNA fragments according to a standardized table (http://www.MIRU-VNTRplus.org). The results can be expressed in digital format where each number represents the number of repeat copies at a particular locus. Phylogenetic analysis and creation of dendrograms can then be done using MIRU-VNTR*plus* (http://www.MIRU-VNTRPlus.org/) to generate a categorical-based NJ-Tree dendrogram to enable comparison of strain genotypes [44]. Creation of dendrogram from phylogenetic analysis enables the establishment of transmission links.

3.4. Sputum sniffing by African giant pouched rats

In recent years diagnosis of pulmonary TB in humans has taken a turn to involve Trained African giant pouched rats (*Cricetomys gambianus*). The procedure takes advantage of the ability of the African giant pouched rats to detect and discriminate odor of volatile compounds produced by mycobacteria [45]. Weetjens and his colleges extended the dimension of research from landmine detection to TB diagnosis [46].

The rats are trained to sniff heat-inactivated sputum from presumptive TB patients for the purpose of discriminating positive samples from negatives. According to Weetjens and colleagues [46], the rats are domesticated, bred and that training is done to the young rats. At the age of 4 weeks, young rats are identified and assigned to a specific trainer. This is accompanied by socialization and habituation learning. Then the rats are trained to locate the sniffing hole in the cage and sniffing at the age of 8 weeks. Discrimination of odor is introduced and multiple sample evaluation follows, at the age of 8 months the rats start operational training. During training, the rats learn to associate odor of TB volatiles with reward (food: peanut or banana). The trained rats sniff holes with sputum samples in the cage. The negative samples are sniffed for less than a second and ignored while for the positive samples, the rat will fix their nose at the hole for at least 5 seconds. In this exercise, each of positive sample detection is accompanied by a reward in form of food [46].

The rats can detect *M. tuberculosis*-specific volatiles such as nicotinate, methyl para-anisate, and ortho-phenylanisole from sputum infected with MTBC, *M. avium*, *M. intracellulare* and other NTM [47]. The detection has been reported to be at 80.4% sensitivity, 72.4% specificity,

and 73.9% accuracy [47]. Sample evaluation results are potentiated by use of multiple rats (e.g. group of four) and positive indication by at least one rat criteria has been reported to be more sensitive than multiple positive indications [48].

Cricetomys gambianus are resistant to TB infection [48]. The rats can detect mycobacteria in sputum in as low concentration as a few bacteria in less than 10 sec [49]. In contrast to a trained microscopist who can examine less than 50 samples a day, the rats can detect hundreds of samples per day before exhaustion [50]. These features make sputum sniffing by the giant African rats a potential rapid screening test. Such technique has been used in Tanzania and Mozambique as a second line screening after ZN staining sputum smear microscopy where 44% increase in TB case detection rate has been reported [48]. However, sputum sniffing by the African giant rats does not guarantee 100% accurate TB diagnosis. Although this disqualifies it as a stand-alone TB diagnostic or first line screening test for presumptive test it can be useful in increasing TB cases among smear-negatives especially in low-income TB endemic countries where diagnostics capabilities are limited [51].

Moreover, TB diagnosis by sniffing rats works in active TB, it may not be handy in latent TB cases. Furthermore, the rats are living creature whose health status needs attention and any anatomical, physiological, or functional deformity may impair TB diagnosis.

4. Patho-physiological assessment in TB diagnosis

Pathological and or physiological changes in the body can be used to diagnose mycobacteria infections or TB. Two common in vivo methods, TST, and chest radiography are presented in this chapter.

4.1. Tuberculin skin test (TST)

The TST is the internationally recognized standard method to identify infection with *M. tuberculosis* and *M. bovis*. Intradermal tuberculin tests based on eliciting a delayed-type hypersensitivity response, which is mediated by a population of sensitized T-cells and takes some weeks to develop after infection [52]. The TST use the purified protein derivative (PPD) tuberculins that have been derived from *M. bovis* and *M. tuberculosis* for detection of latent TB in cattle and in human, respectively.

Techniques that are used for tuberculin skin testing in human include; Multiple puncture tests (tine test, heaf test, and MONO-VACC test) and intracutaneous injections either given by the jet injector or by the Mantoux test [53]. Multiple puncture tests introduce tuberculin into the skin through tuberculin-coated prongs while the Mantoux skin test involves the intracutaneous injection of tuberculin into the volar surface of the forearm [54]. The Mantoux test is recommended for clinical assessment or screening since it is more sensitive and specific than the other methods. The reported sensitivity and specificity of TST in human ranges from 59 to 100% and 70 to 100%, respectively [53].

There are several factors that are associated with false negative results of intradermal skin test in humans. These include recent or advanced TB, severe malnutrition, immuno deficiency

due to the HIV or immunosuppressive chemotherapy, co-infections with viral, bacterial and fungal diseases affecting lymphoid organs (lymphoma, chronic lymphoid leukemia sarcoidosis), metabolic derangement (chronic renal failure), stress (surgery, burns, and mental illness), and live vaccination (measles, mumps, and polio) [53, 54]. False positive reactions are more commonly attributed to co-infection or pre-exposure to other related NTM in cattle and human [55] and BCG vaccination in human [56].

Despite of the several major limitations, TST has been, until recently, a useful procedure for detecting LTBI [57] and most widely used test for control of Bovine TB due to the low-cost, low logistical demands, well-documented use, and its ability to screen the entire cattle populations [52]. Due to the limitations of the sensitivity and specificity of the skin tests, newer ancillary in vitro diagnostic assays for latent tuberculosis has been developed, which measures the production of interferons in whole blood upon stimulation with PPD [52].

4.2. Imaging in TB diagnosis

Radiography uses electromagnetic radiations (x-rays) to visualize internal body organs. Chest radiography is used for imaging lungs, airways, ribs, heart, and diaphragm. The rays are allowed to pass through tissues and captured by a film. Variation of absorption of x-rays by different tissues enables contrasting visualization. Hard tissues, for example, bones absorb more rays and appear dense while soft tissues allow more passage of rays and appear light. Knowledge of anatomy is important for diagnostic interpretation.

According to WHO [58] chest radiography is indicated as a pulmonary TB diagnostic tool for triaging, screening and as a diagnostic aid when clinical pulmonary TB cannot be confirmed bacteriologically. Chest radiography can also be used in the evaluation of TB treatment response. It is, therefore, part of an algorithm within health care system. It can be used to distinguish between active and inactive pulmonary TB based on a temporal evaluation of radiographs whereby the latter is characterized by stable radiographic pictures for 6 months [59].

Most common chest radiographic findings of TB include lymphadenopathy, parenchymal opacities, obstructive atelectasis, pleural effusion, cavitations, and tuberculomas [59]. Chest radiography has high-sensitivity (97%, 95% CI 0.90–1.00) and low-specificity (67%, 95% CI 0.64–0.70) [60]. It can detect any abnormality in the chest including those related to other non-TB pathological conditions. Other imaging techniques such as computed tomography (CT) and Magnetic resonance imaging (MRI) are superior to chest radiography and can be used for reference. It is important to note that besides the low specificity, chest radiography is expensive in terms of equipment and skilled labor, especially in low-income endemic areas.

5. Urine sample tests in TB diagnosis

Advocacy toward non-invasive, non-sputum simple TB diagnosis has pushed research into different directions including the use of urine sample from presumptive TB patients. There are a number of target TB biomarkers in urine including volatile compounds, proteins, and TB antigen.

In a study by Cannas and colleagues [61], traces of mycobacteria DNA were detected in the urine of TB patients at 79% while the controls were negative. In addition, proteins produced in lung lesions and excreted in urine were also recognized by immunoglobulin G (IgG) from active TB patients [62]. Other TB protein biomarkers in the urine of TB patients have been reported by Young and colleagues [63]. In a similar study, Lim and friends [64] tested urine for TB by using a colorimetric sensor array (CSA). Urine headspace analysis showed discrimination between TB and control patients with 85.5% sensitivity and 79.5% specificity. Analysis of volatile organic compounds using headspace gas chromatography/mass spectrometry (GC/MS) showed increased levels of o-xylene and isopropyl acetate and decreased levels of 3-pentanol, dimethylstyrene, and cymol in the urine of TB patients compared to controls with respiratory diseases other than TB [65].

Urine-based TB diagnostic procedures can only detect biomarkers related to active TB and not latent TB. However, the promising feature is that it can detect extra pulmonary TB. Nevertheless, more researches are required to unravel the practicability of the tests.

6. Breath tests in TB diagnosis

Volatile organic compounds (VOCs) in breath have shown to contain biomarkers of active pulmonary tuberculosis derived from the infectious organism (metabolites of *M. tuberculosis*) and from the infected host (products of oxidative stress).

6.1. Breath sample collection

According to the method described by Braden and colleague, [66], a portable breath collection apparatus (BCA) is used to capture the VOCs in 1.0 L breath and 1.0 L room air on to separate sorbent traps. The geometry of the breath reservoir of the BCA is set to ensure that the sample comprises >99% alveolar breath. Subjects wear a nose-clip and respire normally for 2.0 min through a disposable valve mouthpiece with a bacterial filter to prevent mycobacterial contamination of the instrument. The mouthpiece and filter present low-resistance to respiration ensuring that samples are collected without causing any discomfort to patients.

6.2. Breath sample analysis

The VOCs captured in the sorbent traps are then analyzed in the laboratory according to the method described by Phillips where an automated thermal desorption, gas chromatography, and mass spectroscopy (ATD/GC/MS) is employed. To quantify peak areas and control for drift in instrument performance, an internal standard is normally run with every chromatographic assay of breath and air (0.25 mL 2 ppm 1-bromo-4-fluorobenzene, Supelco, Bellefonte, and PA).

Chromatographic data collected are then converted into a series of data points by segmenting them into a series of time slices. The alveolar gradient of each time slice is then determined (i.e. abundance in alveolar breath minus abundance in ambient room air) [67, 68].

For each of the time slice, the alveolar gradient is calculated by taking: ¼ Vb /Ib Va/Ia.

Where, Vb is the integrated abundance of analytes detected by mass spectroscopy in a breath, and Ib is the area under the curve (AUC) of the chromatographic peak associated with the internal standard. Va and Ia denote corresponding values derived from the associated sample of room air.

6.3. Identification of biomarker time slices

The alveolar gradients are identified by comparing the patients positive or negative for active pulmonary TB and rank them as candidate biomarkers according to the value of the C-statistic that is, the AUC of the receiver operating characteristic (ROC) curve [69]. The Kovats Index windows for active TB are clearly distinct from controls in a test that takes 6 min. The detection of TB biomarkers is 80% accurate with 71.2% sensitivity and 72% specificity [70].

TB testing using breath provides hope for a future non-invasive diagnostic procedure. Phillips et al. have put effort to assess the presence of volatile organic compounds in the breath of active TB patients. A special device collected and concentrated breath from TB patients and controls at point-of-care centers. However, the procedure requires special set up and equipment and may not be suitable in low-income endemic settings.

7. Challenges in diagnosis of tuberculosis

In the diagnosis of tuberculosis in both humans and animals, the challenges have always been the availability of better and affordable diagnostic methods. While conventional tests such as ZN test for sputum has been in place and are the gold standard, it can miss some cases due to low-sensitivity. Mycobacterial cultures can improve detection but takes longer to get results. Molecular characterization to nucleotide level that is, through sequencing is perfect but usually unaffordable in low-income countries diagnostic settings. In addition, DNA isolation, species identification, and obtaining cultures from a sophisticated system may face limitations as well. All these challenges necessitate for advocacy focusing on innovations that deliver better tools to confidently diagnose TB and at affordable costs [71]. Although, international and national laboratory partnerships are encouraged particularly to boost diagnostic services in resource-poor countries, the need for diagnostic tests that allow rapid testing at point-of-care is necessary [72]. However, all these need acceptance by health authorities in respective countries for incorporation into countries diagnostic algorithms while ensuring inherent and adequate quality assurance programs in dedicated laboratories. In marginalized communities, refusal of diagnosis, high indirect costs, and anticipated treatment side-effects have posed barriers to TB diagnosis [73]. All these need to be considered if we want to perform right diagnosis and management of tuberculosis across endemic regions. Screening programs for both human and animal tuberculosis could cut the costs of modern molecular diagnostics and characterization but these programs are costly and might currently not be of primary priority by responsible authorities. Otherwise, screening programs despite potentially expected high initial capital investment, their value on cutting down diagnostic costs for TB cannot be overemphasized.

8. Features of desired TB diagnostic procedure

Currently, the procedure includes medical history, physical examination, chest radiography, TST, serologic tests (e.g. IGRA), microbiologic smear (e.g. ZN), and culture [59].

The distinction between and transition from LTBI to active TB as well as differentiation of stages of active TB toward recovery are attributes of a nearly perfect TB diagnostic procedure. This should be coupled with the ability to detect early stages of the TB related health status and a short time to diagnosis. Applicability and convenience of diagnostic (set of) procedures in all life settings are factors to consider when planning a TB diagnostic package. It is not easy to find the majority of these merits in one procedure but a combination of both conventional and molecular tools starting from screening to diagnosis can facilitate the mission of fighting TB.

9. Diagnostic research direction

TB diagnostic research course moves toward blood parameters such as immune response components, protein, and pathological parameters or other tissues (cerebral-spinal fluid). Such parameters shed more light for future informed diagnosis including status and stage of infection or treatment. Converging different research routes may provide an outcome, which shows effects of interactions. For sure there may be antagonistic but also synergistic outcome. For instance, an approach that will detect the presence of the pathogen and also inform about immune status, pathological, or prognostic prediction is required. All in all, instituting programs that focus on screening tests for early detection of the disease can perfectly fit in the strategy of disease control and management. In so doing, disease elimination can be made possible.

10. Conclusion

There is no single best diagnostic approach that can suit all settings including low-income endemic areas. Some procedures are weak in some aspects and can be complemented by others synergistically. This calls for research focus on combinations of procedures and programs, which give out quick and discriminatory results. For instance, a diagnostic procedure should be able to detect LTBI, active TB and different stages of recovery during treatment. Nevertheless, having programs that focus on screening of the disease in our population will definitely reduce diagnostic as well as treatment costs. This can be done particularly through active case finding (ACF) that will enable screening of TB by systematically searching individuals who would otherwise spontaneously not nock the door and present for care at health facility. In so doing early case detection will be increased particularly in marginalized populations [73–76]. Such areas are devoid of reliable and dedicated diagnostic facility under normal settings.

Author details

Athumani Msalale Lupindu[1]*, Erasto Vitus Mbugi[2], Jahash Nzalawahe[3] and Abubakar Shaaban Hoza[3]

*Address all correspondence to: amsalale@yahoo.com

1 Department of Veterinary Medicine and Public Health, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, Morogoro, Tanzania

2 Department of Biochemistry, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania

3 Department of Veterinary Microbiology, Parasitology and Biotechnology, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, Morogoro, Tanzania

References

[1] WHO. Global Tuberculosis Report 2017. Geneva: World Health Organization; 2017

[2] Mack U, Migliori GB, Sester M, Rieder HL, Ehlers S, Goletti D, et al. LTBI: Latent tuberculosis infection or lasting immune responses to *M. tuberculosis*? A TBNET consensus statement for the TBNET. European Respiratory Journal. 2009;**33**:956-973. DOI: 10.1183/09031936.00120908

[3] Houben RMGJ, Dodd PJ. The global burden of latent tuberculosis infection: A re-estimation using mathematical modelling. PLoS Medicine. 2016;**13**:e1002152. DOI: 10.1371/journal.pmed.1002152

[4] Getahun H, Matteelli A, Chaisson RE, Raviglione M. Latent *Mycobacterium tuberculosis* infection. The New England Journal of Medicine. 2015;**372**:2127-2135. DOI: 10.1056/NEJMra1405427

[5] Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma interferon release assays for detection of *Mycobacterium tuberculosis* infection. Clinical Microbiology Reviews. 2014;**27**:3-20. DOI: 10.1128/CMR.00034-13

[6] Olea-Popelka F, Muwonge A, Perera A, Dean AS, Mumford E, Erlacher-Vindel E, et al. Zoonotic tuberculosis in human beings caused by *Mycobacterium bovis*—A call for action. The Lancet Infectious Diseases. 2017;**17**:e21-e25. DOI: 10.1016/S1473-3099(16)30139-6

[7] WHO. Gear up to End TB: Introducing the End TB Strategy. Geneva: World Health Organization; 2017

[8] Sandhu G, Battaglia F, Ely BK, Athanasakis D, Montoya R, Valencia T, et al. Discriminating active from latent tuberculosis in patients presenting to community clinics. PLoS One. 2012;**7**:e38080. DOI: 10.1371/journal.pone.0038080

[9] Cliff JM, Kaufmann SHE, McShane H, van Helden P, O'Garra A. The human immune response to tuberculosis and its treatment: A view from the blood. Immunological Reviews. 2015;**264**:88-102. DOI: 10.1111/imr.12269

[10] Tufariello JM, Chan J, Flynn JL. Latent tuberculosis: Mechanisms of host and bacillus that contribute to persistent infection. The Lancet Infectious Diseases. 2003;**3**:578-590. DOI: 10.1016/S1473-3099(03)00741-2

[11] CDC. TB Elimination. Interferon-Gamma Release Assays (IGRAs) – Blood Tests for TB Infection. Natl Cent HIV/AIDS, Viral Hepatitis, STD, TB Prev Div Tuberc Elimin; 2011. pp. 1-3. https://www.cdc.gov/tb/publications/factsheets/testing/IGRA.pdf [Accessed March 21, 2018]

[12] Seyhan EC, Gunluoglu M, Tural S, Sokucu S, Gunluoglu G. Predictive value of the tuberculin skin test and QuantiFERON-tuberculosis gold in-tube test for development of active tuberculosis in hemodialysis patients. Annals of Thoracic Medicine. 2016;**11**: 114-120. DOI: 10.4103/1817-1737.180023

[13] Starke JR. Committee on infectious diseases. Interferon-γ release assays for diagnosis of tuberculosis infection and disease in children. Pediatrics. 2014;**134**:e1763-e1773. DOI: 10.1542/peds.2014-2983

[14] Steffen RE, Caetano R, Pinto M, Chaves D, Ferrari R, Bastos M, et al. Cost-effectiveness of quantiferon®-tb gold-in-tube versus tuberculin skin testing for contact screening and treatment of latent tuberculosis infection in Brazil. PLoS One. 2013;**8**:e59546. DOI: 10.1371/journal.pone.0059546

[15] Nijhawan AE, Iroh PA, Brown LS, Winetsky D, Porsa E. Cost analysis of tuberculin skin test and the QuantiFERON-TB gold in-tube test for tuberculosis screening in a correctional setting in Dallas, Texas, USA. BMC Infectious Diseases. 2016;**16**:564. DOI: 10.1186/s12879-016-1901-8

[16] Berry MPR, Graham CM, McNab FW, Xu Z, Bloch SAA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;**466**:973-977. DOI: 10.1038/nature09247

[17] Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A blood RNA signature for tuberculosis disease risk: A prospective cohort study. Lancet. 2016;**387**:2312-2322. DOI: 10.1016/S0140-6736(15)01316-1

[18] Agranoff D, Fernandez-Reyes D, Papadopoulos MC, Rojas SA, Herbster M, Loosemore A, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. Lancet. 2006;**368**:1012-1021. DOI: 10.1016/S0140-6736(06)69342-2

[19] Liu J, Jiang T, Wei L, Yang X, Wang C, Zhang X, et al. The discovery and identification of a candidate proteomic biomarker of active tuberculosis. BMC Infectious Diseases. 2013;**13**:506. DOI: 10.1186/1471-2334-13-506

[20] Zhang J, Wu X, Shi L, Liang Y, Xie Z, Yang Y, et al. Diagnostic serum proteomic analysis in patients with active tuberculosis. Clinica Chimica Acta. 2012;**413**:883-887. DOI: 10.1016/J.CCA.2012.01.036

[21] ECDC. Use of Interferon-Gamma Release Assays in Support of TB Diagnosis. Stockholm: European Centre for Disease Prevention and Control; 2011. DOI: 10.2900/38588

[22] Clegg TA, Good M, Doyle M, Duignan A, More SJ, Gormley E. The performance of the interferon gamma assay when used as a diagnostic or quality assurance test in Mycobacterium bovis infected herds. Preventive Veterinary Medicine. 2017;**140**:116-121. DOI: 10.1016/J.PREVETMED.2017.03.007

[23] Pardini M, Varaine F, Iona E, Arzumanian E, Checchi F, Oggioni MR, et al. Cetylpyridinium chloride is useful for isolation of *Mycobacterium tuberculosis* from sputa subjected to long-term storage. Journal of Clinical Microbiology. 2005;**43**:442-444. DOI: 10.1128/JCM.43.1.442-444.2005

[24] Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: A systematic review. The Lancet Infectious Diseases. 2006;**6**:570-581. DOI: 10.1016/S1473-3099(06)70578-3

[25] Pal N, Sharma B, Malhotra B, Rishi S. Transport and storage of sputum specimen by using cetylpyridinium chloride for isolation of mycobacteria. Indian Journal of Pathology & Microbiology. 2009;**52**:59-61. DOI: 10.4103/0377-4929.44966

[26] Hiza H, Doulla B, Sasamalo M, Hella J, Kamwela L, Mhimbira F, et al. Preservation of sputum samples with cetylpyridinium chloride (CPC) for tuberculosis cultures and Xpert MTB/RIF in a low-income country. BMC Infectious Diseases. 2017;**17**:542. DOI: 10.1186/s12879-017-2642-z

[27] Das D, Dwibedi B, Kar SK. Transportation of sputum samples in cetylpyridinium chloride for drug resistance studies from remote areas of Odisha, India. Journal of Infection in Developing Countries. 2014;**8**:1059-1062. DOI: 10.3855/jidc.4384

[28] Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology. 2006;**44**:4498-4510. DOI: 10.1128/JCM.01392-06

[29] Brown AC, Bryant JM, Einer-Jensen K, Holdstock J, Houniet DT, Chan JZM, et al. Rapid whole-genome sequencing of *Mycobacterium tuberculosis* isolates directly from clinical samples. Journal of Clinical Microbiology. 2015;**53**:2230-2237. DOI: 10.1128/JCM.00486-15

[30] Witney AA, Cosgrove CA, Arnold A, Hinds J, Stoker NG, Butcher PD. Clinical use of whole genome sequencing for *Mycobacterium tuberculosis*. BMC Medicine. 2016;**14**:46. DOI: 10.1186/s12916-016-0598-2

[31] Walker TM, Kohl TA, Omar SV, Hedge J, Del Ojo Elias C, Bradley P, et al. Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: A retrospective cohort study. The Lancet Infectious Diseases. 2015;**15**:1193-1202. DOI: 10.1016/S1473-3099(15)00062-6

[32] Imaeda T. Deoxyribonucleic acid relatedness among selected strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*. International Journal of Systematic Bacteriology. 1985;**35**: 147-150. DOI: 10.1099/00207713-35-2-147

[33] de Beer JL, Akkerman OW, Schürch AC, Mulder A, van der Werf TS, van der Zanden AGM, et al. Optimization of standard in-house 24-locus variable-number tandem-repeat typing for *Mycobacterium tuberculosis* and its direct application to clinical material. Journal of Clinical Microbiology. 2014;**52**:1338-1342. DOI: 10.1128/JCM.03436-13

[34] Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. Journal of Clinical Microbiology. 1997;**35**:907-914

[35] Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, et al. *Mycobacterium tuberculosis* complex genetic diversity: Mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiology. 2006;**6**:23. DOI: 10.1186/1471-2180-6-23

[36] Vitol I, Driscoll J, Kreiswirth B, Kurepina N, Bennett KP. Identifying *Mycobacterium tuberculosis* complex strain families using spoligotypes. Infection, Genetics and Evolution. 2006;**6**:491-504. DOI: 10.1016/J.MEEGID.2006.03.003

[37] Mbugi EV, Katale BZ, Streicher EM, Keyyu JD, Kendall SL, Dockrell HM, et al. Mapping of *Mycobacterium tuberculosis* complex genetic diversity profiles in Tanzania and other African countries. PLoS One. 2016;**11**:e0154571. DOI: 10.1371/journal.pone.0154571

[38] Gulich GA, Tulu KT, Worku A, Zewude A, Chimidi GA. Molecular characterization of *Mycobacterium tuberculosis* complex in Gambella region, south West Ethiopia. Journal of Medical Diagnostic Methods. 2015;**4**. DOI: 10.4172/2168-9784.1000175

[39] Debebe T, Admassu A, Mamo G, Ameni G. Molecular characterization of *Mycobacterium tuberculosis* isolated from pulmonary tuberculosis patients in Felege Hiwot referral hospital, Northwest Ethiopia. Journal of Microbiology, Immunology, and Infection. 2014;**47**:333-338. DOI: 10.1016/j.jmii.2013.03.012

[40] Gortazar C, Vicente J, Samper S, Garrido JM, Fernández-De-Mera IG, Gavín P, et al. Molecular characterization of *Mycobacterium tuberculosis* complex isolates from wild ungulates in south-Central Spain. Veterinary Research. 2005;**36**:43-52. DOI: 10.1051/vetres:2004051

[41] Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, Locht C. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Molecular Microbiology. 2002;**36**:762-771. DOI: 10.1046/j.1365-2958.2000.01905.x

[42] Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. Journal of Clinical Microbiology. 2001;**39**:3563-3571. DOI: 10.1128/JCM.39.10.3563-3571.2001

[43] Supply P, Gaudin C, Raze D. Optimization of standard 24-locus variable-number tandem-repeat typing of *Mycobacterium tuberculosis* isolates: A multicenter perspective. Journal of Clinical Microbiology. 2014;**52**:3518-3519. DOI: 10.1128/JCM.01790-14

[44] Weniger T, Krawczyk J, Supply P, Niemann S, Harmsen D. MIRU-VNTRplus: A web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. Nucleic Acids Research. 2010;**38**:W326-W331. DOI: 10.1093/nar/gkq351

[45] Mgode GF, Weetjens BJ, Nawrath T, Lazar D, Cox C, Jubitana M, et al. *Mycobacterium tuberculosis* volatiles for diagnosis of tuberculosis by Cricetomys rats. Tuberculosis (Edinburgh, Scotland). 2012;**92**:535-542. DOI: 10.1016/j.tube.2012.07.006

[46] Weetjens BJ, Mgode GF, Machang'u RS, Kazwala R, Mfinanga G, Lwilla F, et al. African pouched rats for the detection of pulmonary tuberculosis in sputum samples. The International Journal of Tuberculosis and Lung Disease. 2009;**13**(6);737-743

[47] Mgode GF, Weetjens BJ, Nawrath T, Cox C, Jubitana M, Machang'u RS, et al. Diagnosis of tuberculosis by trained African giant pouched rats and confounding impact of pathogens and microflora of the respiratory tract. Journal of Clinical Microbiology. 2012;**50**:274-280. DOI: 10.1128/JCM.01199-11

[48] Weetjens BJ, Mgode GF, Machang'u RS, Kazwala R, Mfinanga G, Lwilla F, et al. African pouched rats for the detection of pulmonary tuberculosis in sputum samples. The International Journal of Tuberculosis and Lung Disease. 2009;**13**:737-743

[49] Mahoney A, Edwards TL, Weetjens BJ, Cox C, Beyene N, Jubitana M, et al. Giant African pouched rats (*Cricetomys gambianus*) as detectors of tuberculosis in human sputum: Two operational improvements. Psychological Record. 2013;**63**:583-594. DOI: 10.11133/j.tpr.2013.63.3.012

[50] Mahoney A, Weetjens BJ, Cox C, Jubitana M, Kazwala R, Mfinanga GS, et al. Giant African pouched rats as detectors of tuberculosis in human sputum: Comparison of two techniques for sputum presentation. Psychological Record. 2013;**63**:21-26. DOI: 10.11133/j.tpr.2013.63.1.002

[51] Mulder C, Mgode GF, Ellis H, Valverde E, Beyene N, Cox C, et al. Accuracy of giant African pouched rats for diagnosing tuberculosis: Comparison with culture and Xpert ® MTB/RIF. The International Journal of Tuberculosis and Lung Disease. 2017;**21**:1127-1133. DOI: 10.5588/ijtld.17.0139

[52] Bezos J, Marqués S, Álvarez J, Casal C, Romero B, Grau A, et al. Evaluation of single and comparative intradermal tuberculin tests for tuberculosis eradication in caprine flocks in Castilla y León (Spain). Research in Veterinary Science. 2014;**96**:39-46. DOI: 10.1016/J.RVSC.2013.10.007

[53] Goldstein EJC, Lee E, Holzman RS. Evolution and current use of the tuberculin test. Clinical Infectious Diseases. 2002;**34**:365-370. DOI: 10.1086/338149

[54] Huebner RE, Bass MFS, JB J. The tuberculin skin test. Clinical Infectious Diseases. 1993;**17**:968-975. DOI: 10.2307/4457498

[55] Harboe M. Antigens of PPD, old tuberculin, and autoclaved *Mycobacterium bovis* BCG studied by crossed immunoelectrophoresis. The American Review of Respiratory Disease. 1981;**124**:80-87. DOI: 10.1164/ARRD.1981.124.1.80

[56] Daniel TM, Janicki BW. Mycobacterial antigens: A review of their isolation, chemistry, and immunological properties. Microbiological Reviews. 1978;**42**:84-113

[57] Jasmer RM, Nahid P, Hopewell PC. Latent tuberculosis infection. The New England Journal of Medicine. 2002;**347**:1860-1866. DOI: 10.1056/NEJMcp021045

[58] WHO. Chest Radiography in Tuberculosis Detection: Summary of Current WHO Recommendations and Guidance on Programmatic Approaches. Geneva: WHO; 2016

[59] Piccazzo R, Paparo F, Garlaschi G. Diagnostic accuracy of chest radiography for the diagnosis of tuberculosis (TB) and its role in the detection of latent TB infection: A systematic review. The Journal of Rheumatology. Supplement. 2014;**91**:32-40. DOI: 10.3899/jrheum.140100

[60] den Boon S, White NW, van Lill SWP, Borgdorff MW, Verver S, Lombard CJ, et al. An evaluation of symptom and chest radiographic screening in tuberculosis prevalence surveys. The International Journal of Tuberculosis and Lung Disease. 2006;**10**:876-882

[61] Cannas A, Goletti D, Girardi E, Chiacchio T, Calvo L, Cuzzi G, et al. *Mycobacterium tuberculosis* DNA detection in soluble fraction of urine from pulmonary tuberculosis patients. The International Journal of Tuberculosis and Lung Disease. 2008;**12**:146-151

[62] Kashino SS, Pollock N, Napolitano DR, Rodrigues V Jr, Campos-Neto A. Identification and characterization of *Mycobacterium tuberculosis* antigens in urine of patients with active pulmonary tuberculosis: An innovative and alternative approach of antigen discovery of useful microbial molecules. Clinical and Experimental Immunology. 2008;**153**:56-62. DOI: 10.1111/j.1365-2249.2008.03672.x

[63] Young BL, Mlamla Z, Gqamana PP, Smit S, Roberts T, Peter J, et al. The identification of tuberculosis biomarkers in human urine samples. The European Respiratory Journal. 2014;**43**:1719-1729. DOI: 10.1183/09031936.00175113

[64] Lim SH, Martino R, Anikst V, Xu Z, Mix S, Benjamin R, et al. Rapid diagnosis of tuberculosis from analysis of urine volatile organic compounds. ACS Sensors. 2016;**1**:852-856. DOI: 10.1021/acssensors.6b00309

[65] Banday KM, Pasikanti KK, Chan ECY, Singla R, Rao KVS, Chauhan VS, et al. Use of urine volatile organic compounds to discriminate tuberculosis patients from healthy subjects. Analytical Chemistry. 2011;**83**:5526-5534. DOI: 10.1021/ac200265g

[66] Braden B, Lembcke B, Kuker W, Caspary WF. 13C-breath tests: Current state of the art and future directions. Digestive and Liver Disease. 2007;**39**:795-805. DOI: 10.1016/j.dld.2007.06.012

[67] Phillips M. Method for the collection and assay of volatile organic compounds in breath. Analytical Biochemistry. 1997;**247**:272-278. DOI: 10.1006/ABIO.1997.2069

[68] Phillips M, Greenberg J, Awad J. Metabolic and environmental origins of volatile organic compounds in breath. Journal of Clinical Pathology. 1994;**47**:1052-1053. DOI: 10.1136/JCP.47.11.1052

[69] Cook NR. Statistical evaluation of prognostic versus diagnostic models: Beyond the ROC curve. Clinical Chemistry. 2008;**54**:17-23. DOI: 10.1373/clinchem.2007.096529

[70] Phillips M, Basa-Dalay V, Blais J, Bothamley G, Chaturvedi A, Modi KD, et al. Point-of-care breath test for biomarkers of active pulmonary tuberculosis. Tuberculosis (Edinburgh, Scotland). 2012;**92**:314-320. DOI: 10.1016/j.tube.2012.04.002

[71] Nema V. Tuberculosis diagnostics: Challenges and opportunities. Lung India. 2012;**29**: 259-266. DOI: 10.4103/0970-2113.99112

[72] Parsons LM, Somosközi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: Challenges and opportunities. Clinical Microbiology Reviews. 2011;**24**:314-350. DOI: 10.1128/CMR.00059-10

[73] Lorent N, Choun K, Malhotra S, Koeut P, Thai S, Khun KE, et al. Challenges from tuberculosis diagnosis to care in community-based active case finding among the urban poor in Cambodia: A mixed-methods study. PLoS One. 2015;**10**:e0130179. DOI: 10.1371/journal.pone.0130179

[74] Corbett EL, Bandason T, Duong T, Dauya E, Makamure B, Churchyard GJ, et al. Comparison of two active case-finding strategies for community-based diagnosis of symptomatic smear-positive tuberculosis and control of infectious tuberculosis in Harare, Zimbabwe (DETECTB): A cluster-randomised trial. Lancet. 2010;**376**:1244-1253. DOI: 10.1016/S0140-6736(10)61425-0

[75] Lorent N, Choun K, Thai S, Kim T, Huy S, Pe R, et al. Community-based active tuberculosis case finding in poor urban settlements of Phnom Penh, Cambodia: A feasible and effective strategy. PLoS One. 2014;**9**:e92754. DOI: 10.1371/journal.pone.0092754

[76] Sekandi JN, Neuhauser D, Smyth K, Whalen CC. Active case finding of undetected tuberculosis among chronic coughers in a slum setting in Kampala, Uganda. The International Journal of Tuberculosis and Lung Disease. 2009;**13**:508-513

Chapter 2

Zoonotic Tuberculosis: A Concern and Strategies to Combat

Ravi N. Teppawar, Sandeep P. Chaudhari,
Shilpa L. Moon, Shilpshri V. Shinde,
Wiqar A. Khan and Archana R. Patil

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76802

Abstract

Mycobacterium bovis is the main causal agent of bovine tuberculosis that causes zoonotic tuberculosis in humans. The most common routes of transmission of the agent to human are airborne transmission, consumption of unpasteurized milk, direct contact with infected animals or untreated animal products. Conventional diagnostic methods in combination with modern molecular and immunological techniques should be used for early and accurate diagnosis of the disease. Some of the challenges to tackle and eradicate zoonotic TB in developing countries are having many hosts, absence of early diagnosis, presence of other acute diseases, being economically unable to implement control strategies, and other social and cultural issues. Usually treatment is not recommended in animals but vaccination is carried out in some countries as a preventive measure. Due to the grave consequences of *M. bovis* infection on animal and human health, it is necessary to introduce accurate control measures to reduce the risk of disease in human and animal populations. Proper food hygiene practices, slaughter of the affected animals in developed countries, and segregation of the suspected animals in developing countries along with stronger intersectoral collaboration between the veterinary and medical professions are important for the control of the disease.

Keywords: *M. bovis*, zoonoses, developing countries, challenges, one health

1. Introduction

Livestock plays an important role in the lives of people throughout the world. They provide dietary protein through meat and milk, materials like wool and leather, and draught power for

agricultural activities and contribute to the livelihoods of around 70% of the world's population living in poverty. Livestock is central to survival strategies of poor families, can serve as a repository of a family's wealth, and may be sold as an emergency source of cash, in some settings; their ownership is linked to social status or may also be important for ceremonial, cultural, and religious significance. Due to unavoidable interaction of man and animals; zoonotic diseases remain a genuine threat to public health. Zoonotic diseases are the diseases or infections which are naturally transmitted between animals and humans, for example, tuberculosis, brucellosis, leptospirosis, and so on. In most cases, animals play an essential role in maintaining, distributing, and actually transmitting the infection up to varying degrees into the nature. One of the economically significant zoonotic diseases worldwide is bovine tuberculosis (TB) because of serious public health consequences, high cost of eradication programs mainly in developing countries, and trade restriction on animals and their products [1–4]. It is said to be the leading cause of death by infectious diseases [5]. World Health Organization (WHO) classified bovine tuberculosis among seven neglected zoonotic diseases having the potential to infect man [6].

M. bovis mainly affects cattle, which are the most important animal reservoirs and can be established in wildlife. The link between animal and human tuberculosis has long always been known to be strong, as shown by the works of Villemin [7] and Koch [8], which demonstrated the cross-adaptability of the tubercle bacilli from one species to another and pointed out the danger that tuberculosis could be transmitted from animals to humans [7]. The infection currently poses a major concern in human populations in developing countries, as humans and animals share the same microenvironment. It has been estimated that zoonotic transmission of *M. bovis* is responsible for 10–15% of new human TB cases in developing countries [9]. Bovine TB has been largely eradicated from herds in the developed world by animal TB control and elimination programs, that is, test-and-slaughter programs have drastically reduced the incidence of disease in both animals and humans [10, 11]. However, in developing countries, animal TB is widely distributed and control measures are not applied or are applied sporadically [12, 13].

The human burden of disease cannot be reduced without improving the standards of food safety and controlling bovine TB in the animal reservoir. As with other zoonotic diseases, zoonotic TB cannot be controlled by the human health sector alone. Animal health and food safety sectors must be engaged to address the role of animals in maintaining and transmitting *M. bovis*. The present review highlights chronic multi-species zoonotic TB, its diagnosis prevention and control, veterinary public health challenges, and strategies to combat this important disease.

2. Epidemiology

2.1. The etiological agent

Mycobacteria belong to the order Actinomycetales, family Mycobacteriaceae. The genus *Mycobacterium* includes *Mycobacterium tuberculosis* and *Mycobacterium avium* complexes, other pathogenic *Mycobacteria*, and numerous species of saprophytic microorganisms present in soil and water. The etiologic agents of mammalian tuberculosis are *Mycobacterium tuberculosis*, the main cause of human tuberculosis; *M. bovis*, the agent of bovine tuberculosis; and *M. africanum*,

which causes human tuberculosis in tropical Africa. This last species has characteristics halfway between those of *M. tuberculosis* and *M. bovis*. *M. bovis* is the principal agent of zoonotic tuberculosis. The distribution of *M. bovis* and *M. tuberculosis* is worldwide. *M. africanum* is prevalent in Africa, but it has also been isolated in Germany and England. *M. africanum* strains phenotypically related to *M. tuberculosis* are nitrase positive and are found in Western Africa while those which are similar to *M. bovis* are nitrase negative and are isolated more frequently in Eastern Africa.

The genus mycobacterium is phenotypically characterized as a facultative intracellular microbe, non-capsular, non-spore forming, non-motile, obligate aerobic, and thin-rod bacteria, usually straight or slightly curved having a length of 1–10 length and width of 0.2–0.6 μm. Its cell wall is rich in lipids, that is, mycolic acid, a thick waxy coat responsible for acid fastness, hydrophobicity, greatly contributing to bacterium resistance to many disinfectants, common laboratory stains, antibiotics, and physical injuries [14, 15].

M. bovis is a member of the mycobacterium tuberculosis complex (MTC) and based on 16S ribosomal RNA sequence studies it shared more than 99.95% of identity with other members of MTC [3, 16]. The MTC includes five mycobacterium species, *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. microti*, *M. bovis,* and two subspecies—*M. caprae* and *M. pinnipedii* [17]. In the environment *M. bovis* can survive for various months especially in cold, dark, and moist conditions. The survival period varies from 18 to 332 days at 12–24°C which is dependent on sunlight exposure. It is found that *M. bovis* best survives in frozen tissue and there are adverse effects of tissue preservatives, that is, sodium tetraborate on viability [18]. It has been found that the culture of the organism can be done for approximately 2 years in samples that are stored artificially [14, 18].

2.2. Host range

The most important causes of bovine TB in cattle are *M. bovis* and *M. caprae,* both of which cause infectious diseases [19–22]. *M. bovis* has one of the broadest host ranges of all known pathogens and has been diagnosed worldwide. Cattle are considered to be the true hosts of *M. bovis* [23]. However the isolations of *M. bovis* has also been detected from domestic animals like buffaloes, sheep, goats, pigs, equines, camels, and so on, along with other animals like deer, antelopes, bison, wild boars, primates, llamas, kudus, elephants, foxes, mink, ferrets, rats, elands, tapirs, elks, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, badgers, otters, seals, hares, moles, raccoons, coyotes and lions, tigers, leopards, and lynx [23, 24]. The natural movement of these reservoir animals increases the spread of the disease to domestic animals [25]. *M. caprae* has been reported in many European countries such as Austria, France, Germany, Hungary, Italy, Slovenia, and the Czech Republic. A disease caused by *M. caprae* is not substantially different from that caused by *M. bovis* and the same tests can be used for its diagnosis [26].

2.3. Transmission

Transmission of *M. bovis* can occur between animals, from animals to humans, and vice versa and rarely in between humans [27]. The transmission of *M. bovis* between animals occurs mainly through aerosols. Transmission through other routes like cutaneous, congenital, and genital routes has also been reported. Close contact among animals and intensive breeding

increase the rate of transmission [28]. Other factors like long survival periods of the organism in the environment also contribute to an increased risk of infection [29, 30]. Suckling calves can get the infection through consumption of infected milk. The infected bull semen may transmit diseases through artificial insemination [18].

Humans acquire the *M. bovis* infection from cattle directly by erogenous route or through the direct contact with material contaminated with the secretions of an infected animal or the herd [31, 32]. The individuals at risk are farm workers, zookeepers, milkers, animal dealers, veterinarians, abattoir workers, meat inspectors, autopsy personnel, laboratory personnel, and owners of potential tuberculous pets [33–35]. People in these occupations may develop pulmonary tuberculosis from *M. bovis* and in turn put other humans and susceptible animals at risk [36, 37]. Indirectly, man acquires the disease from animal sources by consumption of unpasteurized infected milk and ingestion of meat and meat products from slaughtered infected cattle [13, 38–41]. Therefore tuberculosis can be foodborne also [42]. The consumption of contaminated milk products possesses more risks than infected meat products because badly infected carcasses are mostly condemned and meat is generally thoroughly cooked [43]. People suffering from *M. bovis* tuberculosis can retransmit the infection to cattle; however, this is not common [44].

2.4. Geographic distribution

Zoonotic TB is distributed globally and is more prevalent in most of Africa, parts of Asia and of the Americas except Antarctica, Caribbean islands, parts of South America and Australia, Iceland, Denmark, Sweden, Norway, Finland, Austria, Switzerland, Luxembourg, Latvia, Slovakia, Lithuania, Estonia, the Czech Republic, Canada, Singapore, Jamaica, Barbados, and Israel [45]. Although most of the developed countries have reduced or eliminated bovine TB from their cattle population, however, the disease is still present in the wildlife of United Kingdom, Canada, the United States, and New Zealand [23]. Eradication programs are in progress in other European countries, Japan, New Zealand, the United States, Mexico, and some countries of Central and South America where it has been eradicated by following strict test-and-slaughter policies [46].

3. Clinical presentation

3.1. In animals

Bovine TB is a chronic debilitating disease usually characterized by formation of nodular granulomas known as tubercles. In many animals the course of the infection is chronic and signs may be absent, even in advanced cases when many organs may be involved. Subclinical signs include weakness, dyspnea, anorexia, emaciation, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. Lesions are commonly observed in the lymph nodes mainly of the head and thorax, lungs, intestines, liver, spleen, pleura, and peritoneum. Head and neck lymph nodes may become visibly affected, sometimes rupture, drain, and in advanced cases may be greatly enlarged and may obstruct air passages, alimentary tract, or

blood vessels. Clinical signs vary with the involvement of the lung manifested through cough, dyspnea, and other signs of low-grade pneumonia which can be induced by changes in temperature or manual pressure on the trachea. Digestive tract involvement is manifested by intermittent diarrhea or constipation, extreme emaciation, and acute respiratory distress may occur during the terminal stages of tuberculosis [26].

3.2. In humans

M. bovis infection in humans has similar clinical forms as those caused by *M. tuberculosis* [27, 34, 44]. Most of the studies have suggested that the common clinical manifestation of *M. bovis* infection in man is associated with the extra-pulmonary form of the disease; however, about half of the post-primary cases involve the lung which is responsible for human-to-human transmission of tuberculosis due to *M. bovis* [13, 31, 44, 47]. The primary infection of the organism in the intestine may heal or it may progress in the intestines or disseminate to other organs [48]. Cervical lymphadenopathy, intestinal lesions, chronic skin tuberculosis, and other non-pulmonary forms are particularly common [13]. Infection due to *M bovis* in humans usually has a prolonged course and symptoms generally takes months or years to appear. Sometimes, the bacteria remain dormant in the host without causing diseases [23]. The common clinical signs of zoonotic TB include loss of appetite, diarrhea, weight loss, intermittent fever, intermittent hacking cough, large prominent lymph nodes, weakness, and so on.

Young children infected with *M. bovis* typically have abdominal infections and older patients suffer from swollen and sometimes ulcerated lymph glands in the neck [49]. Pulmonary disease is more common in people with reactivated infections [50] and this would occur only when some of the animals had active tuberculosis [32]. The symptoms may include fever, cough, chest pain, cavitation, and hemoptysis [50]. The pulmonary form of tuberculosis occurs less frequently and is usually occupationally related [44].

4. Zoonotic TB: a concern

Bovine TB affects the national and international economy in different ways. It is extremely difficult to determine the economic impact of bovine TB on livestock production. The presence of bovine TB infection in livestock reduces the livestock productivity and economically devastates the cattle industry especially the dairy sector. Some losses are related to the animal production, marketing, or trading of the animals as well as the cost involvement while implementing surveillance and control programs. These losses are also extremely important when endangered wildlife species get involved [51, 52]. The direct productivity losses due to bovine TB can be categorized into "on-farm" losses and losses after the slaughtering of animals. On-farm losses consist of the losses from decreased milk and meat production, the increased reproduction efforts, and replacement costs for infected cattle while losses during slaughter consist of the cost of cattle condemnation and retention, with the loss from condemnation being essentially the purchased value of a slaughter animal and the loss [51]. Along with the direct productivity losses, bovine TB has profound economic consequences on international trade; it affects access to foreign markets due to import bans on animals and animal products

from countries where the disease is enzootic. The presence of the disease in wildlife is not only difficult to eradicate and costly but also bovine TB can theoretically affect entire ecosystems with unpredictable impacts in many areas of private interest, for example, tourism [51].

In 2016, WHO estimated 147,000 new human cases of zoonotic TB in people and around 12,500 deaths due to the disease. The implications of zoonotic TB extend beyond human health. Bovine TB threatens the well-being of communities that rely on livestock for their livelihoods. The African region carries the heaviest burden of disease and death due to zoonotic TB, followed by the Southeast Asian region. However, cases of zoonotic TB in people are uncommon in countries where bovine TB in cattle is controlled and where standards of food safety are high. The true burden of zoonotic TB is likely to be underestimated due to a lack of routine surveillance data from most countries. Therefore, the number of people affected by zoonotic TB annually, and thus suffering from health challenges caused by *M. bovis* infection, might be higher than currently estimated in particular, countries where bovine TB is endemic and where laboratory capacity is limited [60].

Current diagnostics for human TB are focused on pulmonary diseases associated with *M. tuberculosis* (sputum smears examination) but zoonotic tuberculosis in human beings is frequently associated with extra-pulmonary tuberculosis and therefore initiation of treatment can be delayed [53, 54]. There is lack of testing to identify the *Mycobacterium* spp.; very few extrapulmonary lesions are being tested, and requirements for mycobacterial culture for diagnostics are often skipped which contribute to under-reporting of human bovine TB cases [55–58]. Determination of species can add important information needed by epidemiological studies to identify sources of infection and routes of transmission [57, 59, 60].

A major challenge in the case of effective treatment and recovery of a patient infected with zoonotic TB is the natural resistance of *M. bovis* to pyrazinamide, one of the four essential medications used in the standard first-line anti-TB treatment regimen [61]. Most of the healthcare providers initiate the treatment without drug susceptibility testing due to which patients with zoonotic TB may receive inadequate treatment. This may lead to development of resistance to other anti-TB drugs. Resistance to additional drugs has also been reported in some *M. bovis* isolates, including rifampicin and isoniazid, and resistance to these two essential first-line drugs is defined as a multidrug-resistant TB, which is a major threat to human health globally. Such a shortcoming has significant implications for the treatment of zoonotic TB. Because most patients worldwide begin tuberculosis treatment without identification of the causative mycobacterium species, the risk of inadequate treatment of patients with undiagnosed *M. bovis* who do not have drug susceptibility testing is increased [62].

5. Diagnosis

Bovine tuberculosis in the live animal is usually diagnosed on the basis of the standard method for the detection of bovine tuberculosis, that is, delayed hypersensitivity reactions. It is done by injecting bovine tuberculin intradermally into the measured area, measuring subsequent swelling at the site of injection after 72 h and measuring skin thickness. Now, purified protein

derivative (PPD) products have been replaced by the heat-concentrated synthetic medium tuberculins due to their higher specificity and easier standardization. The identification of the pathogenic agent is done by the demonstration of acid-fast bacilli by microscopic examination. The *Mycobacterial* isolation on selective culture media and biochemical tests or DNA techniques, such as PCR, confirms infection. A gold standard for routine confirmation of infection is *Mycobacterial* culture method. Animal inoculation is rarely used because of animal welfare considerations. A number of blood tests are also been used for the identification of *M. bovis* [63]. These can be advantageous, especially with intractable cattle, zoo animals, and wildlife [64]. Blood-based laboratory tests now available are gamma-interferon assay, which uses an enzyme-linked immunosorbent assay (ELISA) as the detection method for interferon [65], the lymphocyte proliferation assay, which detects cell-mediated immune responses [66], and the indirect ELISA, which detects antibody responses.

Diagnosis of active TB in people in many parts of the world is based on the sputum smear examination or some rapid assays like Xpert MTB/RIF. But these commonly used tests are not able to differentiate the *M. tuberculosis* complex into the distinct species of *M. tuberculosis* and *M. bovis;* therefore, most cases of zoonotic TB are misclassified. The identification of *M. bovis* can be done by PCR and gene sequencing of culture isolates, but for these tests the proper collection of samples is very essential as zoonotic TB is extra-pulmonary. However, most of the countries lack the capacity to routinely conduct these tests.

6. Treatment

The treatment of animals with tuberculosis is not a favored option in eradication-conscious countries and is not economical. The Bacillus Calmette and Guérin (BCG) vaccine has advantages for use in cattle since the vaccine is safe, inexpensive, and is commercially produced for human application. However, the vaccination of animals with BCG is sensitive to the tuberculin skin test, and animals become test positive in the classical skin test at least for a significant period of post-vaccination. This is the reason why the test-and-slaughter-based control strategies based on tuberculin skin testing were favored above BCG vaccination in many countries [67].

In human tuberculosis, drugs like isoniazid, combinations of streptomycin and para-aminosalicylic, and other acids are commonly used. Long-term therapy requirement of the disease can create the chances of the development of multidrug-resistant (MDR), extremely drug resistant (XDR), and even totally drug resistant (TDR) bacterial strains if treatment regime is not properly followed. BCG vaccine is the only TB vaccine licensed for use in humans. BCG vaccine has variable levels of protection efficacy in humans against pulmonary TB in children and adults, ranging from 0–80% [68]. It is reported that the prevalence of MDR-TB in previously treated cases was 13.9% and in new cases only 2.3%, whereas the overall prevalence of MDR-TB was 5.7% in all cases [69]. Thus, previously treated cases were more vulnerable for being infected by the MDR-TB strain. Therefore, enhanced TB infection control activities, earliest case detection and treatment, strengthening and proper implementation of directly observed treatment, short course (DOTS), are suggested to reduce the burden of MDR-TB [69].

In human medicine, the treatment policy is based on second-line drug susceptibility testing. Most drug regimens currently used to treat MDR-TB include an aminoglycoside (e.g., streptomycin, kanamycin, amikacin) or capreomycin and a fluoroquinolone. The patients' MDR-TB should be managed by or in consultation with physicians experienced in the management of MDR-TB. The internationally recommended highly efficient and cost-effective strategy for TB control is DOTS (directly observed treatment short course). In this strategy, a healthcare worker at a healthcare center or family DOTS supporter at home gives the standard regimen to all MDR-TB confirmed cases daily under direct observation [70]. The regimens consist of the four drugs which are expected to be effective and the duration is a minimum of 18 months. Furthermore, continuous monitoring and capacity building for family DOTS supporters are essential components of the DOTS strategy. Effective treatments of drug susceptible to TB cure the patient, interrupt the TB transmission to other persons, and also prevent the development of drug-resistant strains.

7. Strategies to combat

The epidemiology of zoonotic TB varies throughout the world, depending on the human, livestock, and wildlife populations, and on existing TB control programs, environmental conditions, and the socio-economic status of countries or regions [71]. The relationship between humans, livestock, wildlife, and ecology in the epidemiology of zoonotic TB makes control of the diseases complex [72, 73]. Zoonotic TB is not a new disease but has long been neglected; burden of this disease in humans cannot be fully addressed without considering the animal reservoir and the risk of transmission at the animal-human interface. As with other zoonotic diseases, zoonotic TB cannot be controlled by the human or animal health sector alone. Human, animal health, and food safety sectors must be engaged to address the role of animals in maintaining and transmitting *M. bovis*. Therefore, "One Health" linking human, animal, and environmental health sector of World Health Organization (WHO), the Food and Agricultural Organization of the (FAO), and the World Organization for Animal Health (OIE) together with the International Union against Tuberculosis and Lung launched a comprehensive roadmap for zoonotic TB in people and bovine TB in animals in October 2017. The roadmap is on the basis of "One Health" approach and is centered under three core themes which consist of improvement of scientific evidence base, reduction in disease transmission at the animal-human interface, and strengthening the intersectoral collaboration.

An improvement in the scientific evidence base can be achieved by collecting, analyzing, and recording and a better quality data of the disease, by improving surveillance and reporting bovine TB in humans, livestock, and wildlife. For the better documentation and to generate accurate representative data which can differentiate *M. bovis* and *M. tuberculosis* infections, countries should strive to incorporate zoonotic TB into their routine surveillance activities. A better detection of cases requires health-care provider expertise and strengthened laboratories having improved access to accurate, rapid diagnostic tools coupled with reliable recording and reporting systems, that are case based and preferably electronic. Data regarding consequences of infectiousness, transmission, clinical presentation, and immunologic responses are important for

the development of a vaccine against all forms of TB. But improved surveillance and data quality will be unsuccessful without strengthened laboratory capacity and better access to appropriate diagnostic tools. Coordination and communication across the sectors is critical for investigating disease epidemiology at the human, livestock, and wildlife interface, including the relative importance of direct and indirect routes of transmission in different populations. Sharing the information within different sectors allows for the identification of patients in a particular geographical area which facilitates a target response for the prevention and control of the disease. To interpret multi-species data there is a need for new methodologies for describing multi-species transmission, such as modeling approaches incorporating genetic data. The biological differences in the host-pathogen interaction of *M. tuberculosis* versus *M. bovis* in humans should be further investigated.

Transmission of zoonotic TB at the animal-human interface can be reduced by developing strategies to improve animal health, identifying the pathways for risk and improving food safety. Healthier animal food supply depends on healthier animal population. For the disease-free state of the animal, both government and private veterinary services must be well organized and should be armed with the tools which can detect disease and reduce the disease prevalence. Developed countries can follow the test-and-slaughter programs by giving compensation to the farmers, post-mortem examinations of the carcass, and can trace-back the herds with appropriate tools to identify and implement control strategies. Similarly, in developing countries, a first step could be a target herd to be disease free in a particular zone of a country and this could gradually expand to other herds and zones. While doing this one must ensure the control of livestock movements from endemic areas to disease-free areas. The disease in a people can be prevented by reducing the risk of exposure and transmission of the infectious agent from animals to humans. Along with the knowledge of the principal routes of transmission some other factors such as sociocultural and economic factors should also be taken into the consideration. The use of modern technologies like sequencing, metagenomics, and phylogenetic analyses helps in characterization of sources of infection, mechanism of transmission, and investigation of drug resistance. Food safety practices can be improved by pasteurization of milk and sanitary inspection of carcasses at abattoirs which lead to removal the contaminated animal products from the food chain and also help in the tracing back animals to herds of origin.

Intersectoral collaboration can be achieved by adopting a "One Health" approach which suggests an intersectoral and multidisciplinary approach by engaging both public and private stakeholders. The most relevant sectors include human health sector, veterinary health sector, wildlife authorities, food safety authorities, farming and trade organizations, consumer groups, educational bodies, and financial institutions. Within these sectors, collaborative relationships among farmers, healthcare providers, veterinarians, laboratory experts, epidemiologists, sociologists, economists, wildlife conservationists, and communication specialists must exist. "One Health" approach also addresses that all relevant sectors should work together for developing legislation and policies, designing, and implementing control strategies. Interventions which jointly address human and animal health can increase health and economic benefits for communities, for example, sharing of human resources, equipment, and transport across sectors can reduce operational costs which increase cost-effectiveness.

Disease eradication programs consist of intensive surveillance which includes farm visits, systematic testing of individual cattle, and removal of infected animals along with the segregation of animals in contact with the infected one similarly in control of animal movement. The identification of infected animals or infected carcass prevents unsafe meat from entering the food chain and allows veterinary services trace back to the infected herd. Pasteurization of milk and meat inspection system should be strengthened and designed to prevent the consumption of contaminated products by people. Vaccine is used in human medicine, but it is not widely used as a preventive measure in animals because its efficacy is variable and it can interfere with testing to eliminate the disease. Thus, the establishment of new TB drugs which can be effective within a short term and capable of controlling the emergence of MDR-TB and XDR-TB is critically urgent.

8. Conclusions

Animal and human health is intimately interwoven and food animals serve as a reservoir of diseases of public health significance [74]. Animals with a contagious disease remain in the population and serve as a reservoir of infection for other animals and human beings. Therefore, the development of vaccines for animals against bovine tuberculosis is highly effective for TB control or development of recombinant BCG with expressing luciferase activity can be used as the most effective tool to advance drug development. The screening of TB in human or animal population is a very time-consuming process as *Mycobacterium* grows very slowly; conventional drug screening takes more than 3 weeks and the biosafety level-3 (BSL-3) facility is the basic requirement. Therefore, it is the need of an hour to develop rapid diagnostic procedures which can detect the organism within a short period of time. Some successful efforts are being made for the development of a new screening method to identify TB drug candidates by utilizing luciferase-expressing recombinant *Mycobacterium bovis* bacillus Calmette-Guéren (rBCG) [75].

The risk due to zoonotic TB is significantly less in developed countries than developing countries, which is due to the milk pasteurization and effective bovine tuberculosis control programs. Therefore, food safety of animal-origin food is worth considering. Efforts to improve food safety include scaling up the heat treatment of milk and ante-mortem and post-mortem inspection of all animals entering the food chain which will not only reduce the risk of transmission but also bring substantial benefits for the control of other foodborne diseases. A healthier animal population leads to healthier food supply along with economic benefits and improvements in animal welfare. The epidemiology of bovine TB is well understood and effective control and elimination strategies have been known for a long time but the disease is still widely distributed and often neglected in most developing countries. The increase of this disease in such areas calls for stronger intersectoral collaboration between the medical and veterinary professions to assess and evaluate the scale of the problem, mostly when zoonotic TB could represent a significant risk. Developed countries which follow test-and-slaughter policies still are not able to completely eliminate infection in cattle because of wild animal reservoirs; therefore, they are now focusing on the wild animal vaccination. Therefore, the vaccine research and

development program should be taken into account for possible application of vaccines to the animals, particularly in developing countries. Disease surveillance programs especially in areas where risk factors are present in animals and humans should be considered as a priority.

Author details

Ravi N. Teppawar, Sandeep P. Chaudhari*, Shilpa L. Moon, Shilpshri V. Shinde, Wiqar A. Khan and Archana R. Patil

*Address all correspondence to: vphsandeep@gmail.com

Centre for Zoonoses, Department of Veterinary Public Health, Nagpur Veterinary College, Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra, India

References

[1] Regassa A, Tassew A, Amenu K, Megersa B, Abunna F, Mekibib B, Marcotty T, Ameni G. Erratum to: A cross-sectional study on bovine tuberculosis in Hawassa town and its surroundings, southern Ethiopia. Tropical Animal Health and Production. 2010;**42**(5):1039

[2] Tenguria RK, Khan FN, Pandey SQA. Epidemiological study of zoonotic tuberculosis complex (ZTBC). World Journal of Food Science and Technology. 2011;**1**(3):31-56

[3] Le Roex N, Van Helden PD, Koets AP, Hoal EG. Bovine TB in livestock and wildlife: What's in the genes? Physiological Genomics. 2013;**45**:631-637

[4] Rodriguez-Campos S, Smith NH, Boniotti MB, Aranaz A. Overview and phylogeny of *Mycobacterium tuberculosis* complex organisms: Implications for diagnostics and legislation of bovine tuberculosis. Research in Veterinary Science. 2014;**97**:5-19

[5] Theon C, LoBue P, Enarson D, Kaneene J, de Kantor I. Mint: Tuberculosis a re-emerging disease in animals and humans. Veterinaria Italiana. 2009;**45**:135-181

[6] Ereqat S, Nasereddin A, Levine H, Azmi K, Al-Jawabreh A. First-time detection of *Mycobacterium bovis* in livestock tissues and milk in the West Bank, Palestinian territories. PLOS Neglected Tropical Diseases. 2013:1-7

[7] Davies PDO. Tuberculosis in humans and animals: Are we a threat to each other? Journal of the Royal Society of Medicine. 2006;**99**(10):539-540

[8] Calmette A. In Tuberculosis bacillus infection and tuberculosis in man and animals (Translated by Soper WB, Smith GH). Baltimore: Williams and Wilkins Co.; 1923

[9] Ashford DA, Whitney E, Raghunathan P, Cosivi O. Epidemiology of selected mycobacteria that infect humans and other animals. Revue Scientifique et Technique. 2001;**20**:325-337

[10] Report of a Joint WHO/DFID-AHP Meeting with the participation of FAO and OIE Geneva, 20 and 21 September, 2005. pp. 1-65

[11] Abu Al-Maaly NMH, Abbas MS, Al-Graibawi MAA, Yousif AA. Identification of the Mycobacterium spp. isolated from cows milk samples by using PCR technique. Journal of Veterinary Science & Animal. 2015;**2**:28-31

[12] Bakshi CS, Shah DH, Verma R, Singh RK, Malik M. Rapid differentiation of *Mycobacterium bovis* and Mycobacterium tuberculosis based on a 12.7-kb fragment by a single tube multiplex-PCR. Veterinary Microbiology. 2005;**109**:211-216

[13] Cosivi O, Grange JM, Daborn CJ, Raviglione MC, Fujikura T, Cousins D. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerging Infectious Diseases. 1998;**4**:59-70

[14] Birhanu T, Mezgebu E, Ejeta E, Gizachew A, Nekemte E. Review on diagnostic techniques of bovine tuberculosis in Ethiopia. Report and Opinion Marsland Press. 2015;**7**:7-14

[15] Jemal AM. Review on zoonotic importance of bovine tuberculosis and its control. Open Access Library Journal. 2016;**3**:e2504. http://dx.doi.org/10.4236/oalib.1102504

[16] Smith NH, Hewinson RG, Kremer K, Brosch R, Gordon SV. Myths and misconceptions: The origin and evolution of *Mycobacterium tuberculosis*. Nature Reviews Microbiology. 2009;**7**:537-544

[17] Smith NH, Gordon SV, de la Rua, Domenech R, Clifton-Hadley RS, Hewinson RG. Bottlenecks and broomsticks: The molecular evolution of *Mycobacterium bovis*. Nature Reviews Microbiology. 2006;**4**(9):670-681

[18] Verma AK, Tiwari R, Chakraborty S, Neha Saminathan M, Dhama K, Singh SV. Insights into Bovine tuberculosis (bovine TB), various approaches for its diagnosis, control and its public health concerns: An update. Asian Journal of Animal and Veterinary Advances. 2014;**9**:323-344

[19] Prodinger WM, Brandstatter A, Naumann L. Characterization of Mycobacterium caprae isolates from Europe by mycobacterial interspersed repetitive unit genotyping. Journal of Clinical Microbiology. 2005;**43**(10):4984-4992

[20] Cvetnic V, Katalinic-Jankovic V, Sostaric B. Mycobacterium caprae in cattle and humans in Croatia. The International Journal of Tuberculosis and Lung Disease. 2007;**11**(6):652-658

[21] Javed MT, Usman M, Irfan M, Cagiola M. A study on tuberculosis in buffaloes: Some epidemiological aspects, along with haematological and serumprotein changes. Veterinary Archives. 2006;**76**(3):193-206

[22] Duarte EL, Domingos M, Amado A, Botelho A. Spoligotype diversity of *Mycobacterium bovis* and *Mycobacterium caprae* animal isolates. Veterinary Microbiology. 2008;**130**(3):415-421

[23] World Organization for Animal Health (OIE). Bovine Tuberculosis–General Information 2011. Available At: www.oie.int

[24] O'Reilly LM, Daborn CJM. The epidemiology of *Mycobacterium bovis* infections in animals and man: A review. Tuberculosis and Lung Disease. 1995;**76**:1-46

[25] Anaelom NJ, Ikechukwu OJ, Sunday EW, Nnaemeka UC. Zoonotic tuberculosis: A review of epidemiology, clinical presentation, prevention and control. Journal of Public Health and Epidemiology. 2010;**2**(6):118-124

[26] World Organisation for Animal Health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Chapter 2.4.7: Bovine tuberculosis adopted; 2009

[27] Health Protection Agency (HPA). Reducing the risk of human *M. Bovis* infection: Information for farmers. Bovine TB; 2009. p. 2010

[28] Elias KHD, Asseged B, Wondwossen T, Gebeyehu M. Status of bovine tuberculosis in Addis Ababa dairy farms. Scientific and Technical Review of OIE. 2008;**27**(3):915-923

[29] Ayele WY, Neill SD, Zinsstag J, Weiss MG, Pavlik I. Bovine tuberculosis: An old disease but a new threat to Africa. The International Journal of Tuberculosis and Lung Disease. 2004;**8**(8):924-937

[30] Drewe JA, Pfeiffer DU, Kaneene JB. Epidemiology of Mycobacterium bovis. In: Thoen CO, Steele JH, Kaneene JB, editors. Zoonotic Tuberculosis: Mycobacterium Bovis and Other Pathogenic Mycobacteria. John Wiley & Sons; 2014:63-78

[31] World Health Organisation. Zoonotic tuberculosis (*M. Bovis*): A Memoranda from WHO meeting (with participation of FAO). Bulletin of the WHO. 1994;**72**(6):851-857

[32] Beals FT. The risk of Bovine Tuberculosis from raw milk consumption with a focus on Michigan in wise traditions in food, farming and the healing arts, the quarterly magazine of the Weston A. Price Foundation. 2007. trit.us/farming/raw-milk-and-tb-michigan.html [Accessed: November 29, 2009]

[33] O'Donahue WJ, Bedi S, Bittner MJ, Preheim LC. Short course chemotherapy for pulmonary infection due to M. Bovis. Archives of Internal Medicine. 1985;**145**:703-705

[34] Ofukwu RA. Studies on the epidemiology of bovine and human Tuberculosis in Benue State, Nigeria. A Ph.D Dissertation, Faculty of Veterinary Medicine. Nsukka: University of Nigeria; 2006

[35] Une Y, Mori T. Tuberculosis as a zoonosis from a veterinary perspective. Comparative Immunology, Microbiology and Infectious Diseases. 2007;**30**:415-425

[36] Kleeberg HH. Human tuberculosis of bovine origin in relation to public health. Ruvue Scientique el Technique office international des epizootics. 1984;**3**:11-32

[37] Dankner WM, Waecker NJ, Essey MA, Moser K, Thompson M, Davis CH. Mint: *Mycobacterium bovis* infections in San Diego: A Clinico-epidemiologic study of 73 patients and a historical review of a forgotten pathogen. Medicine (Baltimore). 1993;**72**:11-37

[38] Radostits OM, Gay CC, Blood DC, Hinchelift KW. Disease caused by bacteria – Mycobacterium. In: Veterinary Medicine: A Text Book of Disease of Cattle, Sheep, Pig, Goat and Horses. 9th ed. London: Harcourt Publisher Ltd.; 2000. pp. 909-918

[39] LoBue PA, Betacourt W, Peter C, Moser KS. Epidemiology of *Mycobacterium bovis* disease in San Diego County, 1994–2000. The International Journal of Tuberculosis and Lung Disease. 2003;**7**:180-185

[40] Thoen C, LoBue P, De Kantor I. The importance of *Mycobacterium bovis* as a zoonosis. Veterinary Microbiology. 2006;**112**(2–4):339-345

[41] Saidu AS, Okolocha EC, Dzikwi AA, Gamawa AA, Ibrahim S, Kwaga JK. Public health implications and risk factors assessment of Mycobacterium bovis infections among abattoir personnel in Bauchi state, Nigeria. Journal of Veterinary Medicine. 2015. http://dx.doi.org/10.1155/2015/718193

[42] Michel AL, Mueller B, Van helden PD. Mycobacterium bovis at the animalhuman interface: A problem or not. Veterinary Microbiology. 2010;**140**(1–3):371-381

[43] Konhya LD, Himes EM, Thoen CO. Mint: Bovine tuberculosis. In: Steele J, editor. Handbook Series in Zoonoses, Section A: Bacterial, Rickettsial and Mycotic Diseases. Vol. II. Boca Raton: CRC Press; 1980. pp. 147-150

[44] Kirk JH. Tuberculosis—Human and Cattle. Vol. 93274. Tulare: School of Veterinary Medicine, University of California Davis; 2003. pp. 1-3

[45] Rani N, Tomar P, Kapoor PK, Mahajan NK, Jindal N, Chhabra R. Historical perspectives and epidemiology of bovine tuberculosis. International Journal of Pure & Applied Bioscience. 2017;**5**(5):1406-1414

[46] The centre for food security and public health (CFSPH). Bovine Tuberculosis. College of Veterinary Medicine. Iowa State University; 2009

[47] Myers JA, Steele JH. Bovine Tuberculosis Control in Man and Animals. Missouri: WH. Green; 1969. p. 403

[48] Grange JM, Collins CH. Bovine tubercle bacilli and disease in animals and man. Epidemiology and Infection. 1987;**92**:221-234

[49] Bolognesi N. TB or Not TB: The Threat of Bovine Tuberculosis. 2007. Available At: www.SciDev.Net

[50] Shitaye JE, Getahun B, Alemayehu T, Skoric M, Treml F, Fictum P, Vrbas V, Pavlik I. A prevalence study of bovine tuberculosis by using abattoir meat inspection and tuberculin skin testing data, histopathological and IS6110 PCR examination of tissues with tuberculous lesions in cattle in Ethiopia. Veterinary Medicine. 2006;**51**:512-522

[51] Munagandu HM, Siamudaala VM, Nambota A, Bwalya JM, Munyeme M, Mweene AS. Disease constraints for utilization of the Africanbuffalo (Syncerus caffer) on game ranches in Zambia. The Japanese Journal of Veterinary Research. 2006;**54**:3-13

[52] Zinsstag J, Schelling E, Roth F, Kazwala RR. Economics of Bovine Tuberculosis: *Mycobacterium Bovis* Infection in Animals and Humans. 2nd ed. Ames: Iowa State University Press; 2006. pp. 68-83

[53] Durr S, Muller B, Alonso S. Differences in primary sites of infection between zoonotic and human tuberculosis: Results from a worldwide systematic review. PLoS Neglected Tropical Diseases. 2013;**7**:2399

[54] de Kantor IN, Ambroggi M, Poggi S, Morcillo N, da Silva Telles MA, Osório Ribeiro M, Garzón Torres MC, Llerena Polo C, Ribón W, García V, Kuffo D, Asencios L, Vásquez Campos LM, Rivas C, de Waard JH. Tuberculosis Edinb: Human *Mycobacterium bovis* infection in ten Latin American countries. 2008;**88**:358-365

[55] Banu B, Bulut E, Barış AB, Toksoy B, Dalgıc N, Celikkan C, Sevgi D. Species distribution of the *Mycobacterium tuberculosis* complex in clinical isolates from 2007 to 2010 in Turkey. Journal of Clinical Microbiology. 2011;**49**(11):3837-3841

[56] Sunnetcioglu A, Sunnetcioglu M, Binici I, Baran AI, Karahocagil MK, Saydan MR. Mint: Comparative analysis of pulmonary and extrapulmonary tuberculosis of 411 cases. Annals of Clinical Microbiology and Antimicrobials. 2015;**14**:34

[57] Rodríguez E, Sanchez LP, Perez S, Herrera L, Jimenez MS, Samper S. Human tuberculosis due to Mycobacterium bovis and M. Caprae in Spain, 2004–2007. The International Journal of Tuberculosis and Lung Disease. 2009;**13**:1536-1541

[58] Jenkins AO, Cadmus SI, Venter EH, Pourcel C, Hauk Y, Vergnaud G, Godfroid J. Mint: Molecular epidemiology of human and animal tuberculosis in Ibadan, southwestern Nigeria. Veterinary Microbiology. 2011;**151**(1–2):139-147

[59] Krauss H, Weber A, Appel M, Enders B, Isenberg DH, Schiefer GH, Slenczka W, von Graevenitz A, Zahner H. Zoonoses: Infectious Diseases Transmissible from Animals to Humans. 3rd ed. 2003. p. 213

[60] World Health Organization (WHO). Global Tuberculosis Report; 2017

[61] Cousins DV, Francis BR, Gow BL. Advantages of a new agar medium in the primary isolation of *Mycobacterium bovis*. Veterinary Microbiology. 1989;**20**:89-95

[62] Haagsma J. Working paper on recent advances in the field of tuberculosis control and research. World Health Organization meeting on zoonotic tuberculosis with particular reference to Mycobacterium bovis; 15 November 1993; Geneva

[63] Cousins DV, Florisson N. A review of tests available for use in the diagnosis of tuberculosis in non-bovine species. Revue scientifique et technique (International Office of Epizootics). 2005;**24**:23

[64] Buddle BM, Ryan TJ, Pollock JM, Anderson P, De Lisle GW. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. Veterinary Microbiology. 2001;**80**:37-46

[65] Griffin JFT, Cross JP, Chinn DN, Rogers CR, Buchan GS. Diagnosis of tuberculosis due to M. Bovis in New Zealand red deer (Cervus elaphus) using a composite blood test (BOVINE TB) and antibody (ELISA) assays. New Zealand Veterinary Journal. 1994;**42**: 173-179

[66] Ameni G, Vordermeier M, Aseffa A, Young DB, Hewinson RG. Field evaluation of the efficacy of *Mycobacterium bovis* bacillus calmette-guerin against bovine tuberculosis in neonatal calves in Ethiopia. Clinical and Vaccine Immunology. 2010;**17**:1533-1538

[67] Parlane NA, Buddle BM. Immunity and vaccination against tuberculosis in cattle. Current Clinical Microbiology Reports. 2015;**2**:44-53

[68] Mekonnen F, Tessema B, Moges F, Gelaw A, Eshetie S, Kumera G. Multidrug resistant tuberculosis: Prevalence and risk factors in districts of Metema and west Armachiho, Northwest Ethiopia. BMC Infectious Diseases. 2015;**15**:461

[69] Biadglegne F, Sack U, Rodloff AC. Multidrug-resistant tuberculosis in Ethiopia: Efforts to expand diagnostic services, treatment and care. Antimicrobial Resistance and Infection Control. 2014;**3**:31

[70] World Health Organization (WHO) Report. Global Tuberculosis Control. Geneva; 2013

[71] Marie-France H, Boschiroli ML, Saegerman C. Classification of worldwide bovine tuberculosis risk factors in cattle: A stratified approach. Veterinary Research. 2009;**40**:50

[72] Nishi JS, Shury T, Elkin BT. Wildlife reservoirs for bovine tuberculosis (*Mycobacterium bovis*) in Canada: Strategies for management and research. Veterinary Microbiology. 2006;**112**:325-338

[73] Siembieda J, Kock R, McCracken T, Newman S. The role of wildlife in transboundary animal diseases. Animal Health Research Reviews. 2011;**12**:95-111

[74] Cadmus SIB, Adesokan HK, Adejuwon TA, Adeyemi MO. Retrospective study of bovine tuberculosis and other diseases of public health importance in Oko-Oba abattoir, Lagos state. Book of abstracts. Nigerian Veterinary Medical Association. 2008;**89**:72-73

[75] Ozeki Y, Igarashi M, Doe M, Tamaru A, Kinoshita N, Ogura Y. A new screen for tuberculosis drug candidates utilizing a luciferase-expressing recombinant *Mycobacterium bovis* Bacillus Calmette-Guéren. PLoS One. 2015;**10**(11):e0141658. DOI: 10.1371/journal.pone.0141658

Chapter 3

Nontuberculous Mycobacterial Infections: Negligent and Emerging Pathogens

Thet Tun Aung and Roger W. Beuerman

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80444

Abstract

Nontuberculous mycobacteria (NTM) are a heterogeneous group of microorganisms other than *Mycobacterium tuberculosis* (*M. tuberculosis*) complex and *Mycobacterium leprae.* NTM infections have increased globally and are now considered an emerging infection as they are often encountered in developed countries. NTMs require extended treatment adding considerably to the economic burden. The increasing number of patients with immunocompromised disorders, increasing usage of immunosuppressive agents, general awareness of the NTM diseases due to the advancement in molecular diagnostic techniques and aging of the population increase the prevalence rate of NTM infections. However, several barriers such as the requirement of better diagnostic techniques, settled treatment guidelines, clinician awareness and knowledge of pathogenesis are limiting and NTM infections are often not treated promptly. Etiology and epidemiology of NTM infections [*Mmycobacterium avium* complex (slowly growing mycobacteria, SGM) and rapidly growing mycobacteria (RGM)] are discussed in this chapter. Clinical features, diagnosis and currently available treatment guidelines for these infections in skin, eye and lung are summarized. Suggestions for future research directions are suggested particularly for the better understanding of host-pathogen crosstalk and new therapeutic strategies.

Keywords: nontuberculous mycobacteria, rapidly growing mycobacteria, slowly growing mycobacteria, biofilms, eye, lung, skin

1. Introduction

1.1. Etiology, epidemiology and possible sources of NTM infections

The NTM group of mycobacteria is nonmotile aerobic bacilli, acid-fast (AF) staining organisms [1]. The lipid-enriched hydrophobic cell well is usually thicker than other bacteria

characterized by tolerance to many disinfectants, heavy metals and antibiotics [1, 2]. They are frequently found in the environment such as soil and water. They readily form biofilms, which contributes to their resistance against a variety of antibiotics [3] as well as high temperatures and a wide range of pH [4]. Environmental recovery of these NTM is the same when they do in similar culture techniques in different geographical regions [5]. However, western countries are reporting a greater prevalence of NTM infections compared to Tuberculosis (TB) than most Asian countries due to very stringent prevention and treatment of tuberculosis [6]. Not all the culture-positive samples represent infection and only half of the culture-positive patients have active respiratory infections, highlighting that NTM can be silent in presence of a normal immune response [7]. Reports suggest that older patients and women have higher chances of NTM infections [8]. As an outcome of the Human Immunodeficiency Virus (HIV) epidemic, NTM infections are frequently isolated from the blood of HIV patients [9]. In the United States, NTM cultures (more than 90%) are from pulmonary disease [10]. According to the Infectious Diseases Society of American Emerging Infections Network and Information from referral centers report, NTM infections are emerging pathogens, particularly rapidly growing mycobacteria (RGM) such as *Mycobacterium abscessus* (*M. abscessus*), *Mycobacterium chelonae* (*M. chelonae*) and *Mycobacterium fortuitum* (*M. fortuitum*) [11]. The prevalence and trend of NTM pulmonary infections are increasing, particularly in Florida and New York, calculated from United States census data from 1998 through 2005 [12]. NTM are the most common pathogens after cosmetic surgeries such as tattooing and Laser in situ keratomileusis (LASIK) [13, 14]. Increasing reports of NTM infections are expected in eye, skin, and lung due to the popularity of LASIK, increasing population of immunocompromised patients and older population. NTM pulmonary infections are found in the areas with heavy population, indicating that urban water supply increases individual's exposures to NTM [15]. NTM infections are frequently associated with farmers in Japan, suggesting that soil is the main source of infection there [16]. For NTM lung infections, aerosolization of droplets by bathroom showers may be another route of infection [17]. Water is considered to be a normal habitat for NTM and households with low water heater temperature are found to correlate with NTM infections [18]. Hospital water supply is considered to be vital in controlling NTM infections and dialysis solutions contaminations have led to the NTM outbreaks [19–21]. Contaminated tap water and increased demand of cosmetic surgeries in freestanding health centers that cannot be reviewed frequently by the infectious diseases control center are other concerns for NTM outbreaks [22].

1.2. Runyon's classification

Runyon classified NTM into four groups, I–IV [23–25]. Group I, photochromogens, which usually grow slowly about 2–4 weeks and change to yellow with light exposure. Group II, scotochromogens, consist mainly of *M. gordonae* and appear as yellow colonies at 2–4 weeks in agar plates when cultured in the dark. Group III are nonphotochromogens, slowly growing mycobacteria, which grow slowly over 2–4 weeks. The rapid growers, group IV NTMs are the most pathogenic and important for human disease. They are divided into three subgroups: *M. fortuitum*, *M. chelonae/abscessus*, and *M. smegmatis*. According to the literature, they are susceptible to various antibiotics such as sulfonamide, polymyxin B, and the third- and fourth-generation fluoroquinolones [25]. Group III organisms are lung pathogens and Group IV organisms are the most important and prevalent strains for the eye, lung cutaneous and subcutaneous infections [25].

1.3. Laboratory diagnosis and barriers

Culture technique is the typical standard method for the identification of suspicious NTM. The organisms must be cultured on specific media such as AF smear, Lowenstein-Jensen (LJ) media, Middlebrook media and MacConkey agar since it cannot be differentiated by Gram-stain [26]. The organisms must be cultured in both liquid medium for growing a large amount of organism for other tests and solid medium to observe colony morphology and characteristic [27]. Moreover, the organisms should be further identified into subspecies level for different appropriate antimicrobial therapy. Subspecies level can be achieved by using gene sequencing, high-performance liquid chromatography (HPLC), and molecular-based methods [28]. HPLC is a fast, reliable method for identifying NTM. However, HPLC has limitations: it cannot separate between *M. abscessus* and *M. fortuitum*/*M. chelonae* [29]. Molecular probes, acridinium ester-labeled DNA probes have been made commercially and approved by the U.S. Food and Drug Administration (FDA) for the rapid identification of NTM [30]. MicroSeq 500 16S rDNA Bacterial Sequencing Kit (PE Applied Biosystems, Foster City, CA) has been developed to identify the NTM strain [31]. However, misdiagnoses frequently occur due to the low frequency of these infections, coupled with a lack of diagnostic experience for NTM infections, as well as confusing morphological features in stained smears [28]. Misdiagnosis can be complicated by incorrectly correlating laboratory results by physicians [32]. Misdiagnosis of NTM infections can lead to fatal incidents and NTM often exhibit the microbiological features of *Corynebacterium* species with long filamentous beaded appearances [33, 34]. NTM microscopic features are also similar to *Nocardia* species [35]. Therefore, clinicians are taking note of these emerging infections for prompt and focused diagnosis to initiate effective treatment.

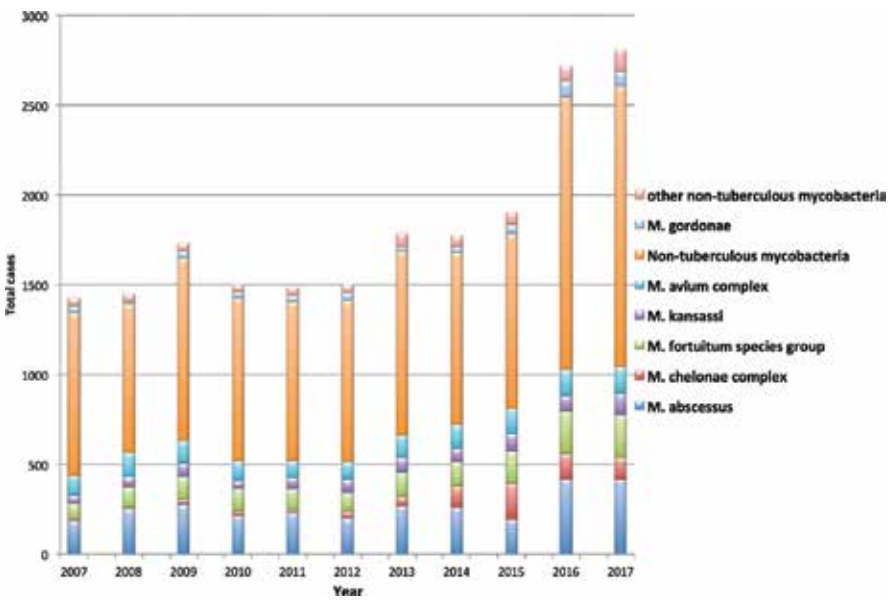

Figure 1. Bar graph showing the incidences of NTM in Singapore (2007–2017). Other NTM consists of *M. szulgai*, *M. terrae* complex, *M. haemophilum*, *M. intracellulare*, *M. marinum*, *M. mucogenicum*, *M. neoaurum*, *M. scrofulaceum*, *M. simiae*, *M. mageritense*, *M. wolinskyi*, *M. asiaticum*, *M. celatum*, *M. chimaera*, *M. duvalli*, *M. cookii*, *M. cosmeticum*, *M. chlorophenolicum*, *M. genavense*, *M. kubicae*, *M. lentiflavum*, *M. mantenii*, *M. obuense*, *M. stomatepiae*, *M. triplex* and *M. xenopi*.

1.4. NTM incidence in Singapore (2007–2017)

The incidences of NTM cases in Singapore are rising in the recent years, about 3000 cases per year [36] (**Figure 1**). Among NTM, *M. abscessus* is responsible for most of the identified NTM cases in Singapore, followed by *M. fortuitum, M. avium* complex and *M. chelonae* (**Figure 1**).

2. NTM cutaneous and subcutaneous infections

2.1. *Mycobacterium abscessus*

M. abscessus, a fast-growing NTM, is commonly found in water drainage systems and sewage. It is a subset of the *M. chelonae* complex and it is vital to segregate from the *M. chelonae* complex due to the dissimilar antibacterial treatment option. It is well known that the clinical success of *M. abscessus* depends on the host's immune defense [37]. It was reported that *M. abscessus* caused posttransplant infection in cystic fibrosis (CF) patients in spite of having antimicrobial treatment [38]. They are responsible for the major causes of skin and soft tissue infections in the literatures [39] and they are the most common cause of identified NTM infections in Singapore (**Figure 1**). The path of entries for this organism is direct inoculation such as skin piercing or injury [40] or secondary involvement from disseminated infection [41]. The most likely source of infection is from tap water. Water and soil are the natural habitats for *M. abscessus* [4, 42]. *M. abscessus* outbreaks have been reported in clinic and hospitals worldwide and the contaminated instruments or disinfectants are the major sources of the outbreaks [41].

2.1.1. Clinical features and causes of M. abscessus cutaneous and subcutaneous infections

M. abscessus infected skin usually presents with painful, swollen and tender to the touch, accompanying with pus-filled vesicles. Nonspecific symptoms of infections may be present such as fever with chills, muscle aches, and malaise. Causes of *M. abscessus* infections include posttraumatic wound infections [20], postinjection wound infections [20] and surgical wound infections (mammoplasties, plastic surgeries, and heart surgeries) [20].

2.2. *Mycobacterium fortuitum*

M. fortuitum is a principal cause of cutaneous and subcutaneous infections associated with catheters [43, 44] as well as post surgical wound infections [45]. The route of entry for *M. fortuitum* is direct inoculation from contaminated water through the lesions.

2.2.1. Clinical features and causes of M. fortuitum cutaneous and subcutaneous infections

Small, erythematous papules are frequent signs of the early stages of infection and large, fluctuant, painful violaceous boils and ulcerations are signs for late stage infections [45, 46]. They can be caused by mesotherapy and present with indurated, erythematous and violaceous papules with 3–20 numbers, the diameter ranging from 0.5 to 6 cm, accompanied by inguinal or axillary lymphadenopathy [47]. *M. fortuitum* can also be recovered from blood and

purulent discharge from patients with venous catheters [43, 44] and is the cause of post surgical wound infections such as liver transplant patients, electromyography and punch biopsy procedures [44, 48, 49].

2.3. *Mycobacterium chelonae*

M. chelonae infections are usually associated with immunocompromised hosts such as HIV patients [50]. It can be seen in postsurgical wounds and can disseminate hematogenoulsy to cause sepsis. Contaminated water is the most common source of infection and the route of entry is direct inoculation.

2.3.1. Clinical features and causes of M. chelonae cutaneous and subcutaneous infections

Circumscribed, red, infiltrative plaques, umbilicated papules, and pustules on the upper part of the body and face are features of *M. chelonae* skin lesions and frequently accompanied by cervical lymphadenopathy [51]. Immunocompromised patients, HIV/AIDS patients often contract *M. chelonae* infections [50]. Kidney transplant patients, liver transplant patients, tattooing, kidney dialysis patients and peritoneal dialysis patients are also frequently associated with *M. chelonae* infections [13, 52, 53]. Reports suggest that immunosuppressive drugs such as prednisolone, methotrexate, and adalimumab [54, 55], and autoimmune diseases such as Cushing's syndrome and rheumatoid arthritis are often associated with *M. chelonae* skin infections [55, 56].

2.4. NTM cutaneous and subcutaneous infections

The correct choice of antimicrobial agent, anatomic locations of the lesions, intracellular uptake and target binding are essential for the management of NTM cutaneous and subcutaneous infections. Moreover, an appropriate route of drug administration (oral, intravenous or intramuscular), acceptable and effective drug concentration is required for the treatment plan. Drug resistance mechanisms for rapidly growing mycobacteria (RGM) involving *erm* gene must be considered due to the prolonged treatment period. Therefore, it is critical to differentiate and identify rapidly growing mycobacterial at the subspecies level [25, 57]. The decision of choosing either surgical debridement in combination with mono or multidrug therapy, or only mono or multidrug therapy depends on the anatomical location and severity of the lesion, patient's immune status with presence of underlying pathology (**Table 1**) and the Minimum Inhibitory Concentration (MIC) breakpoints from the microbiology lab (**Tables 2** and **3**).

2.4.1. M. abscessus cutaneous and subcutaneous infections

Macrolides are the gold standard treatment for *M. abscessus* infections. They exhibit bactericidal actions against *M. abscessus* when the lesion has a small population of bacteria. Reports suggest that azithromycin and clarithromycin are the gold standard for treating *M. abscessus* infections in disseminated cases; however, there are reports suggesting the evolution of resistance against these drugs in prolonged monotherapy [11, 58]. Tigecycline, a new antibiotic,

RGM	Disease pattern	Antimicrobial agents
M. fortuitum	2–8 week duration with significant signs and symptoms	Combination of amikacin, quinolones or tobramycin (imipenem)
	After IV treatment or disease with reduced signs and symptoms	Linezolid
		Doxycycline
		Clarithromycin
		Trimethoprim/sulfamethoxazole
M. abscessus	2–8 week duration with significant signs and symptoms	Combination of clarithromycin, amikacin, cefoxitin (imipenem) or tigecycline
	After IV treatment or disease with reduced signs and symptoms	Linezolid
M. chelonae	2–8 week duration with significant signs and symptoms	Combination of clarithromycin, linezolid (tobramycin, imipenem, tigecycline or oral drug)
	After IV treatment or disease with reduced signs and symptoms	Gatifloxacin
		Doxycycline
		Linezolid
		Clarithromycin

Table 1. Clinicians' choice of antibiotic regimes for different RGM infections [112].

	Susceptible	Intermediate	Resistant
Doxycycline/minocycline	≤1	2–4	≥8
Imipenem/meropenem	≤4	8–16	≥32
TMP/SMX	≤2/38	–	≥4/76
Tobramycin	≤2	4	≥8
Moxifloxacin	≤1	2	≥4

Table 2. MIC breakpoints for RGM [112, 113].

may be another choice for *M. abscessus* infections [59]. Amikacin is known to be the treatment of choice since it is active against all the subspecies of RGM and imipenem or cefoxitin can be added to overcome treatment failures [11, 58]. Surgical debridement plays a role in the better treatment outcomes for *M. abscessus* infections [60].

2.4.2. M. fortuitum cutaneous and subcutaneous infections

M. fortuitum infections are chronic in nature and *in vitro* drug susceptibility tests are required for a guidance of choosing the correct antibiotics. Usually, *M. fortuitum* are sensitive to several oral antimicrobials such as quinolones, sulfonamides, and macrolides [61, 62]. Amikacin is the treatment of choice for *M. fortuitum* with 100% efficacy, while sulfonamide and imipenem/

RGM	Drug	Reporting
M. fortuitum	Clarithromycin	Trailing endpoints, report as resistant
	Imipenem	New breakpoint (8–16 μg/ml) for reproducible MIC
M. abscessus	Amikacin	If MIC is more than 64 μg/ml, need to repeat/confirm
M. chelonae	Tobramycin	If MIC is more than 4 μg/ml, need to repeat/ confirm

Table 3. Reporting MICs of RGM [112].

cilastatin also account for 100%, clarithromycin stands for 80% and linezolid and doxycycline accounts for 50% [63]. Due to rising chances of bacterial resistance to macrolide due to the inducible *erm* gene, clarithromycin uses should be carefully assessed and monitored [52, 63, 64]. Linezolid is another good candidate for *M. fortuitum* in *in vitro*; however, more human clinical studies would be warranted for the future use [65]. The minimum 4 months duration of the combination of two drugs is required for severe or critical *M. fortuitum* cutaneous and subcutaneous infections. Reports are suggested that surgical debridement or surgical drainage is indicated for the better antimicrobial treatment therapy or helping to cure the *M. fortuitum* infections particularly in extensive disease and abscesses [66]. *M. fortuitum* usually possess the *erm* gene, which is inducible to promote resistance to clarithromycin. There was a report showing that sensitivity testing of *M. fortuitum* isolates showed trailing MICs against macrolides [67]. However, the relevance of the *erm* gene in *M. fortuitum* and clarithromycin treatment remains to be determined in clinical management.

2.4.3. M. chelonae cutaneous and subcutaneous infections

Clofazimine is shown to be effective and the addition of sub MIC concentration of amikacin synergies with clofazimine against RGM including *M. chelonae* [68]. Tobramycin has been suggested to be a better treatment option than amikacin [69]. However, *M. chelonae* isolates showed resistant to cefoxitin and imipenem is the alternative option. There is MIC susceptibility of clarithromycin (100%), tobramycin (100%), linezolid (90%), imipenem (60%), amikacin (50%), doxycycline (25%), clofazimine (25%) and ciprofloxacin (20%) [63]. However, *M. chelonae* is susceptible only to clarithromycin, tobramycin, and tigecycline [70]. Monotherapy is not advisable for *M. chelonae* infections due to its facility to acquire drug resistance and combination treatment is advised [71]. Excision and treatment is still the optimal treatment step in combination with antibiotics in treating *M. chelonae* cutaneous and subcutaneous infections [66]. Treatment guidelines are not yet reported; however, current guidelines recommend using antimicrobial susceptibility tests to predict therapeutic efficacy.

3. NTM eye infections

3.1. Clinical features and causes of NTM eye infections

The most prevalent NTM strains causing eye infections are *M. fortuitum* and *M. chelonae* [72, 73]. Keratitis is standing as the most common real situation accounting for 69% of ocular NTM infections (**Table 4**).

Different types of ocular NTM infection	Percentage
1. Ocular surface infections	
a. Keratitis	69
b. Scleritis	4.3
c. Conjunctivitis	0.7
2. Periocular and adnexal infections	13.3
3. Intraocular infections and uveitis	12.6

Table 4. Different types of ocular infection caused by NTM [14].

Late presentation of symptoms and diagnosis was reported in NTM keratitis [74]. Pain, decreased vision, and photophobia were present in gradual increasing patterns in the course of NTM keratitis [75]. The multifocal or single lesion surrounded by radiating corneal infiltrates, 'cracked windshield' appearance, was reported [74, 76, 77]. Infiltrates had irregular margins, mimicking fungal keratitis [78]. Hypopyon is present in untreated or poorly treated cases [74]. There have been reports of infectious crystalline keratopathy, intrastromal opacity and minimal inflammation in some cases of NTM keratitis leading to a misleading diagnosis of herpetic keratitis [79, 80] (**Table 5**).

The most common association of NTM keratitis is LASIK (47.6%), followed by trauma (14.8%), foreign body (17.6%), implants (17.3%) and contact lens (6.4%) [14]. LASIK is the most popular refractive corrective surgery implemented worldwide since it offers less stromal scarring and rapid recovery of visual acuity. The symptoms for post-LASIK mycobacterial keratitis are less severe than other causes [26]. The time frame of 3 to 14 weeks duration is reported to present post-LASIK NTM keratitis. Some cases of post-LASIK mycobacterial keratitis present within 10 days post surgery [26, 81]. The most probable route of entry for post-LASIK NTM keratitis is during the surgery. Corneal infiltrates are within the lamellar flap or interface presenting with either single white lesion or multiple white granular appearances. Anterior extension of corneal infiltrates is common to form a corneal ulceration. Late diagnosis or treatment can result in the posterior extension into the corneal stroma. The anterior chamber is usually silent or has the mild inflammatory reaction [26, 82].

3.2. Treatment of NTM eye infections

Management of NTM keratitis is challenging due to its rarity, potential to acquire antibiotic resistance, natural resistance to a variety of commercially available antibiotics and delayed response to antibiotics. Identification of NTM keratitis can be delayed and one report revealed that the time to identification was delayed for 4 months due to slow growth of the organism [83]. Drug sensitivity tests need to be carried out using a prolonged incubation time, resulting in the delayed treatment of NTM eye infections. Moreover, there are several reports showing that a wide range of antibiotic sensitivities exists in different isolates [84]. Consequently, a combination of two or three drugs helps to prevent acquired antibiotic resistance in long-term

management and clarithromycin, amikacin, and fourth generation fluoroquinolones are mentioned [85]. Topical delivery is the most used method followed by the combination of topical and systemic administration [14]. Amikacin is known to be the treatment of choice for NTM keratitis, however, there have been reports showing corneal toxicity toward the long-term usage of amikacin in high concentration [86]. According to the systemic review, amikacin was given alone in the majority of NTM keratitis cases, followed by amikacin and macrolide (**Table 6**) [14]. Fluoroquinolones, particularly fourth-generation fluoroquinolones, have been accepted as effective for eye infections [3, 86]. Fourth generation fluoroquinolones offer noteworthy benefits over the older generations because of their superior bactericidal activity, decreased risk for resistance and higher corneal concentrations. In contrast, one report suggested that the majority of nontuberculous mycobacteria are resistant to second-generation fluoroquinolones, highlighting the better efficacy properties of fourth generation fluoroquinolones [87].

Recent reports suggest a strong synergism between amikacin and fourth generation fluoroquinolone, gatifloxacin, in treating nontuberculous mycobacteria in *in vitro* and *in vivo* mouse keratitis model [88]. Moreover, it was reported that the NTM habitat in a keratitis infection is in the biofilm mode (**Figure 2**) hindering antibiotic penetration and adding DNase to the antibiotic may make a more effective treatment [88]. Surgical debridement can help to facilitate penetration and lower the bacterial load. Topical steroids are controversial for NTM keratitis and one study suggested that a steroid accelerates the infection [89]. Careful follow up of NTM

Symptoms	Signs
Varying degree of pain	Multiple lesions or single lesion surrounded by the radiating projections
Photophobia	Cracked windshield appearance
Tearing and foreign body sensation	Hypopyon
Decreased visual acuity	Mild or Silent anterior chamber

Table 5. Signs and symptoms of NTM keratitis.

Different antibiotic regimen	Percentage
Amikacin only	29.2
Combination of amikacin and macrolide	14.1
Combination of amikacin and fluoroquinolone	12.5
Combination of amikacin, fluoroquinolone and macrolide	9.4
Combination of fluoroquinolone and macrolide	8.3
Other antibiotics	7.3
Fluoroquinolone only	6.8

Table 6. Different antibiotic regimens for NTM keratitis [14].

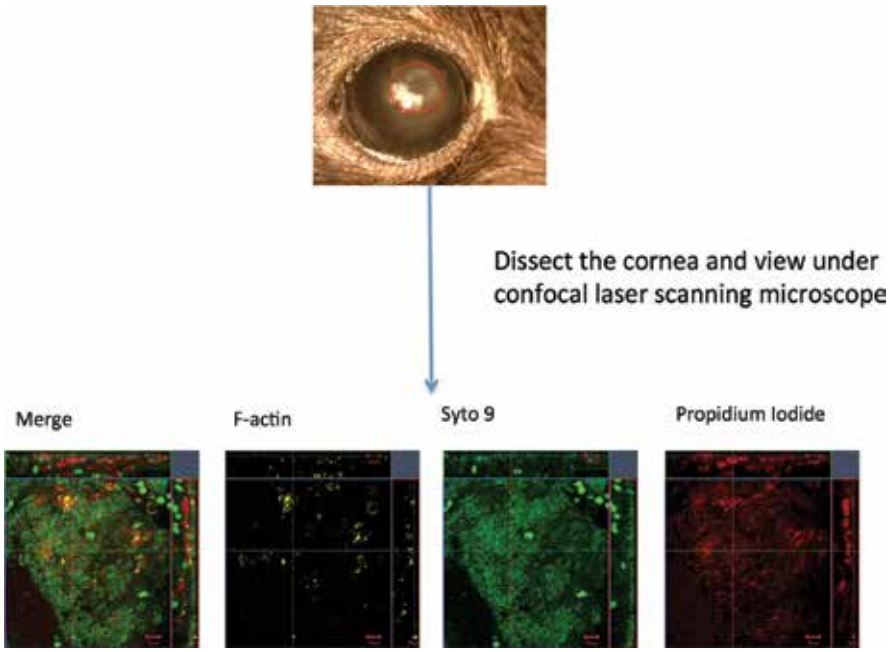

Figure 2. Slit lamp photograph showing central haziness in NTM keratitis mouse model. Confocal microscopy images showing presence of atypical mycobacterial microcolonies biofilm formation (green color) with abundance of extracellular DNA (a major constituent of mycobacterial biofilm matrix in red color) [88].

keratitis patients is suggested; if the lesion is in progression, or stromal thinning and symptoms persistence continues after 2 days of antibiotics, drug sensitivity should be rechecked for an alternative sensitive antimicrobial. However, there is no agreed-upon treatment plan for NTM keratitis and more research including evaluation of new treatment plans and an in-depth knowledge of NTM keratitis pathogenesis is warranted to treat NTM keratitis effectively.

4. NTM lung infections

4.1. Clinical features and causes of NTM lung infections

NTM lung infections are often due to *Mycobacterium avium* complex (MAC) and RGM. NTM lung disease may be misdiagnosed as Tuberculosis and require weeks or months [90]. The clinical diagnosis and treatment remain challenging due to its nonspecific symptoms such as low-grade fever, wet chronic cough, weight loss and malaise similar to *M. tuberculosis* [91]. Radiological imaging is a vital test to screen for NTM lung disease. A broad range of radiological patterns such as bronchiectasis, cavitary lesions, nodular lesions and parenchymal lesions, have been observed in NTM lung disease [92]. However, two major radiological findings have been listed, fibrocavitary and nodular bronchiectatic forms [93]. The first form is similar to pulmonary TB and it usually affects elderly male with underlying pathology of the lung. Cavities with increased opacity are usually seen in the upper parts of the lung in the fibrocavitary form [94]. Thin-walled cavities without lymph node involvement and atelectasis are the common findings in this form [95]. The nodular bronchiectatic form often presents

with bilateral, small nodules and multilobar bronchiectasis in the lower and middle parts of the lung [96]. This disease pattern is associated with elderly nonsmoking women without underlying lung diseases [97]. There is a connection between bronchiectasis and NTM lung diseases [98]. Because of NTM's nonspecific symptoms and similar radiological findings as *M. tuberculosis* and other lung pathologies, it is extremely hard to diagnose NTM lung disease. Risk factors causing NTM lung disease are still poorly understood but immune status is vital for NTM lung disease. A study showed that disseminated NTM infection is often associated in patients with profound immunosuppression [99]. NTM are important pathogens for patients who have undergone or are awaiting lung transplant and cystic fibrosis patients [100]. Defects in the crucial elements of the host defense such as interleukin-12 (IL-12) and interferon-gamma (IF-γ) increase susceptibility to NTM lung infections [101]. Increasing usage of tumor necrosis factor (TNF-α) receptor antagonists usage enhances NTM infections [102]. The rate of NTM prevalence in TNF-α receptor antagonists usage is 74/100,000 persons per year [103].

4.2. Treatment of NTM lung infections

4.2.1. MAC lung infections

Macrolides are the treatment of choices for MAC lung infections [104]. Rifampin or ethambutol can be added to macrolide administration for 18–24 months [63]. Rifampin 600 mg/kg, ethambutol 25 mg/kg with either azithromycin 500 mg/kg or clarithromycin 1000 mg/kg is frequently given as three-times-weekly intermittent therapy for NTM noncavitary lung disease [63]. It has been suggested that intermittent therapy is more efficient and reduced toxicity than daily therapy [105]. A cocktail of rifampin 10 mg/kg/day, ethambutol 15 mg/kg/day with either azithromycin 250 mg/kg/day or 1000 mg/kg is given daily for cavitary nodular bronchiectatic NTM lung disease, with a possibility of adding either streptomycin or amikacin in the first 2 or 3 months of therapy in severe disease [63].

The addition of moxifloxacin to the standard treatment showed a better response if a standard treatment plan fails [106]. Clofazimine has shown that it can be an alternative option to the rifampin or in refractory MAC lung infections [107]. The successful treatment of NTM lung infections totally relies on the prevention of macrolide-resistant MAC infections with the optimal treatment strategies.

4.2.2. RGM lung infections

The management for RGM lung infections typically depends on drug's toxicity and drug sensitivity tests. Treatment for *M. abscessus* lung infection is challenging as shown in previous studies [108]. The recommended guideline for treating RGM lung infection includes a combination of treatment which involves two parenteral antibiotics and an oral macrolide for a relatively long duration (several months) [63]. The most active and useful parenteral agents consist of amikacin 10–15 mg/kg/day, imipenem 500–1000 mg^2, cefoxitin 200 mg/kg/day, and tigecycline 50 mg/day [108]. Moxifloxacin has been shown as an alternative option for treating RGM lung infections [109]. Aggressive parenteral therapy is suggested for initial 4 months of treatment accompanied later by a treatment combination of macrolide and linezolid or clofazimine or fluoroquinolone for coping with toxicity [108]. Treatment with macrolides for

RGM infection should be carefully accessed on the patient's tolerance and treatment compliance due to the possibility of drug resistance evolved [110]. Surgical resection should be considered to combine with chemotherapy in treating RGM lung infections [111].

5. Conclusion

Etiology and epidemiology of NTM infections highlight that NTMs are emerging pathogens, warranting more research. Clinical features, barriers in the diagnosis of NTM and a lack of more effective treatment strategies were discussed for NTM infections in lung, skin and eye system. This overview prompts comments that can be made for NTM infections for future research. (1) NTM infections are considered emerging pathogens around the world including Singapore. (2) Better understanding of microbial life in real human clinical scenarios is important in dealing with the easy biofilm forming NTMs. (3) More research is critically needed to fill a huge gap of host-pathogen interactions in NTM infections. (4) A Multidisciplinary approach, better diagnostic tools, increase public awareness and standard treatment guidelines and new therapeutic research is urgently required.

Conflict of interest

TTA, RWB- Nil.

Notes/thanks/other declarations

The authors would like to acknowledge the Central Tuberculosis Laboratory, Department of Microbiology, Singapore General Hospital, for the contribution of Singapore NTM registry. The authors would like to thank the advices and help from A/Prof. Koh Tse Hsien (Department of Microbiology, Singapore General Hospital), A/Prof. Sng Li-Hwei (Central Tuberculosis Laboratory, Singapore General Hospital), A/Prof. Tan Thuan Tong (Department of Infectious Diseases, Singapore General Hospital), A/Prof. Timothy Mark Sebsatian Barkham (Laboratory Medicine, Tan Tock Seng Hospital) and the support of funding from NMRC/TCR/002-SERI/2012/R1018.

Author details

Thet Tun Aung[1] and Roger W. Beuerman[1,2,3]*

*Address all correspondence to: rwbeuerman@gmail.com

1 Singapore Eye Research Institute, Singapore

2 SRP Neuroscience and Behavioral Disorders, Duke-NUS, Singapore

3 Department of Ophthalmology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

References

[1] Ray C, Ryan K, Enteroviruses. In: Ryan KJ, Ray CG, editors. Sherris Medical Microbiology. 4th ed. New York, USA: The McGraw-Hill Companies; 2004. pp. 531-541

[2] Jarlier V, Nikaido H. Mycobacterial cell wall: Structure and role in natural resistance to antibiotics. FEMS Microbiology Letters. 1994;**123**(1-2):11-18

[3] Aung TT et al. Biofilms of pathogenic nontuberculous mycobacteria targeted by new therapeutic approaches. Antimicrobial Agents and Chemotherapy. 2016;**60**(1):24-35

[4] Falkinham Iii J. Surrounded by mycobacteria: Nontuberculous mycobacteria in the human environment. Journal of Applied Microbiology. 2009;**107**(2):356-367

[5] Von Reyn CF et al. Isolation of *Mycobacterium avium* complex from water in the United States, Finland, Zaire, and Kenya. Journal of Clinical Microbiology. 1993;**31**(12):3227-3230

[6] Cassidy PM et al. Nontuberculous mycobacterial disease prevalence and risk factors: A changing epidemiology. Clinical Infectious Diseases. 2009;**49**(12):e124-e129

[7] Winthrop KL et al. Pulmonary nontuberculous mycobacterial disease prevalence and clinical features: An emerging public health disease. American Journal of Respiratory and Critical Care Medicine. 2010;**182**(7):977-982

[8] Adjemian J et al. Prevalence of nontuberculous mycobacterial lung disease in US Medicare beneficiaries. American Journal of Respiratory and Critical Care Medicine. 2012;**185**(8):881-886

[9] Horsburgh CR Jr, RM Selik. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). The American Review of Respiratory Disease. 1989;**139**(1):4-7

[10] O'Brien RJ, Geiter LJ, Snider DE Jr. The epidemiology of nontuberculous mycobacterial diseases in the United States: Results from a national survey. American Review of Respiratory Disease. 1987;**135**(5):1007-1014

[11] De Groote MA, Huitt G. Infections due to rapidly growing mycobacteria. Clinical Infectious Diseases. 2006;**42**(12):1756-1763

[12] Billinger ME et al. Nontuberculous mycobacteria-associated lung disease in hospitalized persons, United States, 1998-2005. Emerging Infectious Diseases. 2009;**15**(10):1562

[13] Drage LA et al. An outbreak of *Mycobacterium chelonae* infections in tattoos. Journal of the American Academy of Dermatology. 2010;**62**(3):501-506

[14] Kheir WJ et al. Nontuberculous mycobacterial ocular infections: A systematic review of the literature. BioMed Research International. 2015;**2015**:17

[15] Winthrop KL et al. Pulmonary disease associated with nontuberculous mycobacteria, Oregon, USA. Emerging Infectious Diseases. 2011;**17**(9):1760

[16] Maekawa K et al. Environmental risk factors for pulmonary *Mycobacterium avium*-intracellulare complex disease. Chest. 2011;**140**(3):723-729

[17] Falkinham JO et al. *Mycobacterium avium* in a shower linked to pulmonary disease. Journal of Water and Health. 2008;**6**(2):209-213

[18] Falkinham JO. Factors influencing the chlorine susceptibility of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*. Applied and Environmental Microbiology. 2003;**69**(9):5685-5689

[19] Mandel AS et al. State regulation of hospital water temperature. Infection Control & Hospital Epidemiology. 1993;**14**(11):642-645

[20] Uslan DZ et al. Skin and soft tissue infections due to rapidly growing mycobacteria: Comparison of clinical features, treatment, and susceptibility. Archives of Dermatology. 2006;**142**(10):1287-1292

[21] Carson LA et al. Prevalence of nontuberculous mycobacteria in water supplies of hemodialysis centers. Applied and Environmental Microbiology. 1988;**54**(12):3122-3125

[22] Le Dantec C et al. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Applied and Environmental Microbiology. 2002;**68**(11):5318-5325

[23] Runyon EH. Identification of mycobacterial pathogens utilizing colony characteristics. American Journal of Clinical Pathology. 1970;**54**(4):578-586

[24] Murray P, Barron E, Pfaller M. Mycobacterium: General characteristics, islation, and staining procedures. In: Manual of Clinical Microbiology. Washington DC: American Society for Microbiology; 2003

[25] Brown-Elliott BA, Wallace RJ. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clinical Microbiology Reviews. 2002;**15**(4):716-746

[26] Moorthy RS, Valluri S, Rao NA. Nontuberculous mycobacterial ocular and adnexal infections. Survey of Ophthalmology. 2012;**57**(3):202-235

[27] Pfyffer GE, Palicova F. Mycobacterium: General characteristics, laboratory detection, and staining procedures. In: Manual of Clinical Microbiology. 10th ed. Washington DC, USA: American Society of Microbiology; 2011. pp. 472-502

[28] Somoskovi A, Salfinger M. Nontuberculous mycobacteria in respiratory infections: Advances in diagnosis and identification. Clinics in Laboratory Medicine. 2014;**34**(2): 271-295

[29] Butler WR, Guthertz LS. Mycolic acid analysis by high-performance liquid chromatography for identification of Mycobacterium species. Clinical Microbiology Reviews. 2001;**14**(4):704-726

[30] Somoskövi Á et al. False-positive results for *Mycobacterium celatum* with the AccuProbe *Mycobacterium tuberculosis* complex assay. Journal of Clinical Microbiology. 2000;**38**(7): 2743-2745

[31] Hall L et al. Evaluation of the micro Seq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. Journal of Clinical Microbiology. 2003;**41**(4):1447-1453

[32] Somoskovi A et al. Laboratory diagnosis of nontuberculous mycobacteria. Clinics in Chest Medicine. 2002;**23**(3):585-597

[33] Boltin D et al. *Corynebacterium striatum*—A classic pathogen eluding diagnosis. European Journal of Internal Medicine. 2009;**20**(3):e49-e52

[34] Williamson JC et al. Fatal *Mycobacterium abscessus* endocarditis misidentified as *Corynebacterium* spp. Scandinavian Journal of Infectious Diseases. 2010;**42**(3):222-224

[35] Paul J, Baigrie C, Parums D. Fatal case of disseminated infection with the turtle bacillus *Mycobacterium chelonae*. Journal of Clinical Pathology. 1992;**45**(6):528-530

[36] Tang SS et al. Rapidly growing mycobacteria in Singapore, 2006-2011. Clinical Microbiology and Infection. 2015;**21**(3):236-241

[37] Wang H, Jin P, Wu Q. Disseminated cutaneous infection with *Mycobacterium abscessus* in a patient with a low CD4+ T cell count. European Journal of Dermatology. 2008;**18**(3): 337-340

[38] Taylor JL, Palmer SM. *Mycobacterium abscessus* chest wall and pulmonary infection in a cystic fibrosis lung transplant recipient. The Journal of Heart and Lung Transplantation. 2006;**25**(8):985-988

[39] Lee M-R et al. *Mycobacterium abscessus* complex infections in humans. Emerging Infectious Diseases. 2015;**21**(9):1638

[40] Ferringer T, Pride H, Tyler W. Body piercing complicated by atypical mycobacterial infections. Pediatric Dermatology. 2008;**25**(2):219-222

[41] Kothavade R et al. Clinical and laboratory aspects of the diagnosis and management of cutaneous and subcutaneous infections caused by rapidly growing mycobacteria. European Journal of Clinical Microbiology & Infectious Diseases. 2013;**32**(2):161-188

[42] Appelgren P et al. Late-onset posttraumatic skin and soft-tissue infections caused by rapid-growing mycobacteria in tsunami survivors. Clinical Infectious Diseases. 2008;**47**(2):e11-e16

[43] TAZAWA S et al. *Mycobacterium fortuitum* infection caused by the organism in subcutaneous abscess mediated by central venous catheter. Kekkaku (Tuberculosis). 2006;**81**(10):609-612

[44] Zainal MA, Tan A. *Mycobacterium fortuitum* catheter-related sepsis in acute leukaemia. Singapore Medical Journal. 2006;**47**(6):543

[45] Winthrop KL et al. The clinical management and outcome of nail salon—Acquired *Mycobacterium fortuitum* skin infection. Clinical Infectious Diseases. 2004;**38**(1):38-44

[46] Winthrop KL et al. An outbreak of mycobacterial furunculosis associated with footbaths at a nail salon. New England Journal of Medicine. 2002;**346**(18):1366-1371

[47] Quiñones C et al. An outbreak of *Mycobacterium fortuitum* cutaneous infection associated with mesotherapy. Journal of the European Academy of Dermatology and Venereology. 2010;**24**(5):604-606

[48] Ronald Buckley L et al. *Mycobacterium fortuitum* infection occurring after a punch biopsy procedure. Pediatric Dermatology. 1997;**14**(4):290-292

[49] Nolan CM, Hashisaki PA, Dundas DF. An outbreak of soft-tissue infections due to *Mycobacterium fortuitum* associated with electromyography. Journal of Infectious Diseases. 1991;**163**(5):1150-1153

[50] Ferreira O et al. A cutaneous infection by *Mycobacterium chelonae* in a patient with rheumatoid arthritis. Dermatology Online Journal. 2010;**16**(4):3-3

[51] Kullavanijaya P et al. Disseminated *Mycobacterium chelonae* cutaneous infection: Recalcitrant to combined antibiotic therapy. The Journal of Dermatology. 2003;**30**(6):485-491

[52] Patel JB et al. Sequence-based identification of Mycobacterium species using the Microseq 500 16S rDNA bacterial identification system. Journal of Clinical Microbiology. 2000;**38**(1):246-251

[53] Drouineau O et al. Infection cutanée à *Mycobacterium chelonae* en hémodialyse. Néphrologie & Thérapeutique. 2006;**2**(3):136-139

[54] Adenis-Lamarre E et al. Cutaneous infection due to Mycobacterium chelonae during anti-TNF therapy. 2009;**136**(11):811-814

[55] Jankovic M et al. A fatal *Mycobacterium chelonae* infection in an immunosuppressed patient with systemic lupus erythematosus and concomitant Fahr's syndrome. Journal of Infection and Chemotherapy. 2011;**17**(2):264-267

[56] Haas SR, Hodge MB, Duncan RA. Cushing's syndrome presenting as disseminated cutaneous *Mycobacterium chelonae* infection. Clinical Infectious Diseases. 2001;**33**(6):e51-e53

[57] Nash KA, Brown-Elliott BA, Wallace RJ. A novel gene, erm (41), confers inducible macrolide resistance to clinical isolates of *Mycobacterium abscessus* but is absent from *Mycobacterium chelonae*. Antimicrobial Agents and Chemotherapy. 2009;**53**(4): 1367-1376

[58] Colombo RE, Olivier KN. Diagnosis and treatment of infections caused by rapidly growing mycobacteria. In: Seminars in Respiratory and Critical Care Medicine. New York, USA: Thieme Medical Publishers; 2008

[59] Wallace RJ et al. Comparison of the in vitro activity of the glycylcycline tigecycline (formerly GAR-936) with those of tetracycline, minocycline, and doxycycline against isolates of nontuberculous mycobacteria. Antimicrobial Agents and Chemotherapy. 2002;**46**(10):3164-3167

[60] Petrini B. *Mycobacterium abscessus*: An emerging rapid-growing potential pathogen. APMIS. 2006;**114**(5):319-328

[61] Brown B et al. Activities of four macrolides, including clarithromycin, against *Mycobacterium fortuitum*, *Mycobacterium chelonae*, and *M. chelonae*-like organisms. Antimicrobial Agents and Chemotherapy. 1992;**36**(1):180-184

[62] Swenson JM et al. Antimicrobial susceptibility of five subgroups of *Mycobacterium fortuitum* and *Mycobacterium chelonae*. Antimicrobial Agents and Chemotherapy. 1985; **28**(6):807-811

[63] Griffith DE et al. An official ATS/IDSA statement: Diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. American Journal of Respiratory and Critical Care Medicine. 2007;**175**(4):367-416

[64] Chang C-Y et al. Venous catheter-associated bacteremia caused by rapidly growing mycobacteria at a medical center in Central Taiwan. Journal of Microbiology, Immunology, and Infection = Wei mian yu gan ran za zhi. 2009;**42**(4):343-350

[65] Srivastava BK et al. Oxazolidinone antibacterials and our experience. Anti-Infective Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Infective Agents). 2008;**7**(4):258-280

[66] Girard D et al. Comparison of azithromycin, roxithromycin, and cephalexin penetration kinetics in early and mature abscesses. Journal of Antimicrobial Chemotherapy. 1993;**31**(suppl_E):17-28

[67] da Costa ARF et al. Molecular identification of rapidly growing mycobacteria isolates from pulmonary specimens of patients in the State of Pará, Amazon region, Brazil. Diagnostic Microbiology and Infectious Disease. 2009;**65**(4):358-364

[68] Shen G-H et al. High efficacy of clofazimine and its synergistic effect with amikacin against rapidly growing mycobacteria. International Journal of Antimicrobial Agents. 2010;**35**(4):400-404

[69] Chopra S et al. Identification of antimicrobial activity among FDA-approved drugs for combating *Mycobacterium abscessus* and *Mycobacterium chelonae*. Journal of Antimicrobial Chemotherapy. 2011;**66**(7):1533-1536

[70] Regnier S et al. Clinical management of rapidly growing mycobacterial cutaneous infections in patients after mesotherapy. Clinical Infectious Diseases. 2009;**49**(9):1358-1364

[71] Tebas P et al. Rapid development of resistance to clarithromycin following monotherapy for disseminated *Mycobacterium chelonae* infection in a heart transplant patient. Clinical Infectious Diseases. 1995;**20**(2):443-444

[72] Girgis DO, Karp CL, Miller D. Ocular infections caused by non-tuberculous mycobacteria: Update on epidemiology and management. Clinical & Experimental Ophthalmology. 2012;**40**(5):467-475

[73] Turner L, Stinson I. *Mycobacterium fortuitum*: As a cause of corneal ulcer. American Journal of Ophthalmology. 1965;**60**(2):329-331

[74] Huang S et al. Non-tuberculous mycobacterial keratitis: A study of 22 cases. British Journal of Ophthalmology. 1996;**80**(11):962-968

[75] Lalitha P, Rathinam S, Srinivasan M. Ocular infections due to non-tuberculous mycobacteria. Indian Journal of Medical Microbiology. 2004;**22**(4):231

[76] Hu F-R, Huang W-J, Huang S-F. Clinicopathologic study of satellite lesions in nontuberculous mycobacterial keratitis. Japanese Journal of Ophthalmology. 1998;**42**(2):115-118

[77] Lazar M et al. *Mycobacterium fortuitum* keratitis. American Journal of Ophthalmology. 1974;**78**(3):530-532

[78] Brancato R et al. *Mycobacterium chelonae* keratitis after excimer laser photorefractive keratectomy. Archives of Ophthalmology. 1997;**115**(10):1316-1318

[79] Hu F-R. Infectious crystalline keratopathy caused by *Mycobacterium fortuitum* and *Pseudomonas aeruginosa*. American Journal of Ophthalmology. 1990;**109**(6):738-739

[80] Dugel PU et al. *Mycobacterium fortuitum* keratitis. American Journal of Ophthalmology. 1988;**105**(6):661-669

[81] Alvarenga L et al. Infectious post-LASIK crystalline keratopathy caused by nontuberculous mycobacteria. Cornea. 2002;**21**(4):426-429

[82] Chang MA, Jain S, Azar DT. Infections following laser in situ keratomileusis: An integration of the published literature. Survey of Ophthalmology. 2004;**49**(3):269-280

[83] Wunsh SE et al. *Mycobacterium fortuitum* infection of corneal graft. Archives of Ophthalmology. 1969;**82**(5):602-607

[84] Abshire R et al. Topical antibacterial therapy for mycobacterial keratitis: Potential for surgical prophylaxis and treatment. Clinical Therapeutics. 2004;**26**(2):191-196

[85] de la Cruz J, Behlau I, Pineda R. Atypical mycobacteria keratitis after laser in situ keratomileusis unresponsive to fourth-generation fluoroquinolone therapy. Journal of Cataract & Refractive Surgery. 2007;**33**(7):1318-1321

[86] Ford JG et al. Nontuberculous mycobacterial keratitis in South Florida. Ophthalmology. 1998;**105**(9):1652-1658

[87] Alexandrakis G, Alfonso EC, Miller D. Shifting trends in bacterial keratitis in South Florida and emerging resistance to fluoroquinolones. Ophthalmology. 2000;**107**(8):1497-1502

[88] Aung TT et al. Discovery of novel antimycobacterial drug therapy in biofilm of pathogenic nontuberculous mycobacterial keratitis. The Ocular Surface. 2017;**15**:770-783

[89] Aylward G, Stacey A, Marsh R. *Mycobacterium chelonei* infection of a corneal graft. British Journal of Ophthalmology. 1987;**71**(9):690-693

[90] Kwon YS, Koh W-J. Diagnosis of pulmonary tuberculosis and nontuberculous mycobacterial lung disease in Korea. Tuberculosis and Respiratory Diseases. 2014;**77**(1):1-5

[91] Koh W et al. Pulmonary TB and NTM lung disease: Comparison of characteristics in patients with AFB smear-positive sputum. The International Journal of Tuberculosis and Lung Disease. 2006;**10**(9):1001-1007

[92] Jeong YJ et al. Nontuberculous mycobacterial pulmonary infection in immunocompetent patients: Comparison of thin-section CT and histopathologic findings. Radiology. 2004;**231**(3):880-886

[93] Marras TK et al. Opinions differ by expertise in *Mycobacterium avium* complex disease. Annals of the American Thoracic Society. 2014;**11**(1):17-22

[94] Yuan M-K et al. Comparative chest computed tomography findings of non-tuberculous mycobacterial lung diseases and pulmonary tuberculosis in patients with acid fast bacilli smear-positive sputum. BMC Pulmonary Medicine. 2014;**14**(1):65

[95] Chu H et al. Chest imaging comparison between non-tuberculous and tuberculosis mycobacteria in sputum acid fast bacilli smear-positive patients. European Review for Medical and Pharmacological Sciences. 2015;**19**(13):2429-2439

[96] Koh W-J et al. Bilateral bronchiectasis and bronchiolitis at thin-section CT: Diagnostic implications in nontuberculous mycobacterial pulmonary infection. Radiology. 2005;**235**(1):282-288

[97] Kim RD et al. Pulmonary nontuberculous mycobacterial disease: Prospective study of a distinct preexisting syndrome. American Journal of Respiratory and Critical Care Medicine. 2008;**178**(10):1066-1074

[98] Park IK, Olivier KN. Nontuberculous mycobacteria in cystic fibrosis and non–cystic fibrosis bronchiectasis. In: Seminars in Respiratory and Critical Care Medicine. New York, USA: Thieme Medical Publishers; 2015

[99] Horsburgh CR Jr. Epidemiology of disease caused by nontuberculous mycobacteria. 1996;**11**(4):244-251

[100] Olivier KN et al. Nontuberculous mycobacteria: I: Multicenter prevalence study in cystic fibrosis. American Journal of Respiratory and Critical Care Medicine. 2003;**167**(6): 828-834

[101] Dorman SE, Holland SM. Interferon-γ and interleukin-12 pathway defects and human disease. Cytokine & Growth Factor Reviews. 2000;**11**(4):321-333

[102] Winthrop KL et al. Nontuberculous mycobacteria infections and anti–tumor necrosis factor-α therapy. Emerging Infectious Diseases. 2009;**15**(10):1556

[103] Winthrop K et al. Mycobacterial diseases and antitumour necrosis factor therapy in USA. Annals of the Rheumatic Diseases. 2013;**72**(1):37-42

[104] Egelund EF, Fennelly KP, Peloquin CA. Medications and monitoring in nontuberculous mycobacteria infections. Clinics in Chest Medicine. 2015;**36**(1):55-66

[105] Wallace RJ et al. Macrolide/azalide therapy for nodular/bronchiectatic *Mycobacterium avium* complex lung disease. Chest. 2014;**146**(2):276-282

[106] Koh W-J et al. Treatment of refractory *Mycobacterium avium* complex lung disease with a moxifloxacin-containing regimen. Antimicrobial Agents and Chemotherapy. 2013; **57**(5):2281-2285

[107] Jarand J et al. Long-term follow-up of *Mycobacterium avium* complex lung disease in patients treated with regimens including clofazimine and/or rifampin. Chest. 2016;**149**(5): 1285-1293

[108] Kasperbauer SH, De Groote MA. The treatment of rapidly growing mycobacterial infections. Clinics in Chest Medicine. 2015;**36**(1):67-78

[109] Kim SY et al. The drug susceptibility profile and inducible resistance to macrolides of *Mycobacterium abscessus* and *Mycobacterium massiliense* in Korea. Diagnostic Microbiology and Infectious Disease. 2015;**81**(2):107-111

[110] Choi G-E et al. Macrolide treatment for *Mycobacterium abscessus* and *Mycobacterium massiliense* infection and inducible resistance. American Journal of Respiratory and Critical Care Medicine. 2012;**186**(9):917-925

[111] Jarand J et al. Clinical and microbiologic outcomes in patients receiving treatment for *Mycobacterium abscessus* pulmonary disease. Clinical Infectious Diseases. 2011;**52**(5): 565-571

[112] Woods G et al. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard M24-A. Vol. 23, No. 18. Wayne, PA: NCCLS; 2003. pp. 1-56238

[113] Cavusoglu C, Gurpinar T, Ecemis T. Evaluation of antimicrobial susceptibilities of rapidly growing mycobacteria by Sensititre RAPMYCO panel. New Microbiologica. 2012;**35**(1): 73-76

Chapter 4

The Genus *Corynebacterium* in the Genomic Era

Carlos Leonardo Araújo, Jorianne Alves, Alyne Lima, Larissa Dias, Patricia Silva, Joana Marques, Vasco Azevedo, Artur Silva and Adriana Folador

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80445

Abstract

This chapter will address the main omics approaches used in studies involving the genus *Corynebacterium*, Gram-positive microorganisms that can be isolated from many diverse environments. Currently, the genus *Corynebacterium* has more than 130 highly diversified species, many of which present medical, veterinary and biotechnological importance, such as *C. diphtheriae*, *C. pseudotuberculosis, C. ulcerans* and *C. glutamicum*. Due to the wide application in these fields, several omics methodologies are used to better elucidate the species belonging to this genus, such as genomics, transcriptomics and proteomics. The genomic era has contributed to the development of more advanced and complex approaches that enable the increase of generated data, and consequently the advance on the structural, functional and dynamic knowledge of biological systems.

Keywords: *Corynebacterium*, genomic era, genomics, transcriptomics, proteomics

1. Introduction

The genus *Corynebacterium* was proposed by Lehman and Neumann in 1896, to describe a type strain bacillus *Corynebacterium diphtheriae*. However, antecedent to its final taxonomic classification, *C. diphtheriae* had already been described as synonymous species such as *Microsporon diphthericum*, *Bacillus diphtheriae*, and *Pacinia loeffleri*. After its classification, the species was again described as the synonym *Mycobacterium diphtheriae* by Krasil'nikov in 1941 [1]. Afterward, the genus came to accommodate other bacterial species that presented

similar form and/or pathogenicity mechanisms [2]. Currently, the genus has 110 valid species, of which 132 species have synonymous species and 11 subspecies [1].

Frequently, members of the genus *Corynebacterium* have a rod morphology, being Gram-positive, immobile, nonsporulated, presenting an aerobic growth, and producers of the catalase enzyme. They are part of the normal skin microbiota and mucous membranes of several hosts, being present also in the environment (soil, water, among others). Bacteria of this genus still share characteristics like G+C content (47–74%), oxygenase enzyme production, and no production of the enzyme collagenase. In addition, their cell wall is thick, and it has the presence of mycolic acids, peptidoglycan and arabinogalactan, also saturated and unsaturated fatty acids [3].

Bacterial species affiliated with this genus can be classified as: pathogenic, opportunistic and saprophytic. The strains of medical and veterinary interest are commonly divided in two groups: diphtheria and nondiphtheria. The diphtheria group, producer of the diphtheria toxin (TD) encoded by the viral *tox* gene, present in the DNA of β lysogenic bacteriophages [4]. Among this group, we can mention three species: *C. pseudotuberculosis*, *C. diphtheriae* and *C. ulcerans* [5]. Nondiphtheria species, as agents of infection, are considered opportunistic pathogens, because they are present in the skin normal microbiota, and in the human nasopharynx [6]. The species *Corynebacterium jeikeium*, *Corynebacterium urealyticum* and *Corynebacterium resistens* are considered opportunistic [7]. Nonpathogenic strains, such as *Corynebacterium glutamicum*, *Corynebacterium efficiens*, *Corynebacterium crenatum* and *Corynebacterium variabile*, have biotechnological importance in the production of amino acids and in the cheese industry [8].

2. Main species of medical and biotechnological importance

2.1. *Corynebacterium diphtheriae*

Diphtheria is a disease of acute and transmissible evolution within local and systemic manifestations, affecting the upper respiratory tract, and has been one of the main causes of death, especially in children, in different continents, even in countries with immunization programs. *C. diphtheriae* was isolated mainly from humans, however it has been isolated from other hosts, such as horses, cats and dogs [9].

C. diphtheriae is a rod-shaped bacterium, and its cells can measure from 0.5 to 2.0 μm in size. Strains belonging to this species do not produce spores and do not present structures such as capsules and flagella [10]. The strains are classified in four biovars: *mitis*, *gravis*, *intermedius* and *belfanti*, based on the infection severity, morphological pattern of the colonies, carbohydrate fermentation and hemolysis [9]. These biovars resemble the production of cystinase enzyme (Tinsdale medium), and the fermentation of glucose and maltose. Moreover, these biovars produce neither the enzymes pyrazinamidase nor urease, and also they are not capable of fermenting sucrose. In relation to nitrate reduction, the biovars *mitis*, *gravis and intermedius* present a positive reaction, and the *belfanti* biovar presents a negative reaction [11].

In its original form, *C. diphtheriae* does not cause diseases and its pathogenicity is related to infection by a bacteriophage carrying the *tox* gene encoding TD. Thus, the lysogenic cell carries the *tox* gene, highly conserved in the bacterial chromosome through generations [12]. TD is a potent exotoxin of protein nature capable of acting in all tissues with special tropism for the myocardium, nervous system, kidneys and adrenals [6].

TD acts on the inhibition of protein synthesis causing cell death and it is composed of a single polypeptide chain containing two fragments, A and B, connected by a disulfide bond, and both are required for intoxication of tissue culture cells. The fragment A possesses the active site of TD and it is responsible for the enzymatic activity, while the fragment B is responsible for the fixation of the toxin with receptors in the host cells [13].

2.2. *Corynebacterium pseudotuberculosis*

The bacterium *C. pseudotuberculosis* presents characteristics such as the production of the enzyme nitrate reductase (biovar-dependent) and urease, the fermentation of carbohydrates maltose and glucose and the presence of halos of beta-hemolysis in blood agar. As for their colonies, they present a size ranging from 0.5 to 0.6 × 1.0 to 3.0 μm, with a whitish and viscous appearance [14]. Biovar *ovis*, nitrate reductase negative, affects sheep and goats, and occasionally swine, causing caseous lymphadenitis, in humans may cause chronic subacute lymphadenitis [3]. While biovar *equi*, which the isolates can reduce nitrate to nitrite, mainly infects equines, buffaloes and camelids, causing ulcerative lymphangitis and edematous skin disease [15]. Due to its prevalence in animals of economic importance, diseases associated with *C. pseudotuberculosis* strains cause reduction in meat and milk production, wool depreciation, delay in animal development, deficiencies in reproductive indices of the herd, carcass condemnation, early discarding and occasional death of animals, also high treatment costs and veterinary fees [3].

The virulence of *C. pseudotuberculosis* is related to three main factors: the cell wall structure, its intracellular capacity for macrophage persistence and the production of phospholipase D (PLD) as exotoxin, which is considered the main virulence factor of the species [16]. Although its main virulence factor is already well established, toxigenic strains of this species can also produce TD [17, 18].

The diagnosis of *C. pseudotuberculosis* in infected animals is performed by the macroscopic observation of the superficial abscesses formed, associated to laboratory culture in the selective media of tellurian agar, bacterioscopy, catalase test (positive for *Corynebacterium*) and biochemical tests [3]. In addition to serological tests such as seroneutralization, indirect hemagglutination and Enzyme-Linked Immunosorbent Assay (ELISA), allergic tests and tests based on molecular biology such as the polymerase chain reaction (PCR), through the conserved genes *rrs, rpoB* and *pld* in multiplex PCR [19]. Recently, with addition of the *narG* gene, it is possible to distinguish biovars from the capacity to reduce nitrate [20].

The animals affected by caseous lymphadenitis are usually treated through lymph node drainage and isolation of infected animals. Yet, this practice does not completely eliminate bacteria, due to the possibility of dissemination to viscera and other internal organs, as well

to the great potential of contamination of the environment. In addition, antibiotic treatment does not produce satisfactory results due to difficult penetration into the abscess capsule, making the treatment unfeasible, emphasizing that prophylaxis is the best method to combat the disease [3].

2.3. *Corynebacterium ulcerans*

C. ulcerans has been described as the etiological agent of several infections in animals, such as goats, dogs, cats and cattle. The contact with affected animals is the main form of transmission of *C. ulcerans* to human hosts, causing diphtheria of zoonotic nature. The first cases of human infections were related to the consumption of milk contaminated by this microorganism. In the 1990s, it was presented as an emerging pathogen in countries of large animal production, such as England, Japan, Germany, Denmark, and Brazil [21].

As for its biochemical characterization, *C. ulcerans* presents features such as the production of the enzyme gelatinase, inability to reduce nitrate and virulence factors, including toxic lipids associated with the cell wall, which may mediate bacterial resistance to phagocyte attack. *C. ulcerans* is capable of producing PLD, as well as *C. pseudotuberculosis* [22]. The third virulence factor for *C. ulcerans* is the production of the diphtheria toxin. *C. ulcerans* strains infected by bacteriophage carriers of the *tox* gene are the major responsible for clinical cases in humans and animals [23].

The diseases related to these strains show symptoms as frequent nasal bleeding, skin lesions similar to cutaneous diphtheria, necrosis and mucosal ulceration, granulomatous pulmonary nodules, lymph node involvement and the occurrence of cellular death [24].

Although diphtheria by *C. ulcerans* is associated with its TD production, vaccination using the diphtheria toxoid has an unknown efficacy. This fact is due to the knowledge limitation of the bacterium molecular epidemiology. This limitation is mainly related to the structure of the *tox* gene, which has specific differences when compared to both interspecific (*C. diphtheriae tox* gene) and intraspecific in *C. ulcerans* [21].

2.4. *Corynebacterium glutamicum*

Bacterial strains belonging to *C. glutamicum* species are commonly found in the environment, in habitats such as soil. This bacterium is rod-shaped, capable of reducing nitrate to nitrite, facultative, mesophilic anaerobic and capable of carbohydrates fermentation. As a generally recognized as safe (GRAS) microorganism, it is widely used in the biotechnology industry, for its ability to produce amino acids like L-glutamate and L-lysine, used as flavor promoters and food additives [25]. More than 2.5 million tons of lysine is produced annually by mutant strains of *C. glutamicum*, for animal nutrition, applications in the pharmaceutical, cosmetics, fuel and polymer industries [26].

The nutrients used for industrial fermentation by *C. glutamicum* include glucose, fructose and sucrose, derived from corn starch, cassava or wheat, as well as cane molasses and beet molasses. Obtaining sugar from raw materials and agroindustry wastes is very common in

countries with high agricultural production, such as China, the United States and Brazil, its use reduces the industrial production process. Additionally, *C. glutamicum* strains are ideal for large-scale fermentation processes, since they are resistant to oscillations in oxygen tension and in the substrate supply, which often occurs in these industrial processes [25].

One of the factors considered in the selection of strains is the maximum theoretical yield of a cell within production of lysine from glucose. This yield should turn around 75% conversion of carbohydrate into final product. Metabolic flux analysis, considering the main metabolic pathways that can be used by *C. glutamicum* to produce lysine, indicate that the theoretical yield is increasing, producing more than two million tons of amino acids per year [27].

3. Genomics

3.1. The impact of next sequencing technologies on genomics of the genus *Corynebacterium*

Forty years ago, the advent of DNA sequencing by Sanger method began to revolutionize genome studies [28]. The first genomes to be sequenced were viruses and organelles. In 1995, Craig Venter and colleagues published the two first complete bacterial genomes: *Haemophilus influenzae* and *Mycoplasma genitalium* [29, 30]. Later, several sequencing projects were created, which transformed the biology as a whole, by means of allowing to decipher complete genes and later whole genomes using the methodology developed by Sanger and colleagues in 1977 [31].

The publication of the first draft of the human genome in 2001 prompted companies to develop new sequencers that would provide more speed and accuracy, as well as cost and labor savings [32]. Since 2005, new sequencing technologies, called next-generation sequencing (NGS) or high-performance sequencers, have been able to generate gigabases (Gb) of data in a few days, (e.g. Illumina, Ion Torrent, Single Molecule Real Time-SMRT, PacBio, and Oxford Nanopore) [33]. Hence, the public domain databases have had, since registered the emergence of NGS platforms, an exponential increase in the number of deposited biological sequences, with more than 144,000 bacterial genomes already registered [34].

Currently, the genus *Corynebacterium* has more than 265 genome projects registered in public databases. According to the GOLD website, a database that provides project information in all three domains of life, *Corynebacterium* genome deposits date back to 2007 [35]. Since then, the increase of these data positively impacted the development of studies with transcriptomic and proteomic approaches, in order to provide a better understanding of several molecular processes from different corynebacterial species.

3.2. Comparative genomics studies

The remarkable growth of the number of complete genomes provided the advance in the comparative analyzes between genomes, allowing studies in large scale. Comparative genomics

provide a global understanding of the gene repertoire of a given species or genus, in order to elucidate the essential genes that are involved in processes such as replication, transcription and translation, in addition to the genes considered as accessory, that are also important for the characterization of variabilities in their genetic patterns, as well as allows the analysis of the genomic plasticity [36].

In another aspect, comparative analyzes between different strains within the same phylogenetic clade make it possible to recognize similarities and differences among genomes, to clarify which sequences are capable of diverging phenotypic changes in organisms, and to elucidate the mechanisms of virulence among pathogenic organisms or in in the case of environmental microorganisms. From this premise, the pan-genome concept emerged [37].

Regarding *C. ulcerans*, a study was conducted with 19 strains identifying 4120 genes composing the pan-genome, of which 1405 were present in the core genome and 2715 present in the accessory genome, where proteins involved in the pili formation and the *tox* gene were found in a large part of the genomes. Furthermore, variations between the transmembrane proteins and proteins secreted among the different species have been identified, contributing to the variability of the pathogenicity between them. This study made a greater understanding possible, regarding the knowledge around the virulence of this emerging pathogen [38].

The pan-genome is constituted by the core genome, which configures the genes present among all analyzed strains; the accessory genome that shares genes between two or more, but not all, strains and includes the genes the bacteria needs to survive in a specific environment, in addition to species-specific genes belonging to a single lineage, which can be acquired via horizontal transfer [37, 39]. The representatives of the genus *Corynebacterium* become an interesting object of studies of comparative genomics and evolution, due to its diverse lifestyles [40].

This approach was used in *C. jeikeium* by comparing 17 plasmids from different clinical isolates, which identified that plasmid pK43 can act as a natural vehicle for gene transfer conferring antimicrobial resistance between multiresistant strains and possibly between other members of the corynebacteria group, such as *C. diphtheriae* [41].

In *C. pseudotuberculosis*, the pan-genome of 15 strains revealed differences between the biovars of this species, in which the biovar *ovis* presented clonal behavior, while the *equi* group has a greater genetic diversity [42]. Recently, a study with strains isolated from equines was analyzed and corroborated the diversity of the biovar, also presenting a wide repertoire of resistance genes and virulence factors such as: beta-lactamases, recombination endonucleases and phage integrase [43].

In a comparative analysis between *Corynebacterium jeikeium, Corynebacterium urealyticum, Corynebacterium kroppenstedtii, Corynebacterium resistens* and *Corynebacterium variabile,* it was possible to identify 83 regulatory genes, being 56 of transcriptional DNA binding regulators and nine sigma factors. Furthermore, 44 regulatory proteins were identified that were present in the core genome. These genes shared by the strains are involved in the generation of short-chain volatile acids, which are related to the odor formation process of the human body, showing the importance of this approach in lipophilic corynebacteria [44].

Codon deviation studies can aid in the understanding of the evolutionary molecular basis through parameters such as gene expression, amino acid conservation and codon-anticodon interaction. These factors reveal the type of selective pressure in eukaryotic and prokaryotic genes. In order to understand the molecular evolution of the genus *Corynebacterium*, comparative analyzes of G + C content and codon use were carried out relating different species, revealing evolutionary relationships that allowed divergence between the groups of pathogenic and nonpathogenic corynebacteria [40].

4. Transcriptomics

The genomic approach allowed to know the sequence of DNA of a certain organism, though, only this knowledge does not define the gene function to external stimuli. A protein to be synthesized primarily needs the DNA to be transcribed into an RNA molecule, later translated into a protein molecule. However, the genes are not active all the time in the cell, and they are expressed when necessary to act in cellular biological process. The set of genes are expressed in a cell under a certain physiological condition or stage of development at a specific time is called transcriptome [45].

Studies that address the transcriptome technique aim the analysis of the collection of all transcripts and provide information about the regulation of the genes, too allow inferring functions of uncharacterized genes, helping to understand the biology of the organism analyzed. One of the applications obtained by this approach is the usage of the data generated to provide more information about the host defense response to the survival and proliferation of bacterial pathogens, which enables an understanding of the pathogenesis of infectious diseases [46].

Due to the diverse applications of transcriptomics, new technologies and high-throughput methods have been developed for large-scale analysis, such as hybridization-based method (Microarray) and sequencing-based methods such as RNA sequencing [47].

Microarray technology is considered a large-scale method because it generates the expression profile of thousands of transcripts simultaneously. Studies with microarray technology have identified clusters of genes that are involved in specific physiological responses, through the variations of environmental conditions faced by microorganisms [48], such as ammonia limitation. This compound is used as a source of nitrogen that is essential for almost all complex macromolecules in bacteria. A study analyzed the response of *C. glutamicum* in ammonia-limiting medium, demonstrating that there was alteration in the expression of 285 genes, many of which encode transport proteins and proteins involved in metabolism, nitrogen regulation, energy generation and protein turnover [49].

Other studies with *C. glutamicum* were carried out aiming to evaluate the level of gene expression essential to the survival of the bacteria in stress environments. The transcriptional profile of this species under growth conditions with citrate as a source of carbon and energy compared to glucose demonstrated that *citM* and *tctCBA* encoding citrate uptake systems were induced, while the *ptsG*, *ptsS* and *ptsF* genes encoding the glucose capping system were

repressed. Additionally, genes encoding tricarboxylic acid, malic enzyme, PEP carboxykinase, gluconate-glyceraldehyde-3-phosphate dehydrogenase and ATP synthase cycle enzymes were induced [50].

The microarray technique provided an advance in the research with important organisms, such as the members of the genus *Corynebacterium*. Nevertheless, this technique has some limitations, such as high noise interference, inability to detect transcripts with a low number of copies per cell, low coverage of transcripts, and dependence on prior knowledge about the genome for the preparation of the probes, consequently generating little information about the transcript sequence [47].

As a result of these limitations and the advent of NGS platforms, a promising alternative technique was developed, RNA-seq. Through this technique, it was possible to obtain more accurate, fast and reliable analyzes from cDNA sequencing. The advantages of this method are: low occurrence or absence of interference, detection of small transcripts that would not be detected by other methods, low cost and reduction of time and work to prepare the samples. RNA-Seq is considered an ideal tool for the analysis of complete transcriptomes and is applied in the exploration of expression profile, and characterization of differentially expressed genes. Thus, it represents an important tool to uncover the mechanisms of virulence and pathogenicity in microorganisms [51, 52].

Relating to this, two studies with *C. pseudotuberculosis* simulating the stress conditions faced by the bacterium during infection in host were performed. The first study was with strain *C. pseudotuberculosis* 1002, biovar *ovis*, which underwent three stress conditions: thermal, acidic and osmotic. Most of the identified targets were related to oxidation and reduction, cell division and cell cycle, and the *stimulon* of the three stresses presented induced genes that participate in the mechanisms of virulence, defense against oxidative stress, adhesion and regulation, revealing that they have important role in the infection process [53]. The other study, with strain 258, biovar *equi*, was performed using the thermal stress condition, similar to the conditions performed on strain 1002. Herein, 113 genes were considered induced, in which *hspR, grpE, dnaK* and *clpB* were highlighted due to its expression rates and participation in the mechanism of adaptation of the pathogen to high temperatures [54].

Recently, the first analysis of RNA-Seq with *C. diphtheriae* was developed, in which it was sought to investigate the alteration of the transcription profile between a wild strain and a Δ*dtxR* mutant, also to detect the operon structures from the transcriptome data of the wild type strain. The authors revealed that approximately 15% of the genome was differentially transcribed and that DtxR may also play a role in other regulatory functions, in addition to regulating the metabolism of iron and diphtheria toxin. Finally, they identified 471 operons subdivided into 167 sub-operon structures [55].

One of the representatives of the genus that had the gene expression regulation most studied is *C. glutamicum*. The RNA-Seq approach elucidated the regulatory mechanisms of several industrially relevant compounds, such as the dissolved oxygen concentration (DO), which is important in industrial microbial processes, providing new information on the relationship between oxygen supply and bacterial metabolism [56].

In relation to the production of amino acids, L-lysine-producing *C. glutamicum* ATCC21300 obtained 543 differentially expressed genes compared to wild type *C. glutamicum* ATCC13032, highlighting *bioA, bioB, bioD*, NCgl1883, NCgl1884, and NCgl1885 involved in metabolism or transport of the biotin, of which the *bioB* gene was hyper expressed about 20-fold, and when it was discontinued, lysine production was reduced to approximately 76% and the genes NCgl1883, NCgl1884, and NCgl1885 were repressed [57]. Genes involved in the production of L-valine were also analyzed, in which 1155 differentially expressed genes were identified, where *ilvBN, ilvC, ilvD,* and *iLvE* were hyperexpressed, resulting in the improvement of the carbon flux used to produce valine. Thus, the work involving this approach helps to better understand *C. glutamicum* for the generation of biotechnological products [58].

The RNA-Seq technique also can be applied for identification of operon structures, although this approach requires a reliable genome annotation and low gene rate with unknown function. Through these data, transcription initiation sites (TSSs) can be identified and corrected, allowing a more detailed analysis of the promoters and classifying them according to their location in relation to the protein coding regions (CDs). For example, see [59, 60].

5. Proteomics

The central base of molecular biology involves understanding how cells work and interact among each other. These cellular processes occur through the activity of biomolecules that act together throughout specialized mechanisms. This whole process involves storing the genetic information in the DNA molecule and the unidirectional flow of this information to the RNA and proteins. Proteins make up a large part of the cell molecular machinery, and the overall analysis of them provides the information needed to understand how cells work. This analysis is referred to as proteomics [61].

In 1995, the term "proteome" was taken as the set of proteins produced by a cell or tissue at a given time and condition [62]. As early as 1996, the term "proteomics" appeared to define the large characterization of all protein contents of a cell line, tissue or organism [63]. The study of the proteome currently refers not only to the knowledge of the protein content of a given organism in a given condition, but also includes the quantification, location, modifications, interactions and function of these proteins [64, 65].

This area has three strands: expression, structural and functional. Expression proteomics generally involves studies to investigate the pattern of protein expression in abnormal cells. This classification encompasses studies of qualitative and quantitative expression analysis of total proteins under two different conditions. The second analyzes the three-dimensional conformation and structural complexities of functional proteins. This strand makes it possible to identify all the proteins of a complex system and characterize the possible interactions of these proteins and protein complexes. Functional proteomics reveals the function of proteins based on their interactions with specific protein complexes and the detailed description of cell signaling pathways to which they are involved [66].

The most used methods for the identification and quantification of proteins are those based on mass spectrometry (MS). This technique allows the detection of compounds by the separation of the ions through mass-charge ratio. As each compound has a unique fragmentation pattern, the samples are ionized and separated with further identification of this pattern. Generally, two MS-based methods are currently most commonly used. The first method involves two-dimensional electrophoresis (2-DE) followed by staining, selection and MS. The other method involves isotopic markers to label proteins, separation by multidimensional liquid chromatography and MS analysis [65, 67, 68].

A typical proteomic experiment involves the step of preparing the sample, consisting of separating and isolating the proteins from the cell lysates, followed by separation of the protein mixture, and then the individual portions can be analyzed. Analysis may involve the bottom-up strategy and the top-down strategy. The first involves obtaining the peptides by enzymatic digestion of protein solutions and the subsequent separation of these peptides by liquid chromatography and MS analysis. In contrast, the top-down strategy involves the analysis of intact proteins by MS. For the quantitative determination of proteins two approaches are most commonly used: two-dimensional electrophoresis followed by staining, selection and identification by MS; and isotopic markers followed by protein separation by multidimensional liquid chromatography and MS [68, 69].

Proteomics studies involving the genus *Corynebacterium* mainly comprise studies with *C. glutamicum*, due to, in large part, its industrial importance in the production of amino acids. This species is investigated in relation to its genetics and physiology and, consequently, a diversity of information about its molecular biology and biochemistry available, including a variety of proteomic techniques. In consequence of the membrane organization with high concentration of mycolic acids, *C. glutamicum* has been used as a model for development and new proteomic technologies [70].

Proteomics analysis have also been used as an alternative to traditional molecular methods for the characterization of poorly known bacteria, especially those of clinical interest, by reason of the ability of these methodologies to provide a fast and reliable identification of these species. The matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) technique was able to detect strains of *Corynebacterium argentoratens, Corynebacterium confusum, Corynebacterium coyleae, Corynebacterium imitans, Corynebacterium kroppenstedtii, Corynebacterium mucifaciens, Corynebacterium riegelii* and *Corynebacterium ureicelerivorans* isolated from different clinical samples, such as blood, wounds and abscesses, also from respiratory, genitourinary, digestive tract, among others. These analyses show that there is a tendency for clinical laboratories to integrate proteomics in order to obtain faster and more sensitive results for the diagnosis of infections caused by rare bacteria [71].

In the case of pathogenic corynebacterial species, the repertoire of secreted and surface-exposed proteins, the exoproteome, have been documented because of their potential to act as antigenicity and virulence factors, since these molecules are promptly exposed to the host cells, making they are suitable for the use of vaccine and drug targets. A recent study investigated

both surface and extracellular proteome of two *C. ulcerans* strains, where NanoLC-MS/MS was performed to analyze the set of proteins, which were similar expression patterns of putative virulence factors [72].

The mapping of the extracellular proteome of *C. diphtheriae* through 2-DE and MALDI-TOF-MS detected proteins present in pathogenicity islands. According to these tests, possibly, the exoproteome of this pathogen is constituted of two distinct classes. The first involves molecules that have functions in the cytoplasm related with cell viability, such as protein synthesis and folding and detoxification mechanisms. The second class appears to be actively secreted and includes iron transporters and possible virulence factors that can be used in new vaccines [73].

The exoproteome of *C. pseudotuberculosis* has also been extensively characterized in the past years [74]. The use of transposon-binding proteins was investigated through a method of data-independent LC-MS acquisition (LC-MSE), used for proteins identification and quantification that was applied to compare the exoproteome of two biovar *ovis C. pseudotuberculosis* strains, C231 and 1002, where there were found 44 presents in both isolates in a total of 93 extracellular proteins [75].

Further, the combination of different proteomic methodologies as the 2-DE along with MALDI-TOF/TOF allowed the finding of 11 novel molecules in the *C. pseudotuberculosis* exoproteome, noncharacterized on the first comparative work [76]. The integration with *in silico* approaches also gives important insights about the behavior of the exoproteome. Pan-genome analysis can be performed to predict the set of exported proteins present in a large number of genomes available on public databases [77].

The proteomic map of a *C. jeikeium* strain was examined through 2-DE and MALDI-TOF-MS, through peptide mass fingerprinting (PMF), a high throughput protein identification methodology in which a protein is digested with endoprotease to produce the small constituent peptides. In this investigation, most spots were associated with functions essential for cell viability, such as protein synthesis and energy production, as carbohydrate, lipid and nucleotide metabolism. The surface proteins SurA and SurB, the adhesin CbpA and Che cholesterol esterase, known to act as virulence factors were also identified in the extracellular proteome [78].

In addition to these efforts, structural characterization methods for protein elucidation have also been used. The DtxR repressor is activated by transition metal ions and acts on the modulation of *tox* gene expression in *C. diphtheriae*. Through X-ray crystallography, it was possible to determine the general architecture of this biologically active Ni(II) bound protein with a resolution of 2.4 Å [79]. In *C. pseudotuberculosis*, the ArgR protein that acts as a regulator of arginine biosynthesis, an important metabolic pathway for bacteria, had the C-terminal domain crystal structure determined from X-ray diffraction with a resolution of 1.9 Å. The interest in this molecule lies in the fact that it participates in a pathway that is absent in its hosts, which makes it a potential target for the design of new drugs [80].

One of the greatest challenges of the postgenomic era is the amount of data generated through the different approaches, as well as the functional characterization of proteins. In this context, the analysis of protein-protein interaction networks (PPI) has been used for the identification of essential proteins and discovery of new therapeutic targets. This computational method is based on biological data topology information according to known interaction patterns to predict new interactions between molecules, where nodes represent proteins and the edges represent the predicted interactions [81].

The inter-specific PPI networks of *C. pseudotuberculosis* were constructed from proteins conserved in multiple pathogens, such as *M. tuberculosis, Y. pestis, E. coli, C. diphtheriae* and *C. ulcerans*, where the interaction network of the protein acetate kinase (Ack) was indicated as a possible new broad-spectrum therapeutic target [82]. Later, another study involving the interactome of *C. pseudotuberculosis* was developed, where the networks were constructed, revealing nonhomologous proteins to humans, cattle, goats, sheep and horses. The fact that such proteins predicted by the PPI result are essential to the pathogen, but not to the hosts, makes them important candidates for use as targets for the synthesis of new drugs [83].

6. Conclusion

Corynebacterium comprises several Gram-positive species known mainly for their pathogenic and biotechnological potential. Due to the advent of the NGS platforms, several strains of the genus have had their genomes sequenced in recent years, providing significant advances in the understanding of pathogenic mechanisms, metabolism, regulation, adaptation and evolution, among other aspects of these bacteria behaviors. Through genome projects, it was possible to better understand molecular functions and biological processes of several genes, to know the genomic architecture of different isolates, as well as to compare them at a DNA level, making these studies essential for the execution of more complex approaches. Transcriptomics, for example, has been employed in a wide variety of studies in order to fully and clearly understand the modulation of expression of genes of interest to different stimuli. Also, proteomic analyses provide a more complete and advanced knowledge in the study of biological systems. Hence, the field integration of the genomic era has provided valuable insights, aiming at a deeper understanding of various corynebacteria.

Acknowledgements

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES (88881.068052/2014-2101) for the financial support on this work.

Conflict of interest

The authors declare the absence of any conflict of interest.

Author details

Carlos Leonardo Araújo[1], Jorianne Alves[1], Alyne Lima[1], Larissa Dias[1], Patricia Silva[1], Joana Marques[1], Vasco Azevedo[2], Artur Silva[1] and Adriana Folador[1]*

*Address all correspondence to: carneiroar@gmail.com

1 Laboratory of Genomics and Bioinformatics, Center of Genomics and System Biology, Federal University of Pará, Belém, Pará, Brazil

2 Department of General Biology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

References

[1] List of Prokaryotic names with Standing in Nomenclature—Genus Corynebacterium [Internet]. Available from: http://www.bacterio.net/corynebacterium.html [Accessed: 2018 Apr 19]

[2] Pascual C, Lawson PA, Farrow JAE, Navarro Gimenez M, Collins MD. Phylogenetic analysis of the genus *Corynebacterium* based on 16s rRNA gene sequences. International Journal of Systematic Bacteriology. 1995;**45**(4):724-728

[3] Dorella FA, Carvalho Pacheco L, Oliveira SC, Miyoshi A, Azevedo V. *Corynebacterium pseudotuberculosis*: Microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Veterinary Research. 2006;**37**(2):201-218

[4] Groman N, Schiller J, Russell J. *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* responses to DNA probes derived from corynephage B and *Corynebacterium diphtheriae*. Infection and Immunity. 1984;**45**(2):511-517

[5] Fátima LD, Torres C, Ribeiro D, Jr RH, Gustavo L, Pacheco C, et al. Multiplex polymerase chain reaction to identify and determine the toxigenicity of *Corynebacterium* spp with zoonotic potential and an overview of human and animal infections. Mem Inst Oswaldo Cruz. 2013;**108**(2):272-279

[6] Hoskisson PA. Microbe profile: *Corynebacterium diphtheriae*—An old foe always ready to seize opportunity. Microbiology. 2018;**164**:865-867

[7] Schröder J, Maus I, Meyer K, Wördemann S, Blom J, Jaenicke S, et al. Complete genome sequence, lifestyle, and multi-drug resistance of the human pathogen *Corynebacterium resistens* DSM 45100 isolated from blood samples of a leukemia patient. BMC Genomics. 2012;**13**:141

[8] Nishio Y, Nakamura Y, Kawarabayasi Y, Usuda Y, Kimura E, Sugimoto S, et al. Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of *Corynebacterium efficiens*. Genome Research. 2003;**13**(7):1572-1579

[9] Leggett BA, De Zoysa A, Abbott YE, Leonard N, Markey B, Efstratiou A. *Toxigenic Corynebacterium diphtheriae* isolated from a wound in a horse. Veterinary Record. 2010; **166**(21):656-657

[10] Cerdeño-Tárraga AM. The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129. Nucleic Acids Research. 2003;**31**(22):6516-6523

[11] Funke G, Bernard KA. Coryneform gram-positive rods. In: Manual of Clinical Microbiology. 10th ed. 2011. pp. 413-442

[12] Funke G, von Graevenitz A, Clarridge JE, Bernard KA. Clinical microbiology of Coryneform bacteria. Clinical Microbiology Reviews. 1997;**10**(1):125-159

[13] Selim SA, Mohamed FH, Hessain AM, Moussa IM. Immunological characterization of diphtheria toxin recovered from *Corynebacterium pseudotuberculosis*. Saudi Journal of Biological Sciences. 2016;**23**(2):282-287

[14] Ruiz L, Jeronimo R, Barrera Valle M, Frias MT. Linfadenitis Caseosa I: Aspectos históricos, etiológicos y clínicos. Revista Electrónica de Cínica Veterinaria. 2007;**II**(8):1-22

[15] Pratt SM, Spier SJ, Carroll SP, Vaughan B, Whitcomb MB, Wilson WD. Evaluation of clinical characteristics, diagnostic test results, and outcome in horses with internal infection caused by *Corynebacterium pseudotuberculosis*: 30 cases (1995-2003). Journal of the American Veterinary Medical Association. 2005;**227**(3):441-448

[16] Selim SA. Oedematous skin disease of buffalo in Egypt. Journal of Veterinary Medicine Series B. 2001;**48**(4):241-258

[17] Soares SC, Trost E, Ramos RTJ, Carneiro AR, Santos AR, Pinto AC, et al. Genome sequence of *Corynebacterium pseudotuberculosis* biovar *equi* strain 258 and prediction of antigenic targets to improve biotechnological vaccine production. Journal of Biotechnology. 2013;**167**(2):135-141

[18] Viana MVC, Figueiredo H, Ramos R, Guimarães LC, Pereira FL, Dorella FA, et al. Comparative genomic analysis between *Corynebacterium pseudotuberculosis* strains isolated from buffalo. PLoS One. 2017;**12**(4):1-24

[19] Pacheco LGC, Pena RR, Castro TLP, Dorella FA, Bahia RC, Carminati R, et al. Multiplex PCR assay for identification of *Corynebacterium pseudotuberculosis* from pure cultures and for rapid detection of this pathogen in clinical samples. Journal of Medical Microbiology. 2007;**56**(4):480-486

[20] Almeida S, Dorneles EMS, Diniz C, Abreu V, Sousa C, Alves J, et al. Quadruplex PCR assay for identification of *Corynebacterium pseudotuberculosis* differentiating biovar *Ovis* and *Equi*. BMC Veterinary Research. 2017;**13**(1):290):1-8

[21] Cassiday PK, Pawloski LC, Tiwari T, Sanden GN, Wilkins PP. Analysis of toxigenic *Corynebacterium ulcerans* strains revealing potential for false-negative real-time PCR results. Journal of Clinical Microbiology. 2008;**46**(1):331-333

[22] Ott L. Adhesion properties of toxigenic corynebacteria. AIMS Microbiology. 2018; **4**(1):85-103

[23] Hogg RA, Wessels J, Hart J, Efstratiou A, Zoysa A, De MG, et al. Possible zoonotic transmission of toxigenic *Corynebacterium ulceans* from companion animals in a human case of fatal diphteria. Veterinary Record. 2008;**5**(165(23)):691-692

[24] Meinel DM, Konrad R, Berger A, König C, Schmidt-Wieland T, Hogardt M, et al. Zoonotic transmission of toxigenic *Corynebacterium ulcerans* strain, Germany, 2012. Emerging Infectious Diseases. 2015;**21**(2):356-358

[25] Buchholz J, Graf M, Freund A, Busche T, Kalinowski J, Blombach B, et al. CO_2/HCO_3 perturbations of simulated large scale gradients in a scale-down device cause fast transcriptional responses in *Corynebacterium glutamicum*. Applied Microbiology and Biotechnology. 2014;**98**(20):8563-8572

[26] Eggeling L, Bott M. A giant market and a powerful metabolism: L-lysine provided *by Corynebacterium glutamicum*. Applied Microbiology and Biotechnology. 2015;**99**(8):3387-3394

[27] Ntui CM. Structural and Functional Analysis of Thiamine-Phosphate and Homoserine Kinases from *Mycobacterium tuberculosis* [Thesis]. Pretoria: University of Pretoria; 2016

[28] Van Dijk EL, Jaszczyszyn Y, Naquin D, Thermes C. The third revolution in sequencing technology. Trends in Genetics. 2018:xx

[29] Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, et al. The minimal gene complement of *Mycoplasma genitalium*. Science. 1995;**270**(5235):397-404

[30] Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, et al. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science. 1995;**269**(5223):496-512

[31] Vincent AT, Derome N, Boyle B, Culley AI, Charette SJ. Next-generation sequencing (NGS) in the microbiological world: How to make the most of your money. Journal of Microbiology Methods. 2017;**138**:60-71

[32] Liu L, Li Y, Li S, Hu N, He Y, Pong R, et al. Comparison of next-generation sequencing systems. Journal of Biomedicine and Biotechnology. 2012;**2012**

[33] Mahato NK, Gupta V, Singh P, Kumari R, Verma H, Tripathi C, et al. Microbial taxonomy in the era of OMICS: Application of DNA sequences, computational tools and techniques. Antonie Van Leeuwenhoek. 2017;**110**(10):1357-1371

[34] Genomes—National Center for Biotechnology Information [Internet]. Available from: https://www.ncbi.nlm.nih.gov/genome/ [Accessed: 2018 Jun 28]

[35] GOLD—Genomes OnLine Database [Internet]. Available from: https://gold.jgi.doe.gov/ [Accessed: 2018 Jun 23]

[36] Medini D, Serruto D, Parkhill J, Relman DA, Donati C, Moxon R, et al. Microbiology in the post-genomic era. Nature Reviews Microbiology. 2008 Jun;**6**(6):419-430

[37] Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Medini D, Ward NL, et al. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: Implications for the microbial "pan-genome". Proceedings of the National Academy of Sciences. 2005; **102**(39):13950-13955

[38] Subedi R, Kolodkina V, Sutcliffe IC, Simpson-Louredo L, Hirata R, Titov L, et al. Genomic analyses reveal two distinct lineages of *Corynebacterium ulcerans* strains. New Microbes and New Infections. 2018;**25**:7-13

[39] Mira A, Martín-Cuadrado AB, D'Auria G, Rodríguez-Valera F. The bacterial pan-genome: A new paradigm in microbiology. International Microbiology. 2010;**13**(2):45-57

[40] Pal S, Sarkar I, Roy A, Mohapatra PKD, Mondal KC, Sen A. Comparative evolutionary genomics of *Corynebacterium* with special reference to codon and amino acid usage diversities. Genetica. 2018;**146**(1):13-27

[41] Tauch A, Bischoff N, Pühler A, Kalinowski J. Comparative genomics identified two conserved DNA modules in a corynebacterial plasmid family present in clinical isolates of the opportunistic human pathogen *Corynebacterium jeikeium*. Plasmid. 2004;**52**(2):102-118

[42] Soares SC, Silva A, Trost E, Blom J, Ramos R, Carneiro A, et al. The pan-genome of the animal pathogen *Corynebacterium pseudotuberculosis* reveals differences in genome plasticity between the biovar *ovis* and *equi* strains. PLoS One. 2013;**8**(1):1-14

[43] Baraúna RA, Ramos RTJ, Veras AAO, Pinheiro KC, Benevides LJ, Viana MVC, et al. Assessing the genotypic differences between strains of *Corynebacterium pseudotuberculosis* biovar *equi* through comparative genomics. PLoS One. 2017;**12**(1):e0170676

[44] Barzantny H, Schröder J, Strotmeier J, Fredrich E, Brune I, Tauch A. The transcriptional regulatory network of *Corynebacterium jeikeium* K411 and its interaction with metabolic routes contributing to human body odor formation. Journal of Biotechnology. 2012;**159**(3):235-248

[45] Wang Z, Gerstein M, Snyder M. RNA-Seq: A revolutionary tool for transcriptomics. Nature Reviews Genetics. 2009;**10**(1):57-63

[46] Lowe R, Shirley N, Bleackley M, Dolan S, Shafee T. Transcriptomics technologies. PLoS Computational Biology. 2017;**13**(5):1-23

[47] Rani B, Sharma VK. Transcriptome profiling: Methods and applications—A review. Agricultural Reviews. 2017;**38**(04):271-281

[48] Hüser AT, Becker A, Brune I, Dondrup M, Kalinowski J, Plassmeier J, et al. Development of a *Corynebacterium glutamicum* DNA microarray and validation by genome-wide expression profiling during growth with propionate as carbon source. Journal of Biotechnology. 2003;**106**(2-3):269-286

[49] Silberbach M, Schäfer M, Hüser AT, Pühler A, Krämer R, Scha M, et al. Adaptation *of Corynebacterium glutamicum* to ammonium limitation: A global analysis using transcriptome and proteome techniques. Applied Environmental Microbiology. 2005;**71**(5): 2391-2402

[50] Polen T, Schluesener D, Poetsch A, Bott M, Wendisch VF. Characterization of citrate utilization in *Corynebacterium glutamicum* by transcriptome and proteome analysis. FEMS Microbiology Letters. 2007;**273**(1):109-119

[51] Morozova O, Marra MA. Applications of next-generation sequencing technologies in functional genomics. Genomics. 2008;**92**(5):255-264

[52] Skvortsov TA, Azhikina TL. A review of the transcriptome analysis of bacterial pathogens *in vivo*: Problems and solutions. Russian Journal of Bioorganic Chemistry. 2010; **36**(5):550-559

[53] Pinto A, de Sá PHCG, Ramos RTJ, Barbosa S, Barbosa HPM, Ribeiro A, et al. Differential transcriptional profile of *Corynebacterium pseudotuberculosis* in response to abiotic stresses. BMC Genomics. 2014;**15**(1):14

[54] Gomide ACP, de Sá PG, Cavalcante ALQ, de Jesus Sousa T, Gomes LGR, Ramos RTJ, et al. Heat shock stress: Profile of differential expression in *Corynebacterium pseudotuberculosis* biovar *Equi*. Gene. 2018;**645**:124-130

[55] Wittchen M, Busche T, Gaspar AH, Lee JH, Ton-That H, Kalinowski J, et al. Transcriptome sequencing of the human pathogen *Corynebacterium diphtheriae* NCTC 13129 provides detailed insights into its transcriptional landscape and into DtxR-mediated transcriptional regulation. BMC Genomics. 2018;**19**(1):1-17

[56] Liu X, Yang S, Wang F, Dai X, Yang Y, Bai Z. Comparative analysis of the *Corynebacterium glutamicum* transcriptome in response to changes in dissolved oxygen levels. Journal of Industrial Microbiology and Biotechnology. 2017;**44**(2):181-195

[57] Lee JY, Na YA, Kim E, Lee HS, Kim P. The actinobacterium *Corynebacterium glutamicum*, an industrial workhorse. Journal of Microbiology and Biotechnology. 2016;**26**(5):807-822

[58] Zhang H, Li Y, Wang C, Wang X. Understanding the high l-valine production in *Corynebacterium glutamicum* VWB-1 using transcriptomics and proteomics. Scientific Reports. 2018;**8**(1):1-18

[59] Pfeifer-Sancar K, Mentz A, Rückert C, Kalinowski J. Comprehensive analysis of the *Corynebacterium glutamicum* transcriptome using an improved RNAseq technique. BMC Genomics. 2013;**14**(1):888

[60] Albersmeier A, Pfeifer-Sancar K, Rückert C, Kalinowski J. Genome-wide determination of transcription start sites reveals new insights into promoter structures in the actinomycete *Corynebacterium glutamicum*. Journal of Biotechnology. 2017;**257**:99-109

[61] Altelaar AFM, Munoz J, Heck AJR. Next-generation proteomics: Towards an integrative view of proteome dynamics. Nature Reviews Genetics. 2013;**14**(1):35-48

[62] Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, et al. Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. Electrophoresis. 1995;**16**(1):1090-1094

[63] Anderson NG, Anderson NL. Review twenty years of two-dimensional electrophoresis: Past, present and future. Electrophoresis. 1996;**17**:443-453

[64] Gu Q, Yu LR. Proteomics quality and standard: From a regulatory perspective. Journal of Proteomics. 2014;**96**:353-359

[65] Chandrasekhar K, Dileep A, Lebonah DE, Kumari JP. A short review on proteomics and its applications. International Letters of Natural Sciences. 2014;**17**:77-84

[66] Marklund EG, Degiacomi MT, Robinson CV, Baldwin AJ, Benesch JLP. Collision cross sections for structural proteomics. Structure. 2015;**23**(4):791-799

[67] Joshi K, Patil D. Proteomics. In: Patwardhan B, Chaguturu R, editors. Innovative Approaches in Drug Discovery: Ethnopharmacology, Systems Biology and Holistic Targeting. 1st ed. New York: Elsevier Inc.; 2016. p. 273-294

[68] Schubert OT, Röst HL, Collins BC, Rosenberger G, Aebersold R. Quantitative proteomics: Challenges and opportunities in basic and applied research. Nature Protocols. 2017;**12**(7):1289-1294

[69] Van Oudenhove L, Devreese B. A review on recent developments in mass spectrometry instrumentation and quantitative tools advancing bacterial proteomics. Applied Microbiology and Biotechnology. 2013;**97**(11):4749-4762

[70] Poetsch A, Haußmann U, Burkovski A. Proteomics of corynebacteria: From biotechnology workhorses to pathogens. Proteomics. 2011;**11**(15):3244-3255

[71] Seng P, Abat C, Rolain JM, Colson P, Lagier JC, Gouriet F, et al. Identification of rare pathogenic bacteria in a clinical microbiology laboratory: Impact of matrix-assisted laser desorption ionization-time of flight mass spectrometry. Journal of Clinical Microbiology. 2013;**51**(7):2182-2194

[72] Bittel M, Gastiger S, Amin B, Hofmann J, Burkovski A. Surface and extracellular proteome of the emerging pathogen *Corynebacterium ulcerans*. Proteomes. 2018;**6**(2):1-11

[73] Hansmeier N, Chao TC, Kalinowski J, Pühler A, Tauch A. Mapping and comprehensive analysis of the extracellular and cell surface proteome of the human pathogen *Corynebacterium diphtheriae*. Proteomics. 2006;**6**(8):2465-2476

[74] Paule BJA, Meyer R, Moura-Costa LF, Bahia RC, Carminati R, Regis LF, et al. Three-phase partitioning as an efficient method for extraction/concentration of immunoreactive excreted-secreted proteins of *Corynebacterium pseudotuberculosis*. Protein Expression and Purification. 2004;**34**(2):311-316

[75] Pacheco LG, Slade SE, Seyffert N, Santos AR, Castro TL, Silva WM, et al. A combined approach for comparative exoproteome analysis of *Corynebacterium pseudotuberculosis*. BMC Microbiology. 2011;**11**(12):1-14

[76] Silva WM, Seyffert N, Santos AV, Castro TLP, Pacheco LGC, Santos AR, et al. Identification of 11 new exoproteins in *Corynebacterium pseudotuberculosis* by comparative analysis of the exoproteome. Microbial Pathogenesis. 2013;**61-62**:37-42

[77] Santos AR, Carneiro A, Gala-García A, Pinto A, Barh D, Barbosa E, et al. The *Corynebacterium pseudotuberculosis in silico* predicted pan-exoproteome. BMC Genomics. 2012; **13**(Suppl 5):S6

[78] Hansmeier N, Chao TC, Daschkey S, Müsken M, Kalinowski J, Pühler A, et al. A comprehensive proteome map of the lipid-requiring nosocomial pathogen *Corynebacterium jeikeium* K411. Proteomics. 2007;**7**(7):1076-1096

[79] White A, Ding X, VanderSpek JC, Murphy JR, Ringe D. Structure of the metal-ion-activated diphtheria toxin repressor/*tox* operator complex. Nature. 1998;**394**(6692):502-506

[80] Mariutti RB, Ullah A, Araujo GC, Murakami MT, Arni RK. Tyrosine binding and promiscuity in the arginine repressor from the pathogenic bacterium *Corynebacterium pseudotuberculosis*. Biochemical and Biophysical Research Communications. 2016;**475**(4):350-355

[81] Kovács IA, Luck K, Spirohn K, Wang Y, Pollis C, Schlabach S, et al. Network-based prediction of protein interactions. bioRxiv. 2018;275529

[82] Barh D, Gupta K, Jain N, Khatri G, León-Sicairos N, Canizalez-Roman A, et al. Conserved host-pathogen PPIs: Globally conserved inter-species bacterial PPIs based conserved host-pathogen interactome derived novel target in *C. pseudotuberculosis, C. diphtheriae, M. tuberculosis, C. ulcerans, Y. pestis*, and *E. coli* targeted by *Piper betel* compounds. Integrative Biology. 2013;**5**(3):495-509

[83] Folador EL, Vinícius P, Daltro S, Silva WM, Ferreira RS, Silva A, et al. *In silico* identification of essential proteins in *Corynebacterium pseudotuberculosis* based on protein-protein interaction networks. BMC Systems Biology. 2016;**10**(103):1-9

Chapter 5

Clade-Specific Distribution of Antibiotic Resistance Mutations in the Population of *Mycobacterium tuberculosis* - Prospects for Drug Resistance Reversion

Karen van Niekerk, Rian Pierneef, Oleg N. Reva, Ilya S. Korostetskiy, Aleksandr I. Ilin and Gulshara K. Akhmetova

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75181

Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis* (Mtb), is a leading cause of death in humans worldwide. The emergence of antibiotic-resistant strains of Mtb is a threat to tuberculosis control. A general belief is that drug resistance is acquired by Mtb during antibiotic treatment by accumulation of spontaneous mutations. Also, it is known that the drug resistance mutations (DRM) have an associated fitness cost, reducing the transmissibility and virulence of resistant strains. In this work we show that many canonical DRM are clade specific; i.e. they occur only in specific genetic lineages of Mtb and depend on a specific genetic context necessary for the reduction of the fitness cost and sustainability of the drug resistance phenotype. Dependence of the drug resistance on occurrence of genetic variants of multiple genes and specific activities of the encoded proteins allows combating the drug resistance by impairing the global genetic context. A new drug, FS-1, reverses antibiotic resistance by compromising this genetic context and aggravating the fitness cost of DRM.

Keywords: antibiotic resistance, drug resistance mutation, genomic polymorphism, drug resistance reversion

1. Introduction

Tuberculosis (TB), the infectious disease caused by *Mycobacterium tuberculosis* (Mtb), is the leading cause of death from a single infectious disease in humans worldwide. Roughly

one-third of the world's population has latent TB, providing a source of infection. Efforts to curb TB have resulted in 2% annual decline in the global incidence of TB, except in sub-Saharan Africa [1]. However, the emergence of antibiotic-resistant strains of Mtb is a threat to TB control. If antibiotic-resistant TB is not rapidly and appropriately diagnosed, it may lead to an increase in mortality rates and the spread of resistant strains in the population.

The first line antibiotics rifampicin (RIF) and isoniazid (INH), were developed against Mtb in the 1950s and 1960s, and are still the most effective treatments for TB. An estimated 20% of all Mtb isolates are resistant to at least one of the major antibiotics [2]. Multidrug-resistant tuberculosis (MDR-TB) is defined as TB that does not respond to at least rifampicin (RIF) and isoniazid (INH), while extensively drug-resistant TB (XDR-TB) is defined as TB resistant to INH and RIF in addition to resistance to any of the fluoroquinolones (FLQ) and to at least one of the three second-line injectable drugs: amikacin (AMK), capreomycin (CAP) or kanamycin (KAN). Antibiotic resistance arises when bacteria acquire mutations in drug target genes in an infected patient receiving antibiotics, usually as a result of mismanagement of treatment. Primary resistance arises when resistant strains are transmitted from one patient to another.

Efforts to control drug-resistant TB have relied on two beliefs: that most drug resistance is acquired *de novo* during Mtb treatment regiments, i.e., secondary resistance, and that drug-resistance mutations would have an associated fitness cost reducing the transmissibility and virulence of resistant strains [2]. Therefore, TB control has focused on increasing the effectiveness of the first line treatment and of drug-susceptibility tests only in patients who have received anti-TB medication previously. An added challenge is that diagnosing MDR- and XDR-TB requires drug-susceptibility testing with six different drugs, which can take several weeks to months [3].

Results from improved molecular diagnostic methods have challenged these two beliefs. First, an increase in the prevalence of MDR- and XDR-TB appeared to be driving the spread of TB in some areas. For example, primary transmission of MDR- and XDR-TB is the main driving force of drug-resistant TB spread in sub-Saharan Africa [2]. Second, drug-resistance mutations have variable effects on fitness and transmissibility. Mutations associated with resistance to INH, RIF, and streptomycin (SM) have even been associated with low or no fitness costs [4]. Secondary mutations that compensate for drug resistance mutations appear rapidly after the emergence of drug resistance, in the same gene or in genes involved in linked metabolic pathways, and act to restore virulence and may even increase transmissibility [2].

The WHO recommends the Xpert MTB/RIF assay for the diagnosis of rifampicin resistance, and molecular line probe assays for the detection of resistance to first and second line drugs. Many countries with a high TB burden now implement the Xpert MTB/RIF assay, which can be used as a marker for MDR-TB, as INH resistance generally precedes RIF resistance [5]. Microbiological culture is still the reference standard for diagnosis of TB and of drug-resistance. TB remains very difficult to manage in resource-poor areas. Whole-genome sequencing (WGS) and detection of variants holds great promise for characterizing all of the resistance markers (as opposed to a limited range of mutations) as well as genotyping the strain of Mtb, but relies on a more complete understanding of the relationship between genotype, specific drug resistance mutations, activity states of multiple genes and encoded proteins, and the

drug-resistance phenotype [6]. A new promising drug, FS-1, consisting of a nano-molecular complex of iodine atoms ligated to a dextrin-polypeptide network, was reported to cause antibiotic resistance reversion in MDR-TB by compromising the genetic context of the drug resistance phenotype and by aggravating the fitness cost of the drug resistance mutations [7].

2. Genetic mechanisms of drug resistance in Mtb

The major antibiotics for the treatment of TB have four different mechanisms of action: (i) inhibition of RNA synthesis; (ii) inhibition of protein synthesis; (iii) inhibition of cell wall biosynthesis; and (iv) by interfering with the synthesis of cell membranes [8].

Since the early 1990s, numerous studies have described the genetic mechanisms of drug resistance in Mtb, and there is a quantity of data on the polymorphisms found in isolates resistant to specific antibiotics. Mtb is highly clonal, and as such there is little or no horizontal gene transfer, implying that antibiotic resistance is due to point mutations or deletions. Drug-resistance mutations occur in genes coding for the antibiotic target itself (e.g., *gyrA*, *gyrB*, *rrs*), in genes that code for enzymes needed for activating the antibiotic (e.g., *katG*, *inhA*, *rpoB*, *pncA*, *embB*), or in promoter regions of these genes [2, 9]. To date, there are 1031 mutations in the Mtb genome believed to be associated with resistance to nine major groups of antibiotics, with different combinations of mutations causing MDR-TB [10]. Many of the mutations identified are thought to play roles other than causing resistance directly, e.g., compensatory or adaptive roles, to increase fitness, which is being reduced by the drug-resistance mutations [8].

Researchers have not fully elucidated the mechanisms by which drug resistance emerges and is preserved in Mtb populations [11]. Early mathematical models of MDR-TB suggested that DR mutations would impose fitness costs that would tend to select against the mutation in the population and thus limit the spread of TB [12]. However, current research has shown that DR mutations have a variable effect on fitness and transmissibility. INH, RIF and SM resistance have even been associated with low or no fitness costs [2, 4].

Table 1 summarizes the literature data [7, 13–15] on the roles of the major antibiotics used to treat TB and known genes involved in drug-resistance, as well as the mechanisms thought to be responsible for drug-resistance. Drug resistant phenotype in Mtb is associated exclusively with mutations at specific positions in bacterial genomes. No events of a horizontal acquisition of drug resistance genetic determinants were reported for Mtb. Mutations in protein coding genes either alter drug target molecules or reduce activity of enzymes converting pro-drug molecules into active antibiotics, e.g., *katG* gene, which encodes a catalase converting isoniazid to an active isonicotinoyl-NAD adduct [16]. Mutations affecting activities of bacterial enzymes usually reduce viability of bacteria. This phenomenon is known as the fitness cost of drug resistance. Overcoming of the fitness cost requires from bacteria an acquisition of secondary mutations to compensate the side effects of DR mutations. We hypothesized in this paper that the necessity for bacteria to compensate the side effects of DR mutations potentially opens new ways to identify molecular targets for new drugs to induce the reversion of antibiotic resistance in bacterial populations.

	Antibiotic name	Mechanism of action	Some polymorphisms in Mtb causing resistance	Mechanism of drug resistance
First line drugs	Rifampicin, RIF	Inhibits bacterial RNA polymerase by binding it. When RIF binds to the RpoB target, hydroxyl radicals are formed and this has a cytotoxic effect.	Most mutations occur in cluster I of *rpoB*(β subunit of RNA pol), in the 81 bp rifampicin resistance determining region (RRDR)	Drug target is altered. In resistant bacteria, hydroxyl radicals are not formed when RIF binds to RpoB, so cells do not die.
	Ethambutol, EMB	Affects several cellular pathways, mostly arabinogalactan biosynthesis through inhibition of cell wall arabinan polymerization; RNA metabolism, transfer of mycolic acid into cell wall, phospholipid synthesis, spermidine synthesis	Point mutations in the *embCAB* operon or the *emb* genes, affecting expression of the *embA*, *embB*, and *embC* genes	Alteration of the drug target
	Isoniazid, INH	INH is a pro-drug, activated by the catalase-peroxidase enzyme KatG and then binds to InhA. Disrupts multiple pathways, mainly interferes with synthesis of mycolic acid.	Mutations to *katG* gene (50–80%): Mostly S315 T. Mutations to *inhA*, or the promoter region Mutations in *ndh* gene (NADH dehydogenase), *kasA* and *ahpC* genes Mutations in *kasA* gene	*katG*: mutations decrease catalase and peroxidase activity, so reduce activation of INH *inhA* promoter: overexpression of the enzyme
	Pyrazinamide, PZA	Activated by enzyme pyrazinamidase (PZase). Mechanism poorly understood. Disruption of the proton motive force required for essential membrane transport functions by POA at acidic pH.	Mutations in the *pncA* gene encoding PZase, most are in 561-bp region of the open reading frame or in an 82-bp region of its promoter.	Pro-drug cannot be converted to its active form
	Aminoglycosides: streptomycin, SM	Binds to the small 16S rRNA of the 30S subunit of bacterial ribosome, interfering with the binding of tRNA to the 30S subunit	Mutation of the ribosome target binding sites: 50% in the *rpsL* gene, which encodes the ribosomal protein S12, usually K43R 20% mutations to the *rrs* gene. Also mutations in *gidB*, which encodes 16S rRNA methyltransferase	Alteration of the drug target

	Antibiotic name	Mechanism of action	Some polymorphisms in Mtb causing resistance	Mechanism of drug resistance
Second line drugs	Aminoglycosides: kanamycin KAN, amikacin AMK	Binds to the small 16S rRNA of the 30S subunit of bacterial ribosome, interfering with the binding of tRNA to the 30S subunit	Mutation of the ribosome target binding sites genes *rrs*, but not cross-resistant with streptomycin	Alteration of drug target
	Capreomycin, CAP	Inhibits protein synthesis through modification of ribosomal structures at the 16S rRNA	Mutations in the *rrs* gene encoding 16S rRNA mutations in the gene *tlyA* encoding a 2′-O-methyltransferase of 16S rRNA and 23S rRNA	Alteration of drug target
	Ethionamide, ETH	ETH requires activation by monooxygenase EthA, inhibits mycolic acid synthesis by binding the ACP reductase InhA	70% due to mutations in *ethA* or *inhA*	Similar to INH: *inhA* promoter: overexpression of the enzyme
	Fluoroquinolones (FLQ), e.g., ofloxacin (OFX), moxifloxacin (MOX)	Trapping gyrase on DNA as ternary complexes, thereby blocking the movement of replication forks and transcription complexes	Usually multiple mutations in conserved quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB*, most often at positions Ala-90 and Asp-94 in *gyrA* [Mutations at position 80 of *gyrA* cause hypersusceptibility to fluoroquinolones]	FLQ traps the DNA-gyrase complex in which the DNA is broken. Resistant GyrA prevents chromosome breakage.
	Para-aminosalicylic acid, PAS	PAS is a prod-drug and thymidylate synthase A is required for conversion to active form PAS inhibits folic acid biosynthesis and uptake of iron	Mutations in the *thyA* gene encoding the enzyme thymidylate synthesis of the folate biosynthesis pathway, mostly Thr202Ala Also: mutations in *folC*, *ribD*, *dfrA*	Pro-drug cannot be converted to active drug
	Cycloserine, CS	Interrupts peptidoglycan synthesis (for cell wall) by inhibiting the enzymes d-alanine racemase (AlrA) and d-alanine:d-alanine ligase (Ddl)	To be determined *alr*, *ddl*, *cycA*	Unknown

Table 1. The main anti-TB drugs, mechanisms of actions and resistance-conferring polymorphisms.

3. Drug-resistance against the background of Mtb genetic clades and current diagnostic approaches

The disease TB first appeared roughly 70,000 years ago [17]. Studies show that Mtb arose as an obligate human pathogen and that different strains co-evolved with humans, migrated out of Africa, and that the populations expanded with their human hosts [18]. The migrations of modern humans out of Africa and the increased population density during the Neolithic period could be at the origin of its expansion. This theory is consistent with the bacterium's phylogeny and phylogeography [19].

Genetic analyses of global strains have revealed that distinct lineages of Mtb have emerged in different regions of the world. The considerable genetic diversity between these lineages is linked to ancient human migrations out of Africa and to more recent movements and population growth [20]. Hershberg *et al*. demonstrate that there is a greatly reduced selection pressure in Mtb, owing to factors including high clonality of Mtb and serial transmission bottlenecks, both of which reduce the effective population size, increasing the effects of genetic drift [20]. Mutations can reach high functional diversity without being eliminated, which has implications for the emergence of MDR-TB.

During diagnostic procedures, it is helpful to find the lineage of the infecting Mtb strain(s), because some lineages might have acquired specific virulence and/or resistance features before expanding [21]. Clades differ by growth rate and in patterns of host-pathogen interaction in terms of cytokine induction and rate of uptake by macrophages [22]. Lineage 2 (Beijing clade) also is associated with hyper-virulence and with an extended drug resistance pattern [23].

Here we discuss research papers investigating the feasibility of replacing phenotypic drug testing of Mtb with molecular diagnostic techniques. All of them rely on understanding the genetic mechanisms underlying the development and persistence of drug-resistance in Mtb strains, including the context of lineages with varying evolutionary histories.

Köser *et al*. were among the first to publish a method for rapid WGS analysis of an Mtb clinical specimen to reduce the time of XDR-TB diagnosis. They used SNPs to identify lineages, combined with a catalog of well-described DR polymorphisms, demonstrating that WGS is superior to current genotypic tests, but not yet as reliable as phenotypic testing [24].

Rodwell *et al*. of the Global Consortium for Drug-Resistant TB Diagnostics (GCDD) investigated whether a certain collection of mutations can be used as markers of drug resistance in a molecular diagnostic test. They studied a collection of MDR and XDR-TB strains from different regions. Their approach was to select eight genes (*katG*, *inhA*, *rpoB*, *gyrA*, *gyrB*, *rrs*, *eis*, and *thyA*) in which mutations are known to be strongly associated with resistance to the antibiotics INH, RIF, FLQ, AMK, KAN, and CAP. In each specimen, the eight genes were amplified and sequenced, and variants were detected against the H37Rv reference strain. The specificity and sensitivity of the identified variants for drug resistance were determined. They concluded that about 30 mutations in six genes predicted XDR-TB phenotypes with 90–98% sensitivity and almost 100% specificity [3]. However, using these 30 mutations diagnostically would rely on purifying mycobacterial DNA from clinical samples and amplifying the genes of interest before identifying the presence of the mutations. Such a test would rely on broad sequencing coverage and accurate base calling

for the mutations of interest. The study used samples from four geographic regions, but the results do not specify the lineages of the resistant strains. This is problematic, because some DR mutations are lineage specific. More details on the mechanisms of drug resistance summarized from literature sources are in **Table 1**. It should also be noted that the mutations in the identified target genes do not explain all cases of drug resistance.

Genome-wide association studies (GWAS) exploit the rapid turnover and high throughput of NGS, identifying variants in natural populations linked to phenotypic traits by statistical association. Bacterial GWAS have not been frequently used because their population structures reduce the power of association or produce false positives [25]. The clonal nature of bacterial reproduction—especially prevalent in Mtb—means that spurious variations can be strongly associated with particular phenotypes [26]. However, Earle *et al*. have successfully used a linear mixed model approach to perform GWAS on four species of bacteria, including Mtb, to show associations between genetic variation and antibiotic resistant phenotypes. The success of this approach depended on "controlling population structure and boosting power by recovering signals of lineage-level associations" [27]. This method allows the researcher to eliminate signals due only to population, while preserving strain-specific signals that contribute to the DR phenotype.

Coll *et al*. identified a proposed minimum set of SNPs that can be used to differentiate all seven Mtb lineages and 55 sublineages [28]. They identified 88 SNPs in DR candidate regions (two promoters, 21 genes). However, this list of SNPs is aimed at identifying lineages, and is not necessarily informative about drug-resistance in the strains.

Feuerriegel *et al*. showed that many polymorphisms in Mtb previously known to be associated with DR are useful for distinguishing clades, which indicates a lineage specificity of drug resistance [29]. The same team designed the first available web-based drug resistance analysis tool, the Phylo-Resistance Search Engine (PhyResSE) [30]. The tool was evaluated by testing 92 Mtb strains from Sierra Leone with known drug resistance phenotypes, either mono-resistant (RIF, INH or SM) or poly-resistant (RIF, INH, SM, ethambutol (EMB) or pyrazinamide (PZA)). The major advantage of this tool is that it forms a complete analysis pipeline, taking FASTQ files as input: including quality control, mapping and base recalibration prior to genotyping. Thus the end-user need not do complex bioinformatic analysis. This requires considerable computational power. PhyResSE uses a variant catalog based on validated resistance-SNPs from literature as well as from their own experimental data for phylogenetic and drug resistance diagnosis, including lineage-specific resistance mutations. The paper does not give detailed descriptions of the methods used for inclusion or exclusion of specific mutations, or how the sensitivity and specificity were calibrated for mutations, as some mutations may confer only low-level resistance. The program returns a plain-language output which cites the experimental support for the result and also states whether or not there is a high degree of confidence in a particular polymorphism conferring drug resistance. The strains used to evaluate the tool in the paper do not include MDR or XDR strains. Excessive contamination and/or poor sequencing coverage would provide a barrier to correct diagnosis.

One of the few studies using gene pairs associated with drug resistance was by Cui *et al*. [31]. The rationale for the study was that evolution of transmissible drug-resistant Mtb is caused by multiple mutations, many of which interact with each other. This study used nearly 300 Mtb genome sequences from public datasets and their phenotypic drug-sensitivity testing results.

The variants were identified using a standard variant-calling approach. The resulting variants were first filtered using PLINK to remove phylogenetically related variants. The remaining mutations were analyzed using the program GBOOST, which performs a Chi-square test to confirm associations between two variants and phenotype. The resulting gene pairs were screened for the presence of drug target genes and further filtered by non-synonymous mutations. The resulting gene pairs were: one for INH, one for RIF, four for EMB and five for ethionamide (ETH). The authors reported that most of the identified gene pairs containing drug targets consisted of the unique mycobacterial Pro-Pro-Glu (PPE) family proteins, and from this they infer that PPE family proteins play an important role in Mtb drug resistance [30]. The identified mutations were not validated in this study, but the study does show the potential for using pairs of mutations in the diagnosis of drug resistance rather than single mutations. It should be noted that the PPE family proteins make up 10% of the Mtb genome, and they are highly polymorphic, so associations with these genes might occur as a result of genetic drift rather than selection pressure [32]. The value of removing population-specific mutations is unclear, as some lineages of Mtb are strongly associated with drug resistance.

Mortimer *et al*. proposed a method of distinguishing DR loci under positive selection [33]. The rationale behind this is that methods for identifying advantageous mutations usually depend on recombination to differentiate target loci from neutral variants, which is not feasible in the case of Mtb. They analyzed over 1000 Mtb genomes from Russia and South Africa, mostly Lineages 2 (Beijing) and 4, and examined the frequency of different mutations in the populations. They found that resistant sub-populations are less diverse than susceptible subpopulations, which is consistent with the ongoing transmission of resistant Mtb. They classified the DR mutations as either "tight targets" or "sloppy targets" based on their diversity. The authors also noted that lipid metabolism genes are enriched in the list of DR loci under positive selection. This approach has potential for understanding the genetics of resistance in clonal bacteria.

A variety of bioinformatic approaches have been useful for resolving the evolution of the various lineages of *Mtb* over time, for tracing the emergence of pathogenic and more virulent strains, and for identifying variants in Mtb genomes responsible for the development of antibiotic resistance [28, 34–36]. In tandem with methods for rapid identification of drug resistance, researchers are also investigating methods of exploiting our understanding of the evolution of drug resistance. Treatment of TB with antibiotics has had an overall effect of selecting for drug resistance, rather than having the hoped-for effect of selecting DR variants with reduced fitness. Baym *et al*. have reviewed possible mechanisms of selection for drug resistance inversion [37]. These rely on the concept of using combinations of antibiotics and other compounds to inhibit bacterial growth and at the same time reversing the selection for resistance, similar to the combination of penicillin and clavulanic acid to block bacterial β-lactamase, while minimizing or reversing selection for resistance. This avenue shows promise, particularly in combination with quick genotyping of clinical samples.

4. Non-random associations between polymorphic sites in genomes of *M. tuberculosis*

Data for this research was sourced from the GMTV database [17], which consists of SNPs and indels for a large number of Mtb strains for which whole genome sequencing was

performed. Also, this database integrates clinical, epidemiological and microbiological data for all the recorded Mtb isolates. Analysis of this study compared distribution patterns of 58,025 amino acid substitutions in 1089 Mtb strains from the GMTV database. The polymorphisms were determined relative to the H37Rv reference strain [38]. Frequencies of all polymorphisms were calculated for the entire set of 1089 Mtb genomes and for Mtb lineages as they were identified in the GMTV database. Analysis of the data showed that many DR polymorphisms were strongly associated with specific Mtb lineages. A mosaic plot of the data is shown in **Figure 1**. Genomes of the Beijing, Haarlem and Lineage 4.3 clades contained numerous DR mutations, while only a few of them were observed in the Lineage 4.1, Ural and X-type. Bacteria of the latter clades appeared to be mostly drug-susceptible. Statistically significant prevalence of DR mutations in bacteria of specific Mtb clades was confirmed by Fisher's exact test with Bonferroni adjustment. Of these, 25 DR-polymorphism/lineage pairs showed an odds ratio above 1.

Co-occurrence of alleles of different polymorphic sites was identified by calculating the linkage disequilibrium (LD) and χ^2-statistics. In total, 288,840 pairs of polymorphisms showing statistically reliable associations (χ^2 above 6.63 corresponds to a p-value ≤ 0.01) were identified between 823 polymorphic sites including 10 DR mutations [10].Functional associations between DR mutations (denoted as mutations from an initial *A* allele to allele *a* conferring DR) and other genetic polymorphisms (denoted as *B* for the most frequent allele and *b* for all other alternative variants in Mtb population) were identified by Levin's attributable risk statistic [39]. Confidence range values of attributable risks were calculated by Eq. (1).

$$\left[1 - EXP\left(ln\left(1 - R_a\right) - 1.96 \times StdErr\right)\right] \; to \; \left[1 - EXP\left(ln\left(1 - R_a\right) + 1.96 \times StdErr\right)\right] \tag{1}$$

In the case of estimation of the risk of DR mutation from *A* to *a* in a subpopulation of organisms having the allele *b* at the secondary polymorphic site, the parameter R_a was calculated by Eq. (2) and the Fleiss' standard error parameter *StdErr* was calculated by Eq. (3).

$$R_a = \frac{P_{AB}P_{ab} - P_{aB}P_{Ab}}{\left(P_{AB} + P_{aB}\right)\left(P_{aB} + P_{ab}\right)} \tag{2}$$

$$StdErr = \sqrt{\frac{P_{Ab} + R_a\left(P_{AB} + P_{ab}\right)}{N \times P_{aB}}} \tag{3}$$

Risks of secondary mutations *B* to *b* in a DR subpopulation with the genotype *a* were calculated by Eq. (1), but in this case the parameters R_a and *StdErr* were calculated by Eqs. (4) and (5), respectively:

$$R_a = \frac{P_{AB}P_{ab} - P_{aB}P_{Ab}}{\left(P_{AB} + P_{Ab}\right)\left(P_{Ab} + P_{ab}\right)} \tag{4}$$

$$StdErr = \sqrt{\frac{P_{aB} + R_a\left(P_{AB} + P_{ab}\right)}{N \times P_{Ab}}} \tag{5}$$

In Eqs. (2)–(5), values P_{AB}, P_{Ab}, P_{aB} and P_{ab} are the frequencies of corresponding allele combinations; and N is the total number of the analyzed Mtb strains = 1089.

The reasoning behind the further analysis is displayed in **Figure 2**, where two contingency tables of co-distribution of an arginine to leucine replacement at position 463 in the protein KatG rendering INH resistance [40] and two other secondary mutations are shown. Both pairs of mutations are characterized by strong linkage disequilibrium above 0.9. First, the co-distribution of the DR mutation KatG R463L and a polymorphism D69Y in a drug efflux protein Stp (Rv2333c) is considered (**Figure 2-1**). The replacement of the aspartate residue by tyrosine at position 69 of the protein Stp is rather common in the Mtb population and it has not been associated with any DR phenotype. However, this study showed that 91–99% of the DR mutation KatG R463L depends on the presence of the Stp D69Y substitution. In contrast, the likelihood of a D → Y replacement in the protein Stp does not depend significantly on the state of the KatG R463L polymorphism. The estimated attributable risk is in the range of 21–27%. The confidence ranges of attributable risks in **Figure 2** are denoted as $A \to a_{|b}$ and $B \to b_{|a}$, respectively.

Let us consider another co-distribution of the same DR-related polymorphism KatG R463L and a leucine to serine substitution at position 896 in PPE35 protein (Rv1918c), which is shown in **Figure 2-2**. These two mutations are strongly associated with each other, but this

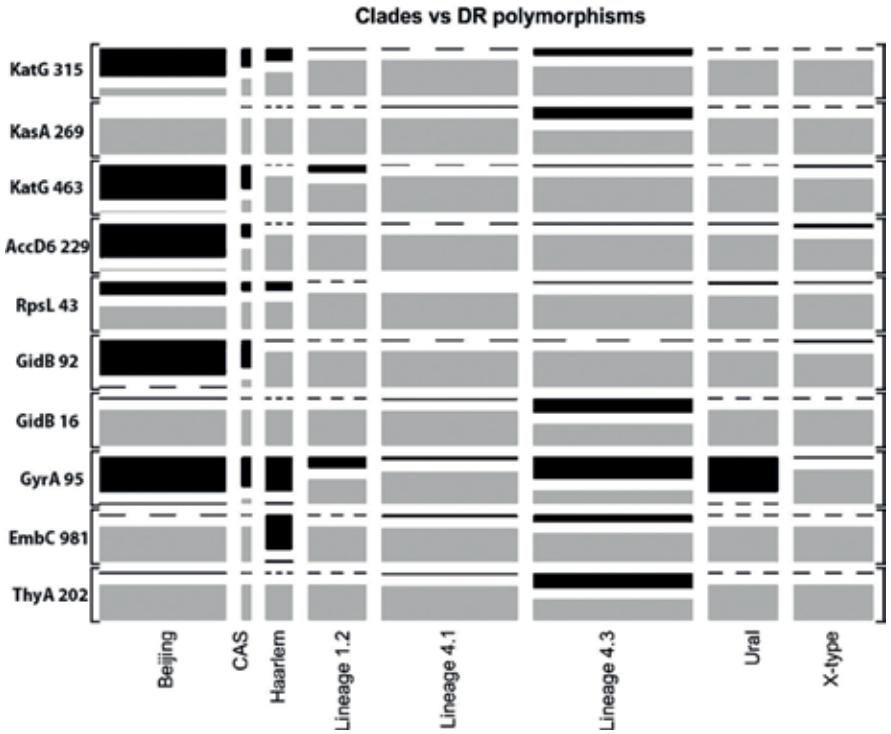

Figure 1. Mosaic plot representing the contingency table for the presence (black) or absence (gray) of each DR polymorphism for each of the 10 loci and the clade for the specimens in the GMTV dataset. The size of the rectangle represents the number of sequences in the category. A dotted line indicates that there were no specimens in that category. Clades with 10 or fewer representatives were omitted.

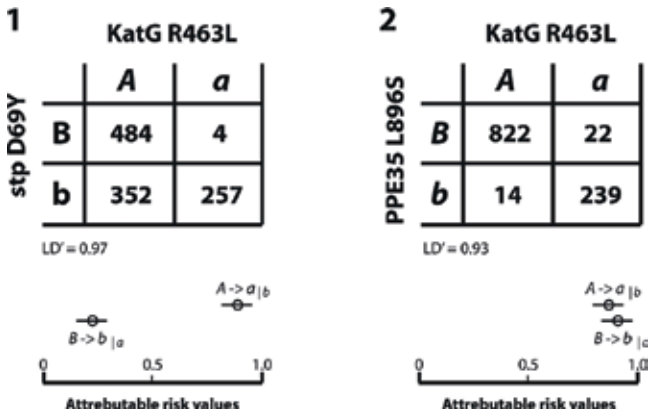

Figure 2. Contingency tables of co-distribution of a DR mutation KatG R463L rendering resistance to INH and two secondary polymorphisms in the (1) efflux drug protein Stp and (2) PPE35 protein. Attributable risks of mutation acquisition were calculated and denoted as $A \rightarrow a_{|b}$ and $B \rightarrow b_{|a}$ for the likelihood of DR mutation acquisition when the secondary site is mutated and the likelihood of secondary mutation in a DR sub-population, respectively.

Secondary mutations	Drug resistance mutations									Annotation
	GyrA S95 T (FLQ)	KatG S315 T,N (INH)	KatG R463L (INH)	AccD6 D229G (INH)	ThyA T202A (PAS)	EmbC V981 L (EMB)	RpsL K43R (SM)	GidB E92D (SM)	GidB L16R (SM)	
Rv0193c K417*,E	86.4 to 94.6	87.5 to 97.7	77.4 to 93.1	71.9 to 90.9	80.7 to 99.6		78.1 to 98.5	79.6 to 99.5	81.1 to 96.0	Hypothetical protein
Rv1186c P207A,T	84.0 to 93.9	82.8 to 96.4	79.8 to 95.7	76.5 to 95.0	64.6 to 94.7		68.7 to 96.6	71.8 to 98.1	74.4 to 93.9	Hypothetical protein
Rv1321 S144R	81.8 to 91.1	84.7 to 96.0	80.6 to 94.5	72.5 to 90.6	67.1 to 92.9		76.7 to 97.4	75.6 to 97.3	76.0 to 92.7	Hypothetical protein
Rv2017 A262E	76.5 to 86.5	83.9 to 95.1	73.5 to 89.4	70.6 to 88.6	72.9 to 95.0		75.7 to 96.4	77.8 to 97.5	76.6 to 92.4	Transcriptional regulator
GalU Q235R	76.6 to 86.6	81.8 to 93.8	74.9 to 90.3	69.4 to 87.8	70.2 to 93.6		72.9 to 95.0	78.0 to 97.6	75.3 to 91.6	UTP-glucose-1-phosphate uridylyltransferase
Rv3204 T34A	74.7 to 84.8	82.5 to 94.1	72.2 to 88.3	69.1 to 87.3	68.5 to 92.4		79.6 to 97.8	75.7 to 96.4	74.8 to 91.0	DNA-methyltransferase
CorA K139*,E	76.2 to 86.1	85.4 to 95.9	70.7 to 87.3	67.3 to 86.1	68.0 to 92.2		73.5 to 95.1	75.4 to 96.3	75.9 to 91.8	Magnesium and cobalt transporter
VapC47 S46 L	74.1 to 84.4	84.5 to 95.3	71.0 to 87.5	71.8 to 89.1	71.2 to 93.8		73.8 to 95.2	75.7 to 96.4	69.3 to 87.4	VapC47 toxin

Secondary mutations	Drug resistance mutations									Annotation
	GyrA S95 T (FLQ)	KatG S315 T,N (INH)	KatG R463L (INH)	AccD6 D229G (INH)	ThyA T202A (PAS)	EmbC V981 L (EMB)	RpsL K43R (SM)	GidB E92D (SM)	GidB L16R (SM)	
EccC3 P214R	77.6 to 87.4	79.7 to 92.5	71.2 to 87.8	65.1 to 84.8	70.2 to 93.6		72.9 to 95.0	74.8 to 96.2	66.8 to 85.9	Type VII secretion protein
Rv2542 T211A	71.8 to 82.4	81.3 to 93.4	66.4 to 84.1	64.9 to 84.4	71.1 to 93.8		73.8 to 95.2	75.6 to 96.4	70.6 to 88.3	Hypothetical protein
PstA1 M5T	87.2 to 95.3	74.6 to 90.6	82.9 to 96.4	84.1 to 97.6	79.8 to 99.5			78.8 to 99.5	82.3 to 96.8	Phosphate-transport integral membrane ABC transporter
TsnR L232P	88.6 to 96.2	72.4 to 89.0	79.9 to 94.7	76.6 to 93.8	80.1 to 99.5				80.6 to 95.9	23S rRNA methyl-transferase
AroG D265E		81.7 to 92.8	81.5 to 93.4	77.3 to 91.6	73.3 to 93.5		68.5 to 90.8	79.5 to 96.9	83.4 to 95.3	Phenylalanine-repressible DAHP synthetase
ProX L85P		61.4 to 75.4	81.2 to 92.1	80.0 to 92.1		76.2 to 94.2			84.9 to 95.1	Osmoprotectant
UspA V127 L		59.2 to 74.3	82.1 to 93.2	78.2 to 91.3		83.3 to 98.1			82.5 to 94.0	Sugar ABC transporter
Stp D69Y		53.8 to 71.1	90.9 to 98.6	90.7 to 99.0		83.1 to 98.9			88.2 to 97.8	Drug efflux protein
AceAa G179D		53.2 to 70.8	90.8 to 98.6	87.9 to 97.8		82.9 to 98.8			89.4 to 98.4	Isocitratelyase
GalTb T174A		54.0 to 71.1	84.6 to 95.3	87.1 to 97.3		85.3 to 99.7			89.7 to 98.5	Galactose-1-phosphate uridylyl-transferase
Rv0324 T168A		54.8 to 72.3	87.2 to 96.9	90.5 to 98.9		82.6 to 98.8			89.2 to 98.4	Transcriptional regulator
EspK C729S		54.0 to 70.5	83.6 to 94.4	85.5 to 96.2		79.5 to 96.9			83.4 to 94.9	ESX-1 secretion-Associated protein

Polymorphic sites are denoted by names of genes and pairs of amino acid substitutions from the most common allele to one or several alternative allelic states.

Deletions are marked by asterisks (*).Values X_{min} *to* X_{max} in cells represent confidence ranges estimated for p-value ≤0.05 (Eq. (1)).

Table 2. Attributable risk of acquisition of DR mutations in sub-populations of Mtb with secondary mutations.

dependence is highly symmetric: in more than 90% of cases both mutations co-occur in the same genomes. It may indicate a genetic drift event when the DR phenotype is characteristic for a sub-lineage of isolates sharing common ancestry and the neutral mutation in the hyper-mutable PPE35 protein is a genetic marker of the sublineage.

For further analysis, only those secondary polymorphisms which influenced the DR mutations significantly, but were independent, were selected; i.e. cases were selected when confidence

ranges of attributable risks $A \rightarrow a_{|b}$ and $B \rightarrow b_{|a}$do not overlap and $A \rightarrow a_{|b} > B \rightarrow b_{|a}$ as in **Figure 2-1**. In total, 554 secondary polymorphisms were found, which increase likelihood of acquisition of 9 out of 10 studied DR mutations. The mutation KasA G269S, rendering resistance to INH [41], was strongly associated only with the GidB L16R mutation rendering SM resistance [42], which indicates that the former polymorphism is most likely a secondary mutation in multidrug resistant Mtb.

A selection of secondary mutations predetermining acquisition of nine of the most widely distributed DR mutations rendering resistance to FLQ, INH, EMB, SM and para-aminosalisylic acid (PAS) in multidrug resistant Mtb are shown in **Table 2**. Values X_{min} *to* X_{max} in **Table 2** represent confidence ranges estimated for p-value ≤ 0.05 (Eq. (1)). It was found that the acquisition of DR mutations require allelic alterations in many other proteins including several transmembrane transporter and efflux proteins, osmoprotectant, transcriptional regulators and some other proteins. Strong cross-associations between DR polymorphisms characteristic for different lineages (**Figure 1**) favors the hypothesis of strong functional associations between these mutations compared to neutral genetic drift. The identified proteins predefining the acquisition of the DR phenotype may be molecular targets for development of new drugs for antibiotic resistance reversion.

5. The concept of the drug resistance reversion and implementation thereof

The concept of drug resistance reversion was applied in recent studies [7, 41]. Drug resistance mutations are often incompatible with one another, as shown by negative linkage disequilibrium values. This suggests that the cumulative fitness cost of mutations is often too high for the resulting strain to be viable. FS-1 is a new drug which seems to exploit this tendency. Active units of FS-1 are aggregated micelles containing complexes of tri-iodide molecules coordinated by metal ions and integrated into a dextrin-polypeptide moiety. The basic formula of the micelle is:

$$\left[\left\{\left(\mathrm{L_n}\left(\mathrm{MeJ_3}\right)^{+}\right)_{\mathrm{y}}\left[\mathrm{Me}\left(\mathrm{L_m}\right)\mathrm{J}\right]^{+}{}_{\mathrm{x}}\right\}\left(\mathrm{Cl}^{-}\right)_{\mathrm{y+x+k}}\right]$$

where L—dextrin-polypeptide ligand; Me—Li/Mg ions; n, m, x, y and k—variable integers ≥1; molecular mass of the micelles is in the range of 30–300 kD. This molecular complex was designed to prolong the residence time of moderately oxidative iodine molecules in an organism and facilitate their transportation to inner tissues.

Studies of XDR-TB infection in animal models showed the reversion of Mtb pathogens to a more drug sensitive phenotype after treatment with FS-1 despite the remaining DR related mutations in their genomes [7]. Drug resistance reversion was also confirmed on an *in vitro* model with a XDR-TB clinical isolate SCAID 187.0 when cultivated for 60 days in six passages on a medium with a sub-lethal dose of ¼ MIC of FS-1. Reduction of the antibiotic resistance of XDR-TB isolates obtained during the clinical trial of FS-1 was consistent with the results of the above-mentioned laboratory experiments. It was concluded that the DR phenotype requires multiple genes to be in specific activity states controlled either by transcription regulation or resulting from specific mutations. A combination of genetic variants creates a genomic context of drug resistance.

Clinical trials of FS-1 has been undertaken in Kazakhstan and registered in the Clinical Trial database (www.ClinicalTrials.gov) under an accession number NCT02607449. It was found that FS-1 had a high absorption rate after per-oral administration, which was not affected by food intake. Peak plasma concentration of FS-1 was observed within 1–2 h after administration. Gastric juice activated the infusion of FS-1 in stomach. Pharmacokinetic study of FS-1 showed a long residence time of the drug in the blood stream and an elevated accumulation in the liver. The drug was excreted from the test organism mainly by the kidneys.

The preclinical trial of FS-1 included pharmacological studies (primary and secondary pharmacokinetics); general toxicity determination; tests for mutagenesis, inhibition of reproductive performance, immune toxicity, mucous membrane irritation and several other general physicochemical studies of the compound. FS-1 caused no irritation of the stomach mucosa when applied in concentrations of up to 5.0 mg/kg. No ulcerogenic, allergenic, immune toxic, mutagenic or carcinogenic side effects were observed after repeated administration of FS-1. Also, no cytotoxicity or embryonic toxicity was observed. Toxicological studies attributed FS-1 to low toxicity compounds with a reduced accumulation in an organism (drug accumulation coefficient was 1.85). The maximum endurable dose of FS-1 identified in rats was 496 mg/kg, and in mice, 993 mg/kg. The average lethal dose (LD50) in rats was found to be 992 mg/kg for both male and female individuals. Therapeutic doses of FS-1 in clinical trials on humans for the treatment of patients with lung XDR-TB infection ranged from 1.0 to 5.0 mg/kg. During the clinical trials, FS-1 was administrated for up to 6 months in combination with the antibiotics commonly prescribed for XDR-TB treatment. Currently, in the third stage of the clinical trials, FS-1 is administrated at a concentration of 2.5 mg/ml for 6 months. Clinical studies complied with the regulations and recommendations of the Ministry of Health of Kazakhstan and were approved by the respective committees of the Ministry.

The first phase of clinical trials was undertaken in 2009–2010 at the Central Clinical Hospital of the Executive Officer of the President of Kazakhstan. During this phase, the drug tolerance and safety of a unitary and repeated per-oral intake of the drug by healthy volunteers were determined. Hematological parameters, including measuring the concentrations of important microelements, i.e., potassium, sodium, magnesium and calcium; functions of liver and kidney, electro-physiological parameters of myocardium, metabolism of proteins, hydrocarbons and lipids, were monitored. Biochemical parameters of the blood plasma of volunteers remained normal during the study. It was found that the administration of FS-1 activated cellular immunity and synthesis of γ-interferon.

The second phase of clinical trials was conducted in 2010–2012 at the Municipal anti-tuberculosis clinic in Almaty, at the National Centre of Tuberculosis in Almaty and at the Regional anti-tuberculosis clinic of the Karaganda region in Kazakhstan. In total, 220 volunteer patients with active XDR-TB lung tuberculosis were involved in this phase of trials. The volunteers ranged from 18 to 65 years old, with a body mass within 10% of the average body weight of male and female adults, with no contraindications to the common MDR-TB antibiotic therapy. Informed consent principles, which imply voluntariness of participation and understanding of the matter of the trial, were complied with. Contraindications to participation in the trial were: pregnancy; oncological diseases; HIV; 3-fold higher than normal ALT/AST or increased

creatinine in blood; dermatomycosis; mental disorders; hypothyroidism; any allergies, especially an allergy to iodine-containing preparations; and any other cardiovascular, kidney or liver decompensated concomitant diseases.

The therapeutic efficacy of the drug was evaluated by bacteriological examination of sputum samples of patients on Lowenstein-Jensen medium for the presence of Mtb isolates. Other tests performed during the trial were: microscopic examination of sputum smears; controlling the positive dynamics of recovery by regular X-ray examinations and by general clinical tests; positive body weight dynamics; and the efficacy of prevention of disease relapses. The efficacy and safety of the regimen of per-oral administration of FS-1 in concentrations of 2.0–5.0 mg/kg during the 6 months in combination with commonly prescribed antibiotics against XDR-TB were confirmed in the second phase of the trial. No serious side effects of the treatment were recorded. In particular, thyroid gland function was monitored for adverse effects. No statistically reliable alterations in the concentration of thyroid hormones in blood were observed, which indicated no deleterious effect of this iodine-containing drug on thyroid gland functions. The time of complete recovery from XDR-TB was reduced, with no disease relapses during the 12 months surveillance, resulting in a significant reduction of the average cost of XDR-TB treatment (**Table 3**).

Mtb isolates were collected on a regular basis during the second phase of the FS-1 clinical trials. It was found that the percentage of drug resistant isolates decreased continuously during the treatment course with FS-1 despite the administration of the antibiotics. It was hypothesized that the therapeutic activity of FS-1 may be associated with the reversion of antibiotic resistance [37]. This hypothesis was then confirmed in an *in vivo* experiment on guinea pigs, which has been recently published [7].

The third phase of clinical trials began in 2014 and is still in progress. The drug FS-1 has been approved as an antibacterial medicine for per-oral administration in a complex of commonly prescribed anti-tuberculosis drugs for the treatment of XDR-TB in Kazakhstan (approval certificate РК-ЛС-5 №021305 from 08-04-2015).

XDR TB treatment expenses	Conventional antibiotic therapy	Combined therapy by antibiotics with FS-1
Time of 100% sanation from *M. tuberculosis* isolation from sputum	12–24 months	3–6 months
Percentage of relapses in 12 month surveillance period	46.1%	Not observed
Daily therapy cost in clinics of Kazakhstan	$ 11.7	$ 12.5
Full cost of the treatment course including the treatment of disease relapses	$ 4274 or up to $ 8548 in the case of TB relapses	$ 2256 (no TB relapses were recorded)

Table 3. Summarized efficacy of application of FS-1 in the second phase of the clinical trial in terms of reduction of the treatment course duration and cost.

6. Conclusion

The idea that the DR phenotype is determined by multiple genes was supported in a review by Trauner *et al*. and Müller *et al*. [9, 13]. They argued that the genetic background of Mtb is important in determining the phenotypic effect of DR mutations. Epistasis—the genetic interactions that determine a phenotype—and bacterial fitness are the two factors that determine the evolution of drug resistance. It is thus important to study genes that are directly involved in drug metabolism as well as genes that could play a compensatory role, such as those involved in aspects of cell physiology, e.g., permeability of the cell. The complexity of the genetic and epistatic determination of the DR phenotype allows the development of new drugs to induce reversion of drug resistance in populations of pathogens. The phenomenon of DR reversion was defined in a review by Baym *et al*. as an active drug-induced counterselection of resistant variants from populations of pathogens [37]. Several theoretical assumptions were discussed in this paper to explain the resistance reversion despite the presence of selective antibiotics in a medium. Practical application of the antibiotic resistance reversion approach to combat multidrug resistant tuberculosis was exemplified in this work by an overview of the clinical trial of the new drug FS-1.

Acknowledgements

This research was funded by grant 105996 provided by the National Research Foundation of South Africa and by grant 0115PK00389 of the program "Study on the reversion of antibiotic resistance in pathogenic microorganisms" provided by the Ministry for Investments and Development of Kazakhstan.

Conflict of interest

No conflict of interest was reported by the authors.

Author details

Karen van Niekerk[1], Rian Pierneef[1], Oleg N. Reva[1]*, Ilya S. Korostetskiy[2], Aleksandr I. Ilin[2*] and Gulshara K. Akhmetova[2]

*Address all correspondence to: oleg.reva@up.ac.za and ilin_ai@mail.ru

1 Centre for Bioinformatics and Computational Biology, Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa

2 Scientific Center for Anti-Infectious Drugs, Almaty, Kazakhstan

References

[1] WHO. Global Tuberculosis Report. 2017. DOI: WHO/HTM/TB/2017.23

[2] Dheda K, Gumbo T, Maartens G, Dooley KE, McNerney R, Murray M, Furin J, Nardell EA, London L, Lessem E, Theron G, van Helden P, Niemann S, Merker M, Dowdy D, Van Rie A, Siu GKH, Pasipanodya JG, Rodrigues C, Clark TG, Sirgel FA, Esmail A, Lin H-H, Atre SR, Schaaf HS, Chang KC, Lange C, Nahid P, Udwadia ZF, Horsburgh CR Jr, Churchyard GJ, Menzies D, Hesseling AC, Nuermberger E, McIlleron H, Fennelly KP, Goemaere E, Jaramillo E, Low M, Jara CM, Padayatchi N, Warren RM. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. The Lancet Respiratory Medicine. 2017;**5**:291-360. DOI: 10.1016/S2213-2600(17)30079-6

[3] Rodwell TC, Valafar F, Douglas J, Qian L, Garfein RS, Chawla A, Torres J, Zadorozhny V, Kim MS, Hoshide M, Catanzaro D, Jackson L, Lin G, Desmond E, Rodrigues C, Eisenach K, Victor TC, Ismail N, Crudu V, Gler MT, Catanzaro A. Predicting extensively drug-resistant *Mycobacterium tuberculosis* phenotypes with genetic mutations. Journal of Clinical Microbiology. 2014;**52**:781-789. DOI: 10.1128/JCM.02701-13

[4] Naidoo CC, Pillay M. Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*. Clinical Microbiology and Infection. 2014;**20**:O361-O369. DOI: 10.1111/1469-0691.12415

[5] Manson AL, Cohen KA, Abeel T, Desjardins CA, Armstrong DT, Barry CE 3rd, Brand J, TBResist Global Genome Consortium, Chapman SB, Cho SN, Gabrielian A, Gomez J, Jodals AM, Joloba M, Jureen P, Lee JS, Malinga L, Maiga M, Nordenberg D, Noroc E, Romancenco E, Salazar A, Ssengooba W, Velayati AA, Winglee K, Zalutskaya A, Via LE, Cassell GH, Dorman SE, Ellner J, Farnia P, Galagan JE, Rosenthal A, Crudu V, Homorodean D, Hsueh PR, Narayanan S, Pym AS, Skrahina A, Swaminathan S, Van der Walt M, Alland D, Bishai WR, Cohen T, Hoffner S, Birren BW, Earl AM. Genomic analysis of globally diverse *Mycobacterium tuberculosis* strains provides insights into emergence and spread of multidrug resistance. Nature Genetics. 2017;**49**:395-402. DOI: 10.1038/ng.3767

[6] Cabibbe AM, Cirillo DM. Best approaches to drug-resistance surveillance at the country level. International Journal of Mycobacteriology. 2016;**5**:S40-S41. DOI: 10.1016/j.ijmyco.2016.09.010

[7] Ilin AI, Kulmanov ME, Korotetskiy IS, Islamov RA, Akhmetova GK, Lankina MV, Reva ON. Genomic insight into mechanisms of reversion of antibiotic resistance in multidrug resistant *Mycobacterium tuberculosis* induced by a nanomolecular iodine-containing complex FS-1. Frontiers in Cellular and Infection Microbiology. 2017;**7**:151. DOI: 10.3389/fcimb.2017.00151

[8] Zhang Y, Yew W-W. Mechanisms of drug resistance in *Mycobacterium tuberculosis*: Update 2015. The International Journal of Tuberculosis and Lung Disease. 2015;**19**:1276-1289. DOI: 10.5588/ijtld.15.0389

[9] Trauner A, Borrell S, Reither K, Gagneux S. Evolution of drug resistance in tuberculosis: Recent progress and implications for diagnosis and therapy. Drugs. 2014;**74**:1063-1072. DOI: 10.1007/s40265-014-0248-y

[10] Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. PLoS Medicine. 2009;**6**:e2. DOI: 10.1371/journal.pmed.1000002

[11] Fonseca JD, Knight GM, McHugh TD. The complex evolution of antibiotic resistance in *Mycobacterium tuberculosis*. International Journal of Infectious Diseases. 2015;**32**:94-100. DOI: 10.1016/j.ijid.2015.01.014

[12] Cohen T, Murray M. Modeling epidemics of multidrug-resistant *M. tuberculosis* of heterogeneous fitness. Nature Medicine. 2004;**10**:1117-1121. DOI: 10.1038/nm1110

[13] Müller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant *Mycobacterium tuberculosis*. Trends in Genetics. 2013;**29**:160-169. DOI: 10.1016/j.tig.2012.11.005

[14] Kolyva A, Old KP. New TB drugs: Mechanisms of action and resistance. In: Understanding Tuberculosis—New Approaches to Fighting against Drug Resistance. Rijeka, Croatia: InTech; 2012. pp. 210-232. DOI: 10.5772/30992

[15] Aldred KJ, Blower TR, Kerns RJ, Berger JM, Osheroff N. Fluoroquinolone interactions with *Mycobacterium tuberculosis* gyrase: Enhancing drug activity against wild-type and resistant gyrase. Proceedings of the National Academy of Sciences. 2016;**113**:E839-E846. DOI: 10.1073/pnas.1525055113

[16] Zhao X, Yu H, Yu S, Wang F, Sacchettini JC, Magliozzo RS. Hydrogen peroxide-mediated isoniazid activation catalyzed by *Mycobacterium tuberculosis* catalase-peroxidase (KatG) and its S315T mutant. Biochemistry. 2006;**45**:4131-4140. DOI: 10.1021/bi051967o

[17] Chernyaeva EN, Shulgina MV, Rotkevich MS, Dobrynin PV, Simonov SA, Shitikov EA, Ischenko DS, Karpova IY, Kostryukova ES, Ilina EN, Govorun VM, Zhuravlev VY, Manicheva OA, Yablonsky PK, Isaeva YD, Nosova EY, Mokrousov IV, Vyazovaya AA, Narvskaya OV, Lapidus AL, O'Brien SJ. Genome-wide *Mycobacterium tuberculosis* variation (GMTV) database: A new tool for integrating sequence variations and epidemiology. BMC Genomics. 2014;**15**:308. DOI: 10.1186/1471-2164-15-308

[18] Gagneux S. Host-pathogen coevolution in human tuberculosis. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences. 2012;**367**:850-859. DOI: 10.1098/rstb.2011.0316

[19] Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, Parkhill J, Malla B, Berg S, Thwaites G, Yeboah-Manu D, Bothamley G, Mei J, Wei L, Bentley S, Harris SR, Niemann S, Diel R, Aseffa A, Gao Q, Young D, Gagneux S. Out-of-Africa migration and Neolithic co-expansion of *Mycobacterium tuberculosis* with modern humans. Nature Genetics. 2014;**45**:1176-1182. DOI: 10.1038/ng.2744

[20] Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, Homolka S, Roach JC, Kremer K, Petrov DA, Feldman MW, Gagneux S. High functional diversity in *Mycobacterium*

tuberculosis driven by genetic drift and human demography. PLoS Biology. 2008;**6**:e311. DOI: 10.1371/journal.pbio.0060311

[21] Banuls A-L, Sanou A, Van Anh NT, Godreuil S. *Mycobacterium tuberculosis*: Ecology and evolution of a human bacterium. Journal of Medical Microbiology. 2015;**64**:1261-1269. DOI: 10.1099/jmm.0.000171

[22] Reiling N, Homolka S, Walter K, Brandenburg J, Niwinski L, Ernst M, Herzmann C, Lange C, Diel R, Ehlers S, Niemann S. Clade-specific virulence patterns of *Mycobacterium tuberculosis* complex strains in human primary macrophages and aerogenically infected mice. MBio. 2013;**4**:pii: e00250-pii: e00213. DOI: 10.1128/mBio.00250-13

[23] Hanekom M, Gey van Pittius NC, McEvoy C, Victor TC, Van Helden PD, Warren RM. *Mycobacterium tuberculosis* Beijing genotype: A template for success. Tuberculosis. 2011;**91**:510-523. DOI: 10.1016/j.tube.2011.07.005

[24] Köser CU, Bryant JM, Becq J, Török EM, Ellington MJ, Marti-Renom M, Carmichael AJ, Parkhill J, Smith GP, Peacock J. Whole-genome sequencing for rapid susceptibility testing of *M. tuberculosis*. The New England Journal of Medicine. 2013;**369**:290-292. DOI: 10.1056/NEJMc1215305

[25] Falush D, Bowden R. Genome-wide association mapping in bacteria? Trends in Microbiology. 2006;**14**:353-355. DOI: 10.1016/j.tim.2006.06.003

[26] Falush D. Bacterial genomics: Microbial GWAS coming of age. Nature Microbiology. 2016;**1**:16059. DOI: 10.1038/nmicrobiol.2016.59

[27] Earle SG, Wu CH, Charlesworth J, Stoesser N, Gordon NC, Walker TM, Spencer CCA, Iqbal Z, Clifton DA, Hopkins KL, Woodford N, Smith EG, Ismail N, Llewelyn MJ, Peto TE, Crook DW, McVean G, Walker AS, Wilson DJ. Identifying lineage effects when controlling for population structure improves power in bacterial association studies. Nature Microbiology. 2016;**1**:16041. DOI: 10.1038/nmicrobiol.2016.41

[28] Coll F, McNerney R, Guerra-Assunção JA, Glynn JR, Perdigão J, Viveiros M, Portugal I, Pain A, Martin N, Clark TG. A robust SNP barcode for typing *Mycobacterium tuberculosis* complex strains. Nature Communications. 2014;**5**:4812. DOI: 10.1038/ncomms5812

[29] Feuerriegel S, Koser CU, Niemann S. Phylogenetic polymorphisms in antibiotic resistance genes of the *Mycobacterium tuberculosis* complex. The Journal of Antimicrobial Chemotherapy. 2014;**69**:1205-1210. DOI: 10.1093/jac/dkt535

[30] Feuerriegel S, Schleusener V, Beckert P, Kohl TA, Miotto P, Cirillo DM, Cabibbe AM, Niemann S, Fellenberg K. PhyResSE: A web tool delineating *Mycobacterium tuberculosis* antibiotic resistance and lineage from whole-genome sequencing data. Journal of Clinical Microbiology. 2015;**53**:1908-1914. DOI: 10.1128/JCM.00025-15

[31] Cui ZJ, Yang QY, Zhang HY, Zhu Q, Zhang QY. Bioinformatics identification of drug resistance-associated gene pairs in *Mycobacterium tuberculosis*. International Journal of Molecular Sciences. 2016;**17**:1417-1425. DOI: 10.3390/ijms17091417

[32] Mukhopadhyay S, Balaji KN. The PE and PPE proteins of *Mycobacterium tuberculosis*. Tuberculosis. 2017;**91**:441-447. DOI: 10.1016/j.tube.2011.04.004

[33] Mortimer TD, Weber AM, Pepperell CS. Signatures of selection at drug resistance loci in *Mycobacterium tuberculosis*. mSystems 2018;**3**:e00108-17. DOI: 10.1128/mSystems.00108-17

[34] Coll F, Prestona M, Guerra-Assunção JA, Hill-Cawthorn G, Harris D, Perdigão J, Viveiros M, Portugal I, Drobniewski F, Gagneux S, Glynn JR, Pain A, Parkhill J, McNerney R, Martin N, Clark TG. PolyTB: A genomic variation map for *Mycobacterium tuberculosis*. Tuberculosis. 2014;**94**:346-354. DOI: 10.1016/j.tube.2014.02.005

[35] Faksri K, Tan JH, Chaiprasert A, Teo Y-Y, RT-H O. Bioinformatics tools and databases for whole genome sequence analysis of *Mycobacterium tuberculosis*. Infection, Genetics and Evolution. 2016;**45**:359-368. DOI: 10.1016/j.meegid.2016.09.013

[36] Aminian M, Couvin D, Shabbeer A, Hadley K, Vandenberg S, Rastogi N, Bennett KP. Predicting *Mycobacterium tuberculosis* complex clades using knowledge-based Bayesian networks. BioMed Research International. 2014;**2014**:1-11. DOI: 10.1155/2014/398484

[37] Baym M, Stone LK, Kishony R. Multidrug evolutionary strategies to reverse antibiotic resistance. Science. 2016;**351**:aad3292

[38] Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. 1998;**393**:537-544. DOI: 10.1038/31159

[39] Fleiss JL, Levin B, Paik MC. Statistical methods for rates and proportions. In: Shewart WA, Wilks SS, editors. Wiley Series in Probability and Statistics. 3rd ed. Wiley Online Library. Hoboken, New Jersey: John Wiley and Sons, Inc; 2004

[40] Heym B, Alzari PM, Honoré N, Cole ST. Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. Molecular Microbiology. 1995;**15**:235-245. DOI: 10.1038/31159

[41] Mdluli K, Slayden RA, Zhu Y, Ramaswamy S, Pan X, Mead D, Crane DD, Musser JM, Barry CE 3rd. Inhibition of a *Mycobacterium tuberculosis* beta-ketoacyl ACP synthase by isoniazid. Science. 1998;**280**:1607-1610. DOI: 10.1126/science.280.5369.1607

[42] Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, Suzuki Y, Ochi K. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Molecular Microbiology. 2007;**63**:1096-1106. DOI: 10.1111/j.1365-2958.2006.05585.x

Chapter 6

Streptomyces Secondary Metabolites

Mohammed Harir, Hamdi Bendif,
Miloud Bellahcene, Zohra Fortas and Rebecca Pogni

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.79890

Abstract

Actinobacteria are found spread widely in nature and particular attention is given to their role in the production of various bioactive secondary metabolites. Tests on soil samples show that there can be a diversity of actinomycetes depending on the climate, the area it is growing in, how dry the soil is, and the quality of the soil. However, it was agreed after tests in Yunnan, China, that the genus *Streptomyces* sp. is most important in ecological function, representing up to 90% of all soil actinomycetes, and therefore helping to show the important characteristics needed of the soil actinomycete population. Streptomycete compounds are used for other biological activities, not just for antibiotics. It has been found that metabolites can be broadly divided into four classes: (1) regulatory activities in compounds, these include consideration of growth factors, morphogenic agents and siderophores, and plants promoting rhizobia; (2) antagonistic agents, these include antiprotozoans, antibacterials, antifungals, as well as antivirals; (3) agrobiologicals, these include insecticides, pesticides, and herbicides; and (4) pharmacological agents, these include neurological agents, immunomodulators, antitumorals, and enzyme inhibitors. It is found that *Streptomyces hygroscopicus* is one of the very best examples because it secretes in excess of 180 secondary metabolites to locate simultaneous bioactivities for a given compound. Increasingly, both its agricultural and pharmacological screenings are being used in conjunction with antimicrobial tests and have revealed several unusual aerobiological and therapeutic agents, which were hitherto unknown for biological use as antibiotics. Since streptomycetes are now being used increasingly to screen for antimicrobial activity, reports show the existence of secondary metabolites with other activities that may have been missed. Currently, nearly 17% of biologically active secondary metabolites (nearly 7600 out of 43,000) are known from streptomycetes. It has been found that soil streptomycetes are the main source used by bioactive secondary metabolites. However, recently there have been many and varied types of structurally unique and biologically active secondary metabolites found and obtained from marine actinomycetes, including those from the genus *Streptomyces*. Also, compounds that are synthesized by streptomycetes exhibit extreme chemical diversity. Diverse form made from from simple amino acid

derivatives to high molecular weight proteides, and macrolactones from simple eight membered lactones to different condensed macrolactones. Berdy (1974) introduced the first classification scheme for antibiotics referring to the chemical structure. On the basis of Berdy's scheme, (1996) recognized that both low and high molecular weight compounds from 63 different chemical classes are produced by streptomycetes.

Keywords: antibiotics, PKS, NRPS, *Streptomyces*, secondary metabolites, antibacterial

1. Introduction

Streptomyces are Gram-positive, filamentous bacteria belonging to the group actinomycetes, a group that encompasses the majority of soil bacterial species. It is estimated that a gram of soil contains 109 CFU (colony-forming units) and out of these 109 CFUs, 107 are Actinobacteria [1]. They are ubiquitous soil bacteria, which are also found in the marine environment such as sediments [2]. Some are symbionts of sponges, for example, or insects like the ant *Acromyrmex octospinosus*, which lives in symbiosis with *Streptomyces* (*Streptomyces* S4)-producing antifungals, which help protect fungi cultivated by phytopathogenic ants [3]. *Streptomyces* have a particular development cycle. This cycle begins with a spore that germinates forming vegetative hyphae very little septate that will be structured in a network, the vegetative mycelium whose role is to explore the environment in search of nutrients. The bacterium will form aerial hyphae compartmentalized during a deficiency in element nutrients; these hyphae will then differentiate into spores, which are the form of resistance and dissemination of this bacterium [4].

The production of many secondary metabolites, including antibiotics, is coupled with morphological differentiation. Indeed, we observe a greater production of secondary metabolites during the transition from vegetative growth to aerial growth [5]. During this change in growth type, partial lysis of the mycelium vegetation takes place to provide the necessary nutrients for the creation of aerial mycelium; this release of nutrients could attract competitors. This synchronization of the cycle of development and production of secondary metabolites could be a way for the bacteria to dispel the invaders to keep these nutrients, or else kill the surrounding bacteria to feed them.

The secondary metabolite-producing microorganisms synthesize these bioactive and complex molecules at the lag phase and stationary phase of their growth (**Figure 1a**). However, regarding actinomycetes and *Streptomyces* especially, secondary metabolites can be produced at exponential, stationary, and death phases [6, 7]. It appears in times of environmental issues that nutrient depletion-limiting growth conditions allow formation of secondary metabolites. These are mostly found in fungi, plants, soil, and marine environments and organisms. Its has also been found that different organisms can produce metabolites that have various biological abilities, which include metal transporting agents, sex hormones, toxins, pigments, pesticides, immunosuppressants, anticancer agents, antibacterial agents, immunomodulating agents, antagonists, and receptor antagonists. The intermediate or finished products of primary metabolic pathways are obtained from their own systematic pathways for the synthesis

http://dx.doi.org/10.5772/intechopen.79890

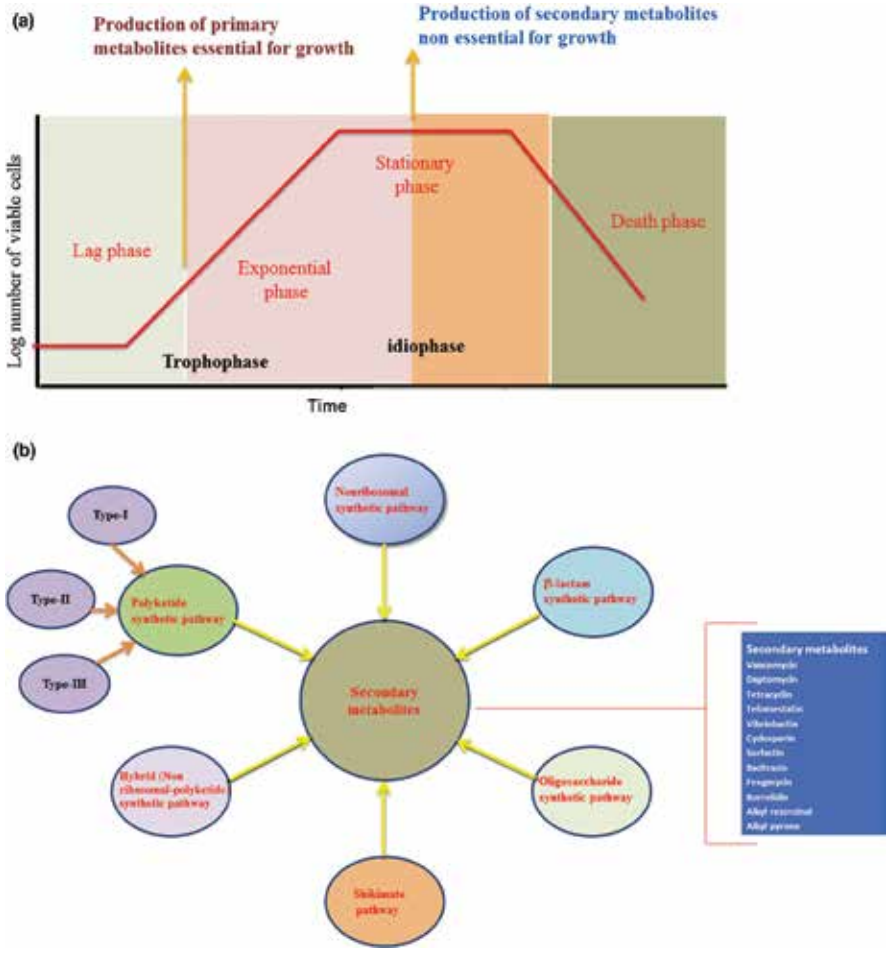

Figure 1. (a) Phases of bacterial growth and metabolite production. Overall, the major metabolites can be produced at the late interval phase and center of exponential phase, since the minor metabolites can be produced at the end of the stationary phase and during the constant phase. (b) Various pathways responsible for the assembly of secondary metabolites.

of secondary metabolites. To be able to obtain secondary metabolites, metabolic pathway reaction methods are conducted using multienzyme complexes or an individual enzyme. Genes that encode the synthetic pathway enzyme in general are within chromosomal DNA mostly arranged in cluster formation. As an example, *Streptomycetes griseus* and *Streptomyces glaucescens* chromosomal DNA contain 30 or more str/sts and blu genes that participate in streptomycin biosynthesis.

There are many varieties of known secondary metabolites synthesized by six pathways of different biosynthesis (**Figure 1b**): the peptide pathway, the polyketide synthase (PKS) pathway, the nonribosomal polypeptide synthase (NRPS) pathway, the hybrid (nonribosmial polyketide synthetic) pathway, the shikimate pathway, the β-lactam synthetic pathway, and the carbohydrate pathway. The pathway peptide concerns a part of the protein secondary

metabolites: they are synthesized by simple translation of mRNAs into peptides by ribosomes. NRPSs are enzymes capable of condensing amino acids to form peptides without going through the ribosomal synthesis pathway. PKSs are enzymes capable of synthesizing a particular family of secondary metabolites: polyketides. The enzymes necessary for the synthesis of these polyketides are homologous to fatty acid synthase (FAS), which is responsible for the synthesis of fatty acid chains. Like the FASs these enzymes can couple precursors to form a chain. This chain will then undergo eight post-PKS changes before becoming active. Regarding the carbohydrate (known scientifically as oligosaccharide) route, it is based on the use of enzymes capable of coupling different sugars to form a carbohydrate precursor; this chain will then undergo modifications that will make the precursor active [8].

2. Bioactivity of *Streptomyces*

Streptomyces produce 70–80% of the natural bioactive substances known for their pharmaceutical or agrochemical applications [9, 10]. Continuously new metabolites with different biological activities are isolated from *Streptomyces* strains [11–14]. The first and most important product of *Streptomyces* is antibiotics [15]. From 1955 the genus *Streptomyces* has been the major supplier of new antibiotics [16]. They are the source of antibacterial, antifungal, antitumor, antiparasitic [17–19], antiviral, insecticide, pesticide, and herbicide substances, in addition to pharmacological substances such as immunomodulators (immunosuppressive and immunostimulatory agents), vasoactive substances, and neurological agents [20].

Enzymes are the most important products of *Streptomyces* after antibiotics [21], such as proteases, lipases, cellulases, amylases, pectinases, and xylanases [22, 23].

2.1. Production of antibiotics by *Streptomyces*

2.1.1. General

Antibiotics are produced by a wide range of fungal microorganisms and bacteria, and inhibit or kill other microorganisms at low concentrations [24]. A large number of antibiotics have been identified in natural environments, but less than 1% are medically useful. Many antibiotics have been structurally modified in the laboratory to increase their effectiveness, forming the class of semisynthetic antibiotics [25].

The history of antibiotics began with the discovery of penicillin by Fleming in the 1940s. The antimicrobial activities of antibiotics produced by microorganisms have been extensively studied, and the research undertaken has allowed completion of the antibacterial arsenal available to doctors and the general public.

Microorganisms producing chloramphenicol, neomycin, tetracycline, and terramycin were isolated in 1953. The discovery of chemotherapeutic agents and the development of new, more powerful drugs revolutionized medicine and have greatly reduced human suffering [26]. It is very well known that the genus *Streptomyces* produces the majority of antibiotics and biologically active secondary metabolites. Nearly 50% of the species *Streptomyces*

isolated are recognized as producers of antibiotics [25]. Actinomycetes synthesize two-thirds of the microbial antibiotics of which about 80% are isolated from the genus *Streptomyces*. Even if other secondary metabolites are included, the actinomycetes remain the largest suppliers with about 60% (*Streptomyces* always have the biggest part with 80%). More than 60 substances with antibiotic activity produced by *Streptomyces* species are used not only in the world of veterinary and human medicine, but also in the field of agriculture and industry. The capacity of the members of the genus *Streptomyces* [27, 28] to produce commercially significant compounds, especially antibiotics, remains unsurpassed, possibly because of the extra-large DNA complement of these bacteria [17]. Antibiotics that come from Actinobacteria are grouped together so that they belong in their major structural classes. Examples of these are ansamycins (ritamycin), macrolides (erythromycin, azithromycin, and clarithromycin), aminoglycosides (streptomycin, kanamycin, tobramycin, gentamicin, and neomycin), tetracyclines, anthracyclines (doxorubicin), and β-lactam (penicillin, cephalosporin, carbapenems, and monobactams). Streptomycin and its varying species strains have been responsible for the production of most antibiotics and it appears that these organisms produce antibiotics to kill off potential competitors [29]. Streptomycin was one of the first antibiotics found. It is produced by *S. griseus* [30]. Today, various *Streptomyces* species are responsible for approximately 75% of both medical and commercial antibiotics and work very well in these areas. Due to the need for new antibiotics, studies have steered towards the isolation of streptomycetes and the careful screening of different habitats in which they are used. It has also been found through research that different conditions such as nutrients, culturing, and other factors may affect how *Streptomyces* develop to form antibiotics. With this in mind the medium constitution along with metabolic capacity of any organism production can affect antibiotic biosynthesis. Research into actinomycetes has found that they are capable of producing more one antibiotic (e.g. *S. griseus and S. hygroscopicus*) and also the same antibiotic can produce various species of Actinobacteria (e.g. streptothricin and actinomycin). Therefore, an antibiotic may be exactly the same with the same chemical composition and antibiotic spectrum as a produced Actinobacterium (**Table 1**). The table gives a list of antibiotics produced by variations of Actinobacteria and how the antimicrobial application has had a profound impact on the medical world where previously cancers, tumors, and even malaria could not be treated.

2.2. Production of enzymes

Research has reported that there are a great variety of enzymes that can be applied to biomicrobial fields and biotechnological industries from different genera of actinomycetes. Using the information available from genome and protein sequencing data, actinomycetes are constantly screened and used for producing amylases, xylanases, proteases, chitinases, cellulases, and other enzymes. Industrial applications, for example, the pronase of *S. griseus* and the kerase of *Streptomyces fradiae*, are used for the commercial production of biotechnology products such as hydrolysate proteins from different protein sources [31]. The proteases of *Streptomyces* have the advantage of easy elimination of the mycelium by filtration or simple centrifugation [32]. Similarly, Actinobacteria have been revealed to be an excellent resource for L-asparginase, which is produced by a range of Actinobacteria, mainly those from soils such as *S. griseus*, *Streptomyces karnatakensis, Streptomyces albidoflavus*, and *Nocardia* spp. [33, 34] (**Table 2**).

Antibiotic compound	*Streptomyces* species	Application
1,4-Dihydroxy-2-(3-hydroxybutyl)-9,10-anthraquinone 9,10 anthrac	*Streptomyces* sp. RAUACT-1	Antibacterial
1,8-Dihydroxy-2-ethyl-3-methylanthraquinone	*Streptomyces* sp.	Antitumor
2-Allyloxyphenol	*Streptomyces* sp.	Antimicrobial; food preservative; oral disinfectant
Anthracyclines	*S. galileus*	Antitumor
Arenimycin	*S. arenicola*	Antibacterial; anticancer
Avermectin	*S. avermitilis*	Antiparasitic
Bafilomycin	*S. griseus*, *S. halstedii*	ATPase; inhibitor of microorganisms, plant and animal cells
Bisanthraquinone	*Streptomyces* sp.	Antibacterial
Carboxamycin	*Streptomyces* sp.	Antibacterial; anticancer
Chinikomycin	*Streptomyces* sp.	Anticancer
Chloramphenicol	*S. venezuelae*	Antibacterial; inhibitor of protein biosynthesis
Chromomycin B, A2, A3	*S. coelicolor*	Antitumor
Daryamides	*Streptomyces* sp.	Antifungal; anticancer
Elaiomycins B and C	*Streptomyces* sp. BK 190	Antitumor
Frigocyclinone	*S. griseus*	Antibacterial
Glaciapyrroles	*Streptomyces* sp.	Antibacterial
Hygromycin	*S. hygroscopicus*	Antimicrobial; immunosuppressive
Lajollamycin	*S. nodosus*	Antibacterial
Lincomycin	*S. lincolnensis*	Antibacterial; inhibitor of protein biosynthesis
Mitomycin C	*S. lavendulae*	Antitumor; binds to double-stranded DNA
Pacificanones A and B	*S. pacifica*	Antibacterial
Piericidins	*Streptomyces* sp.	Antitumor
Proximicins	*Verrucosispora* sp.	Antibacterial; anticancer
Pristinamycine	*S. pristinaespiralis*	Antibacterial
Rapamycin	*S. hygroscopicus*	Immunosuppressive; antifungal
Resistoflavin methyl ether	*Streptomyces* sp.	Antibacterial; antioxidative
Saliniketal	*S. arenicola*	Cancer; chemoprevention
Salinispyrone	*S. pacifica*	Unknown
Salinispyrone A and B	*S. pacifica*	Mild cytotoxicity
Salinosporamide A	*Salinispora tropica*	Anticancer; antimalarial
Salinosporamide B and C	*S. tropica*	Cytotoxicity

Antibiotic compound	*Streptomyces* species	Application
Sesquiterpene	*Streptomyces* sp.	Unknown
Staurosporinone	*Streptomyces* sp.	Antitumor; phycotoxicity
Streptokordin	*Streptomyces* sp.	Antitumor
Streptomycin	*S. griseus*	Antimicrobial
Streptozotocin	*S. achromogenes*	Diabetogenic
Tetracyclines	*Streptomyces achromogenes*; *S. rimosus*	Antimicrobial
Tirandamycins	*Streptomyces* sp.	Antibacterial
Valinomycin	*S. griseus*	Ionophor; toxic for prokarotes, eukaryotes

Table 1. List of antibiotics produced by different Actinobacteria and their applications.

Enzyme	Industry	Use	*Streptomyces* strains
Aminoacylase	Pharmaceuticals	Production of semisynthetic penicillins and celpholosorin	*S. olivaceus*
			S. roseiscleroticus
			S. sparsogenes
Amylase	Detergent	Removal of stains	*Streptomyces* sp.
	Baking	Softening of bread; volume	*S. erumpons*
	Paper and pulp	Deinking	
		Drainage improvement	
	Starch	Production of glucose, fructose, syrups	
	Textile	Removal of starch from woven fabrics	
Cellulase	Detergent	Removal of stains	*S. thermobifida*,
	Textile	Denim finishing, softening of cotton	*halotolerans*, *S.*
	Paper and pulp	Deinking, modification of fibers	*thermomonospora*, *S. ruber*
Chitinase	Bioremediation	Utilization of chitin waste	*S. griseus*
			S. antibioctius
Glucose oxidase	Baking	Strengthening of dough	*S. coelicolor*
Keratinase			
Laccase	Bleaching	Clarification (juice), flavor (beer), cork stopper treatment	*S. brahimensis*
L-Asparaginase	Medicine	The treatment of acute lymphoblastic leukemia	*S. karnatakensis*
			S. halstedii

Enzyme	Industry	Use	*Streptomyces* strains
Lipase	Detergent	Removal of stains	*S. griseus*
	Baking	Stability of dough	
	Dairy	Cheese flavoring	
	Textile	Deinking, cleaning	
N-Acetylmuramidase	Bacteriology	Bacteriostatic enzymes	*S. globisporus*
Neuraminidase	Medical research	Cell surface and clinical studies	*Streptomyces* sp.
Pectinase	Beverage	Clarification, mashing	*S. lydicus*
	Textile	Scouring	
Penicillin amidase	Commercial significance	Production of 6-aminopenicillanic acid on an industrial scale	*Streptomyces* sp.
Peptide hydrolase	Pharmaceuticals	Industrial biosynthesis of oxytetracycline	*S. rimosus*
Phytase	Animal feed	Phytate digestibility	*S. luteogriseus* R10
Protease	Food	Cheese making	*S. pactum*, *S.*
	Brewing	Clarification; low calorie beer	*thermoviolaceus*, *Streptomyces* sp.
	Leather	Dehiding	
	Medicine	Treatment of blood clot	
Tyrosinase	Pharmacy	L-Dopa synthesis	*S. cyaneofuscatus*
Xylanase	Baking	Conditioning of dough	*Streptomyces* spp.
	Animal feed	Digestibility	
	Paper and pulp	Bleach boosting	
β-*N*-Acetyl-D-glucosaminidase	Studying their biochemical functions	Structural determination of the carbohydrate moiety of several glycoproteins	*S. griseus*

Table 2. List of enzymes produced by various Actinobacteria and their industrial application.

2.3. Bioherbicides

Secondary metabolites of Actinobacteria are used as herbicides against unwanted herbs and weeds (**Table 3**).

2.4. Probiotics

The use of *Streptomyces* sp. on the growth of tiger shrimp has been previously documented. Also, it was found that antibiotic product extracted from marine Actinobacteria and supplemented in feed was efficient in exhibiting the in vivo effect on feed and the detection of the efficient effect of in vivo white spot syndrome virus in black tiger shrimp. The murine actinomycete

Bioherbicides	**Biocontrol**	***Streptomyces* strains**
Anisomycin	Inhibitor of growth of annual grassy weeds such as barnyardness and common crabgrass and broad-leaved weeds	*Streptomyces* sp.
Bialaphos	Control of annual and perennial grassy weeds and broad-leaved weeds	*S. viridochromogenes*
Carbocyclic coformycin and hydantocidin	Control of several weeds	*S. hygroscopicus*
Herbicidines and herbimycins	Monocotyledonous and dicotyledonous weed	*S. saganonensis*
Phthoxazolin, hydantocidin, and homoalanosin	Control of several weeds	*Streptomyces* sp.

Table 3. Exemles of herbicides produced by actinobacteria used against unwanted herbs and weeds.

activity was found to be an effective microorganism against biofilms resulting from *Vibrio* spp., suggesting therefore the potential preventive effect of Actinobacteria against *Vibrio* deseases [35]. Moreover, Latha [36] identified 18 Actinobacteria with probiotic properties isolated from chicken, and their results support the potential preventive effect of *Streptomyces* sp. JD9 as probiotic agents against deseases.

2.5. Aggregative peptide pheromones

The production of pheromone is considered to have important criteria: it is used as a defense against predators, in mate selection, and to conquer host-habitats through mass attack. Sex pheromone peptides in culture supernantrants were mainly found to support aggregation together by the same related species [37, 38]. A good example for aggregative peptide pheromones is *Streptomyces werraensis* LD22, which secretes a heat-stable, acidic pH resistant, low molecular weight peptide pheromone that promotes aggregation propensity and enhances the biofilm-forming ability of other Actinobacterial isolates.

2.6. Biosurfactants

Microbially derived compounds that share hydrophilic and hydrophobic moieties are surface active biosurfactants that are independent of mineral oil as a feedstock compared with chemically derived surfactants.

Biosurfactants are widely used in scientific research topics (nutrients, cosmetics, textiles, varnishes, pharmaceuticals, mining, and oil recovery) [39, 40]. The lipopeptide antibiotic daptomycin has received great interest as a treatment for Gram-positive bacterial infections; it is marketed as Cubicin by Cubist Pharmaceuticals. Various biosurfactant drugs or bioemulsifiers have been described as a class of Actinobacteria. The best described biosurfactants include a class of glucose-based glycolipids, most of which have a hydrophilic backbone, including glycosides associated with glucose units forming a trehalose moiety.

2.7. Vitamins

Vitamin B12 or cobalamine can be synthesized through the fermentation of Actinobacteria [41, 42], and has aroused considerable interest in the possible production of vitamins through microbial fermentation. In addition, cobalt salts in media act as a general Actinobacteria precursor in producing vitamins. Because cobalt is a rather effective bactericidal agent, this precursor must be added carefully. The fermentations producing the antibiotics streptomycin, aureomycin, grisein, and neomycin produce vitamin B12 as well if the medium is supplemented with cobalt without affecting the yields of antibiotic substances.

2.8. Pigments

Microbe-oriented pigments are of great concern. Especially, Actinobacteria are characterized by the production of various pigments on natural or synthetic media and are considered an important cultural characteristic in describing the organisms. Generally, the morphological features of colonies and production of different pigments and aerial branching filaments are known as hyphae, giving them a fuzzy appearance. These pigments are usually various shades of blue, violet, red, rose, yellow, green, brown, and black, which can be dissolved in the medium or may be retained in the mycelium. These microbes also have the ability to synthesize and excrete dark pigments, melanin or melanoid, which are considered useful criteria for taxonomical studies in the textile industry (**Table 4**).

2.9. Nanoparticle synthesis

The chemical techniques of nanoparticle preparation are less expensive when produced in high quantities; however, the nanoparticles may be contaminated by precursor chemicals, toxic solvents, and risky by-products. As a result, the development of high-yield, low-charge, nontoxic effects, and beneficial environmental procedures for metallic nanoparticle synthesis, and thus the biological method of nanoparticle synthesis, is considered important. Actinobacteria are actually effective nanoparticle producers, showing a number of biological properties, including antibacterial, antifungal, anticancer, antibiofouling, antimalarial, antiparasitic, and antioxidant activities. *Streptomyces* and *Arthrobacter* genera have proved to be "nanofactories" for developing clean and nontoxic procedures for the preparation of silver and gold nanoparticles (**Table 5**).

Pigments	***Streptomyces* strain**	**Class**
III Undecylprodigiosin	*S. longispororuber* DSM 40599	Prodigiosin
IV Metacycloprodigiosin		
Actinomycin	*Streptomyces* sp.	Phenoxazinone
Granaticin	*S. litmocidin* DSM 40164	Naphthoquinone
Rhodomycin	*Synodontis violaceus* DSM 40704	Anthracycline glycoside

Table 4. Exemples of pigments produced by some streptomyces species and their classification.

***Streptomyces* strains**	**Nanoparticles**
Streptomyces sp. GRD, *Streptomyces* sp., *S. albidoflavus*, *S. hygroscopicus*, *S. rochei*	Silver
S. aureofaciens, *S. glaucus*, *S. viridogens*, *S. hygroscopicus*	Gold
Streptomyces sp.	Zinc, copper, manganese

Table 5. Exemples of nanoparticules produced by some streptomyces species.

2.10. Bioremediation

Streptomyces have an important role in the recycling of organic carbon and are able to degrade complex polymers [43]. As reported, the wide use of petroleum hydrocarbons as chemical compounds and fuel in everyday life was considered well-known pollutants of large soil surfaces, causing serious environmental damage. Some studies proved the possible beneficial role of *Streptomyces* flora in the degradation of hydrocarbons [44, 45]. Many Actinobacterial strains are able to solubilize lignin and break down lignin-related compounds following the production of cellulose and hemicellulose-degrading enzymes and extracellular peroxidase [46]. Actinobacteria species are able to grow and live in oil-rich environments, and thus they could be in bioremediation to reduce oil contaminants.

2.11. Control of plant diseases

Results of new approaches to control plant diseases. Actinobacteria are potentially used in the agro-industry as a source of agroactive compounds of plant growth (rhizobacteria (polyglycerol polyricinoleate, PGPR) promoting) and for biocontrol [47, 48]. Approximately 60% of the new insecticides and herbicides derived from *Streptomyces* were discovered in the last 5 years. Kasugamycin, a bactericidal and fungicidal metabolite discovered *in Streptomyces kasugaensis* [49], inhibits protein biosynthesis in microorganisms but not in mammals, since its toxicological features are excellent. Inhibition of plant pathogenic *Rhizoctonia solani* under in vitro conditions was assessed with the culture supernatant of *Streptomyces* sp., which showed that the tested Actinobacteria had the ability to reduce damping-off severity in tomato plants (**Table 6**).

2.12. Nematode control

The majority of microorganisms were identified as antagonists of plant-parasitic nematodes, in particular Actinobacteria, which are effectively used in biological control because of their ability to produce antibiotics. The *Streptomyces* species-producing avermectins show that high nematicidal compounds can be produced by soil-borne organisms. *Streptomyces avermitilis* produces ivermectin, having an efficient activity against *Wucheria bancroftii* [50]. Similarly, various other antiparasitic compounds are produced from various *Streptomyces* sp.

2.13. Enhancement of plant growth

PGPR can directly or indirectly affect the growth of plants in two common ways. Indirect growth happens when PGPR decreases or prevents the harmful effects of one or more damaging

Disease	*Streptomyces* strains	Antibiotic produced
Asparagus root diseases	*S. griseus*	Faeriefungin
Blotch of wheat	*S. malaysiensis*	Malayamycin
Broad range of plant diseases	*S. griseochromogenes*	Blasticidin S
Brown rust of wheat	*S. hygroscopicus*	Gopalamycin
Damping-off of cabbage	*S. padanus*	Fungichromin
Grass seedling disease	*S. violaceusniger* YCED9	Nigericin and guanidylfungin A
Phytophthora blight of pepper	*S. humidus*	Phenylacetic acid
Phytophthora blight of pepper	*S. violaceusniger*	Tubercidin
Potato scab	*S. melanosporofaciens*	Geldanamycin
	EF-76 and FP-54	
Powdery mildew	*Streptoverticillium rimofaciens*	Mildiomycin
Powdery mildew of cucumber	*Streptomyces* sp. KNF2047	Neopeptin A and B
Rice blast disease	*S. kasugaensis*	Kasugamycin
Rice sheath blight	*S. cacaoi* var. *Asoensis*	Polyoxin B and D
Root rot of pea geldanus	*S. hygroscopicus*	Geldanamycin
Sheath blight of rice	*S. hygroscopicus* var. *Limoneus* No. T-7545	Validamycin

Table 6. Antibiotics produced by the Actinobacteria that suppress various plant diseases.

microorganisms. This is mainly researched through biocontrol or the antagonism of soil plant pathogens. Particularly, the effects of pathogen invasion and establishment can be strongly prevented by colonization or the biosynthesis of antibiotics and other secondary metabolites. Direct growth promotes plant growth by PGPR when the plant is supplied with a bacterial synthesized compound, or when PGPR otherwise facilitates plant uptake of soil nutrients. Merriman [51] reported the use of *S. griseus* for seed treatment of barley, oat, wheat, and carrot to increase their growth. The isolate was originally selected for the biological control of *Rhizoctonia solani*. It has been reported that *Streptomyces pulcher*, *Streptomyces canescens*, and *Streptomyces citreofluores* were used in the control of bacterial, *Fusarium*, and *Verticillium* wilts, early blight, and bacterial canker of tomato.

Like most rhizobacteria, it seems highly probable that streptomycetes are capable of directly enhancing plant growth.

2.14. Phytohormone production

Manulis et al. [52] described plant hormone production, including indole-3-acetic acid (IAA), as well as the underlying pathways of synthesis by a variety of *Streptomyces* spp. (*Streptomyces violaceus*, *Streptomyces scabies*, *S. griseus*, *Streptomyces exfoliatus*, *Streptomyces coelicolor*, and *S. lividans*),

***Streptomyces* strain**	**Odor type**	**Secondary metabolite**
Streptomyces sp.	Earthy	Trans-1,10-dimethyl-trans-9-decalol (geosmin)
	Musty	1,2,7,7-Tetramethyl-2-norbornanol
	Potato-like	2-Isobutyl-3-methoxypyrazine or 2-isopropyl-3-methoxypyrazine

Table 7. Odor-producing compounds from Actinobacteria.

since earlier works have studied the IAA synthesis process in *Streptomyces* spp. This was the first investigation confirming IAA production according to new analytical methods, i.e. high-performance liquid chromatography and gas chromatography–mass spectrometry. Furthermore, Manulis et al. [53] described well the biosynthetic pathways of IAA in *Streptomyces.* On the other hand, Aldesuquy et al. [54] studied the effect of streptomycetes culture filtrates on wheat growth, showing a subesquent significant increase in shoot fresh mass, dry mass, length, and diameter statistically exhibited with some bacterial strains at different sample times. *Streptomyces olivaceoviridis* revealed a remarkable effect on yield components (spikelet number, spike length, and fresh and dry mass of the developing grain) of wheat plants. This activity may result from the increase in phytohormone bioavailability defined as PGPR produced, since all PGPR strains (*Streptomyces rimosus*, *Streptomyces rochei*, and *S. olivaceoviridis*) produce significant amounts of auxins (IAA), gibberellins, and cytokinins.

2.15. Biolarvicides

Dhanasekaran et al. [55] obtained that the isolates *Streptomyces* sp., *Streptosporangium* sp., and *Micropolyspora* sp. presented with great larvicidal activity against *Anopheles* mosquito larvae. Rajesh et al. [56] prepared silver nanoparticles from *Streptomyces* sp. GRD cell filtrate and found remarkable larvicidal activity against *Aedes* and *Culex* vectors, causing transmission of dengue and filariasis. In addition, studies carried out on the larvicidal effect of Actinobacterial extracts against *Culex* larvae have shown that a concentration of 1000 ppm of the isolate *Streptomyces* sp. appeared as KA13-3 with 100% mortality and KA25-A with 90% mortality. Other secondary metabolites obtained from Actinobacteria (tetranectin [56], avermectins [57], macrotetrolides [58], and flavonoids [59]) are classified as toxic to mosquitoes.

2.16. Odor and flavor compound production

The work carried out by Gaines and Collins [60] on the metabolites of *Streptomyces odorifer* led them to conclude that the earthy odor is likely due to a combination of trivial compounds (acetic acid, acetaldehyde, ethyl alcohol, isobutyl alcohol, isobutyl acetate, and ammonia). Consequently, other components contributing to the odor could also be produced. Several odor-producing compounds have been defined from Actinobacteria (**Table 7**). Earthy odors in sufficiently treated water supplies led to considerable interest from consumers, who may classify water with these odors as harmful for human drinking needs. These odors are the second most common cause of odor problems recorded by water utilities, behind chlorine.

3. Metabolic pathways in the production of secondary metabolites of bacteria

Secondary metabolic pathway reactions are formed by an individual enzyme or multienzyme complexes. Intermediate or end products of primary metabolic pathways are channeled from their systematic metabolic pathways that lead to the synthesis of secondary metabolites. There are six known pathways: the peptide pathway, the PKS pathway, the NRPS pathway, the hybrid (nonribosomal polyketide) synthetic pathway, the shikimate pathway, the β-lactam synthetic pathway, and the carbohydrate pathway. The genes encoding these synthetic pathway enzymes are generally present in chromosomal DNA and are often arranged in clusters.

3.1. Nonribosomal peptide synthesis pathways

Nonribosomal peptides are peptides that are not synthesized at the level of ribosomes. One of the peculiarities of nonribosomal peptides is their small size. These peptides are not encoded by a gene, and they are not limited to the 20 basic amino acids. Indeed, the peculiarity of the NRPS system is the ability to synthesize peptides containing proteinogenic and nonproteinogenic amino acids. In many cases, these enzymes are activated in collaboration with polyketone synthases giving hybrid products. The products of these multifunctional enzymes have a broad spectrum of biological activities, and some of them have been useful for medicine, agriculture, and biological research [61].

NRPS are organized in a modular way. Each module is responsible for the incorporation of a specific monomer. The modules are subdivided into domains, and each domain catalyzes a specific reaction in the incorporation of a monomer. The number and order of modules and the type of domain present in the modules of each NRPS determine the structural variation of synthesized peptides by dictating the number, order, and choice of amino acid to incorporate during elongation. Four main areas are needed for complete synthesis (**Figure 2**). Each domain has a specific function when incorporating the monomer. Domain A, from 500 to 600 amino acid residues, is necessary for the recognition of the amino acid and its activation.

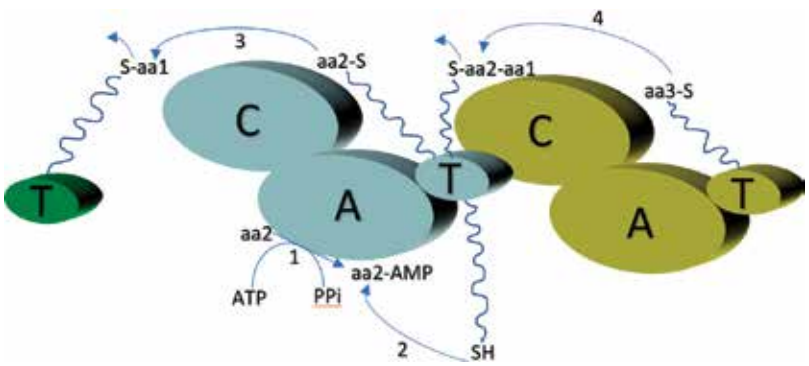

Figure 2. Minimum domains required in an NRPS [62].

The 80–100 amino acid residues of domain T, located downstream of domain A, form a thioester bond (covalent bond) between the activated monomer and the NRPS, and this allows the peptide being synthesized to remain attached to the NRPS throughout the process of elongation. The condensation domain C (450 amino acids) is usually found after each A–T module and catalyzes the formation of peptide bonds between bound residues on two adjacent modules. In general, the number and order of modules present in an NRPS determine the length and the resulting nonribosomal peptide structure. The thioesterase domain, present only in the last module, releases the peptide from the NRPS.

3.2. Polyketide synthase pathways

Polyketides are kown as natural products, having diverse functions in medical applications, and they are assembled by PKS enzymes. PKS enzymes act exactly like fatty acid synthase to generate a diverse extent of polyketides. Also, PKS enzymes start the polyketide assembly by priming the initiator molecule to the catalytic residue, and then making an extender unit for the elongation chain. On the basis of structural architecture and variation in enzymatic mechanism, PKS enzymes have been classified into three types: (1) type I PKS, (2) type II PKS, and (3) type III PKS.

This section describes all three types of PKS enzymes (**Table 8**). Modular PKSs include active sites, called modules; they are polypeptides used to synthesize a string of carbon. The active sites of each module are used only once during assembly of the molecule and determine the choice of units of structure and the level of reduction or dehydration for the cycle of expansion. They catalyze the length of the string of carbon, and the number of cycles of reaction is determined by the number and order of the modules in the polypeptide constituting the PKS [63].

3.2.1. Type I PKS

These are multidomain proteins (containing several domain enzymes on the same polypeptide) that can be modular (**Figure 3**), for example, the modular systems responsible for the synthesis of macrolides (erythromycin, rapamycin, rifamycin B, etc.) in bacteria, which is iterative (**Figure 4**) (for example, lovastatin nonaketide).

Either modular PKS or type I	Either discrete PKS or type II	Either ketosynthase polyketide or type III
Many functional enzymes organized into modules. Each module has a specific function and use; acyl carrier protein (ACP) domain activates acyl-CoA substrates malonyl-CoA or methylmalonyl-CoA or ethylmalonyl-CoA, an extender unit	Includes a series of modular heterodimeric enzymes. Each enzyme has a special function and use; the ACP domain transfers activated acyl-CoA substrate malonyl-CoA, an extender unit	The homodimeric ketosynthase enzyme can carry out various biochemical reactions at a single active site; it acts in the absence of ACP or directly recognizes the acyl-CoA molecules malonyl-CoA or methylmalonyl-CoA, an extender unit

Table 8. Classification of polyketide synthase enzymes and the functional and mechanistic differences between them.

Figure 3. Structure of a modular type I PKS [64]. Note: KS, ketosynthase; AT, acyl transferase; KR, ketoreductase; ACP, acyl carrier protein; TE, Thioesterase; DH, dehydrate.

3.2.2. Type II PKS

These are monofunctional protein complexes (for example, actinorhodin from *S. coelicolor*). These PKSs catalyze the formation of compounds that require aromatization and cyclization steps but no reduction or dehydration. These PKSs are involved in the biosynthesis of aromatic bacterial products such as actinorhodin, tetracenomycin, and doxorubicin [66].

3.2.3. Type III PKS

These have a single active site to catalyze the extension of the polyketide chain and cyclization without the use of an ACP (**Figure 5**). They are responsible for the synthesis of chalcones and stilbenes in plants, as well as polyhydroxy phenols in bacteria. Chalcone synthases are small

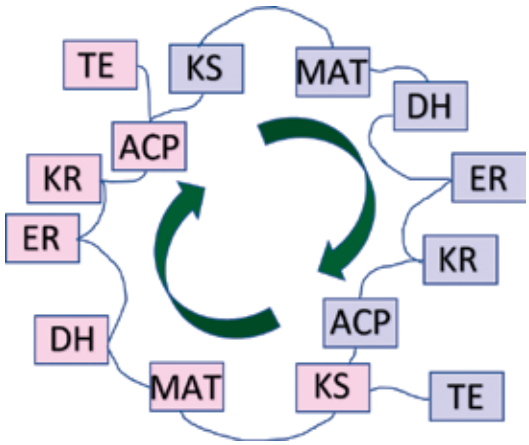

Figure 4. Structure of an iterative type I PKS [65].

http://dx.doi.org/10.5772/intechopen.79890

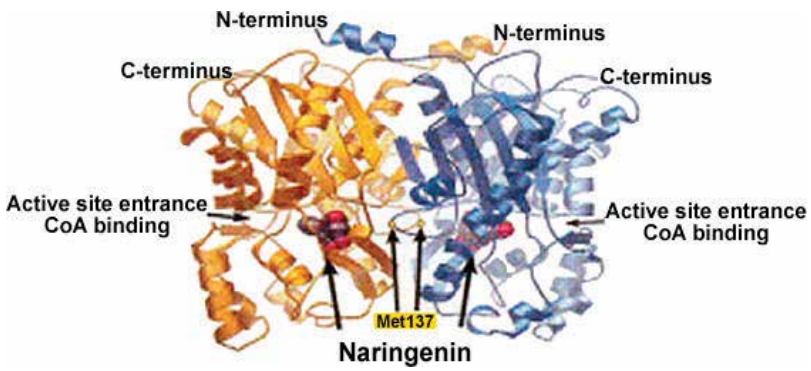

Figure 5. Type III PKS [68].

proteins with a unique polypeptide chain, and are involved in the biosynthesis of flavonoid precursors [67].

The shikimate pathway groups the essential building blocks for a large assembly of aromatic metabolites and amino acids. Metabolites of the aromatic compounds present protection against ultraviolet radiation, electron transport, and signaling molecules, and also act as antibacterial agents. The shikimate pathway enzymes use specific chemical substrates, i.e. erythrose-4-phosphate and phosphoenol pyruvate (primary metabolites), to start the synthesis of aromatic building blocks. Herein, the first seven enzymes catalyze the chemical reactions in a chronological manner to produce chorismate. Two bacterial enzymes are able to transfer a complete enolpyruvoyl moiety to a metabolic pathway. 5-Enolpyruvoyl shikimate 3-phosphate synthase is considered one of the shikimate pathways. Chorismate synthase is an enzyme involved in this pathway, and its function needs the presence of a reduced cofactor, flavin mononucleotide, for its activation [69].

The Gram-positive, filamentous *Streptomyces venezuelae* (soil bacterium) and other actinomycetes gather chloramphenicol with the help of aromatic precursors. Aromatic building blocks originated from the shikimate pathway act as precursors for the phenylpropanoid unit of chloramphenicol. First, chorismic acid branches out from the shikimate pathway to produce *p*-aminophenylalanine, which could afterwards be converted into a *p*-nitrophenylserinol component by an enzymatic reaction. 4-Amino-4-deoxychorismic acid (ADC) was found as a common precursor for both para-aminobenzoic acid and PAPA: a flexible tool for identifying pleiotropic pathways using genome-wide association study summaries pathways. The genetic map reveals that pabAB genes encode enzymes for ADC biosynthesis that are clustered in a distinct region of the *S. venezuelae* chromosome. Echinosporin isolated from *Saccharopolyspora erythraea* has antibacterial and anticancer activities. This molecule has a sole tricyclic acetal-lactone structure, and the main structure does not show its biosynthetic pathway. The shikimate pathway intermediate is guided to group the echinosporin by enzymatic reactions [70].

3.3. Lactam ring synthetic pathways

Cephalosporins belong to the family of β-lactam antibiotics, used for treating bacterial infections for more than 40 years. Interestingly, Gram-positive bacteria, Gram-negative bacteria,

and fungi are the major sources of β-lactam antibiotics. The Gram-positive *Streptomyces clavuligerus* is able to produce both clavulanic acid and cephamycin, since the Gram-negative bacterium *Lysobacter lactamgenus* produces cephabacins. Two hypotheses have been put forward for β-lactam biosynthesis: (1) horizontal gene transfer (HGT) from bacteria to fungi and (2) vertical descent (originated from a common ancestor). Bioinformatics, genetic designs, and sequence identity are more beneficial in HGT.

The production of β-lactam antibiotic occurs through three different steps: prebiosynthetic steps, intermediate formation steps, and late steps (also known as decorating steps) [71–76]. The biosynthesis of building blocks for β-lactam consist of L-α-aminoadipic acid, L-cysteine, and L-valine. L-α-Aminoadipic acid is not a proteinogenic amino acid formed from L-lysine. The actinomycete lysine 6-aminotransferase converts L-lysine into L-α-aminoadipic acid.

The two starting enzyme reactions are omnipresent in fungi and cephalosporin biosynthesis. D-(L-Aminoadipyl)-L-cysteinyl-D-valine synthase is the first enzyme, using all three amino acids gathered into a tripeptide through condensation reaction. This enzyme is NRPS encoded by the acvA (pcbAB) gene. The next step is the synthesis of a bicyclic ring (a four-member β-ring is fused with a five-member thiazolidine ring) through an oxidative reaction, catalyzed by isopencillin N-synthase, and results in the formation of isopenicillin N. Cephalosporin–cephamycin biosynthesis is the development of the five-member thiazolidine ring into a six-member dihydrothiazine ring. Several enzymes consecutively contribute to this ring conversion. β--Lactam biosynthesis is synthesized by a gene, which is usually clustered in the DNA of all reproducing bacteria. Bacterial species capable of producing β--lactam antibiotics exhibit an ecological benefit. In contrast, β-lactam–producing bacteria show low sensitivity to β-lactams on their own, or they have evolved to inactivate β-lactam antibiotics by β-lactamase enzymes.

4. Conclusion

Streptomyces are able to produce a number of antibiotics and other important pharmaceutical drugs to treat infections caused by bacteria and fungi, cancer, and heart-related diseases. Bacterial species reveal a complex lifecycle with physiological and biochemical adaptability, along with the ability to synthesize a large variety of secondary metabolites, presenting complex structures following different metabolic pathways. Understanding the secondary metabolite biosynthesis and pathways would lead to progress in combinatorial biosynthesis in the pharmaceutical and biotechnology industries.

Acknowledgements

We thank Miss Susan Ann Hill for technical assistance and for her useful contribution to the English manuscript checking.

Conflict of interest

The authors declare that no conflicting interest exists.

Author details

Mohammed Harir[1,2]*, Hamdi Bendif[2], Miloud Bellahcene[3], Zohra Fortas[1] and Rebecca Pogni[4]

*Address all correspondence to: mohamedharir31@gmail.com

1 Biology of Microorganisms and Biotechnology Laboratory, University of Oran 1 Ahmed Ben Bella, Oran, Algeria

2 Department of Natural and Life Sciences, Faculty of Sciences, Mohamed Boudiaf University, M'sila, Algeria

3 Department of Natural and Life Sciences, Institute of Sciences, University Center of Ain Temouchent, Temouchent, Algeria

4 Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Siena, Italy

References

[1] Baltz R. Antimicrobials from actinomycetes: Back to the future. Microbe - American Society for Microbiology. 2007;**2**(3):125-131

[2] Selvakumar JN, Chandrasekaran SD, Vaithilingam M. Bio prospecting of marine-derived *Streptomyces spectabilis* VITJS10 and exploring its cytotoxicity against human liver cancer cell lines. Pharmacognosy Magazine. 2015;**11**(44):469. DOI: 10.4103/0973-1296.168974

[3] Seipke RF, Barke J, Brearley C, Hill L, Yu DW, Goss RJM, et al. A single Streptomyces symbiont makes multiple antifungals to support the fungus farming ant *Acromyrmex octospinosus*. PLoS One. 2011;**6**(8):e22028. DOI: 10.1371/journal.pone.0022028

[4] Claessen D, de Jong W, Dijkhuizen L, Wösten HAB. Regulation of Streptomyces development: Reach for the sky! Trends in Microbiology. 2006;**14**(7):313-319. DOI: 10.1016/j.tim.2006.05.008

[5] Granozzi C, Billetta R, Passantino R, Sollazzo M, Puglia AM. A breakdown in macromolecular synthesis preceding differentiation in *Streptomyces coelicolor* A3 (2). Journal of General Microbiology. 1990;**136**(4):713-716. DOI: 10.1099/00221287-136-4-713

[6] Zitouni A. Taxonomic study and antagonistic properties of Nocardiopsis and Saccharothrix isolated from Saharan soil and production of new antibiotics by Saccharothrix sp. 103 p.

[PhD Thesis, specialty: Microbiology]. Tizi Ouzou: University Mouloud Mammeri; 2005. p. 230

[7] Badji B. Etude de la taxonomie et des antibiotiques antifongiques de trois souches d'actinomycètes d'origine saharienne appartenant aux genres Actinomadura et Nonomuraea. Thèse de Doctorat en Microbiologie. Tizi Ouzou, Algerie: Université de Mouloud Mammeri; 2006. p. 226

[8] Clardy J, Fischbach MA, Currie CR. The natural history of antibiotics. Curr. Biol. 2009; **19**(11):R434-R437

[9] Berdy J. Bioactive microbial metabolites and antibiotics. The Journal of Antibiotics. 2005; **58**:1-26

[10] Manteca A, Alvarez R, Salazar N, Yague P, Sanchez J. Mycelium differentiation and antibiotic production in submerged culture of *Streptomyces coelicolor*. Applied and Environmental Microbiology. 2008;**74**:3877-3886

[11] Getha K, Vikineswary S, Wong WH, Seki T, Ward A, Goodfellow M. Evaluation of *Streptomyces* sp. strain g10 for suppression of Fusarium wilt and rhizosphere colonization in pot-grown banana plantlets. Journal of Microbiology and Biotechnology. 2005;**32**:24-32

[12] Dastager GS, Agasar D, Pandey A. Production and partial purification of amylase from a novel isolate *Streptomyces gulbargensis*. Microbial Biotechnology. 2009;**36**:189-194

[13] Oskay M. Antifungal and antibacterial compound from Streptomyces strains. African Journal of Biotechnology. 2009;**8**(13):3007-3017

[14] Kang MJ, Strap JL, Crawford DL. Isolation and characterization of potent antifungal strains of the *Streptomyces violaceusniger* clade active against Candida albinos. Journal of Industrial Microbiology and Biotechnology. 2010;**37**:35-41

[15] Watve MG, Tickoo R, Jog MM, Bhole BD. How many antibiotics are produced by the genus Streptomyces? Archives of Microbiology. 2001;**176**:386-390

[16] Hwang BK, Lim SW, Kim BS, Lee JY, Moon SS. Isolation and in vivo and in vitro antifungal activity of phenyl acetic acid and sodium phenyl acetate from *Streptomyces humidus*. Applied and Environmental Microbiology. 2001;**67**:3730-3745

[17] Kurtboke DI. Biodiscovery from rare actinomycetes: An eco-taxonomical perspective. Applied Microbiology and Biotechnology. 2012;**93**(5):1843-1852

[18] Dietera A, Hamm A, Fiedler HP, Goodfellow M, Muller WE, Brun R, et al. Pyrocoll, an antibiotic, antiparasitic and antitumor compound produced by a novel alkaliphilic Streptomyces strain. The Journal of Antibiotics. 2003;**56**:639-646

[19] Hopwood DA. Forty years of genetics with Streptomyces: From in vivo through in vitro to in silico (review article). Microbiology. 1999;**145**:2183-2202

[20] Petrosyan P, Gartia-Varela M, Luz-Madrigal A, Huitron C, Flores ME. *Streptomyces mexicanus*, a xylanolytic microorganism isolated from soil. International Journal of Systematic and Evolutionary Microbiology. 2003;**53**:269-273

[21] Nascimento RP, Coelho RRR, Marques S, Alves L, Girio FM, Bon EPS, et al. Production and partial characterization of xylanase from *Streptomyces* sp. strain AMT-3 isolated from Brazilian Cerrado soil. Enzyme and Microbial Technology. 2002;**31**:549-555

[22] Vonothini G, Murugan M, Sivakumar K, Sudha S. Optimization of protease production by an actinomycete strain PS-18A isolated from an estuarine shrimp pond. African Journal of Biotechnology. 2008;**7**(18):3225-3230

[23] Syed DG, Dayanand A, Pandey A. Production and partial purification of amylase from a novel isolate *Streptomyces gulbargensis*. Journal of Industrial Microbiology & Biotechnology. 2009;**36**:189-194

[24] Marinelli F. Antibiotics and Streptomyces: The future and antibiotic discovery. Microbiology Today. 2009;**2**:20-23

[25] Madigan MT, Martinko JM. Biologie des microorganismes. 11e éd. ed. Pearson Education France; 2007. pp. 331-423, 686-718

[26] Prescott LM, Harley JP, Klein DA. Microbiologie. Bruxelle: De Boek & Larcier; 2007. pp. 805-825

[27] Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, et al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature. 2002;**417**(6885):141-147

[28] Ikeda H, Ishikawa J, Hanamoto A, Shinose M, Kikuchi H, Shiba T, et al. Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. Nature Biotechnology. 2003;**21**(5):526-531

[29] Laskaris P, Tolba S, CalvoBado L, Wellington L. Coevolution of antibiotic production and counter-resistance in soil bacteria. Environmental Microbiology. 2010;**12**(3):783-796

[30] Schatz A, Bugie E, Waksman SA, Hanssen AD, Patel R, Osmon DR. The classic: Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. Clinical Orthopaedics and Related Research. 2005;**437**:3-6

[31] Hiramatsu A, Ouchi T. On the proteolytic enzymes from the commercial protease preparation of *Streptomyces griseus* (Pronase P). Biochemistry. 1963;**54**:462-464

[32] Phadatare SU, Deshpande VV, Srinivasan MC. High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents. Enzyme Microbe Technol. 1993;**15**:72-76

[33] Dejong PJ. L-Asparaginase production by *Streptomyces griseus*. Applied Microbiology. 1972;**23**(6):1163-1164

[34] Narayana KJ, Kumar KG, Vijayalakshmi M. L-Asparaginase production by *Streptomyces albidoflavus*. Indian Journal of Microbiology. 2008;**48**(3):331-336

[35] You J, Xue X, Cao L, Lu X, Wang J, Zhang L, et al. Inhibition of Vibrio biofilm formation by a marine actinomycete strain A66. Applied Microbiology and Biotechnology. 2007; **76**(5):1137-1144

[36] Latha S, Vinothini G, Calvin DJ, Dhanasekaran D. In vitro probiotic profile based selection of indigenous Actinobacterial probiotic *Streptomyces* sp. JD9 for enhanced broiler production. Journal of Bioscience and Bioengineering. 2016;**121**(1):124-131

[37] Garcia-Bernal M, Medina-Marrero R, Campa-Cordova AI, Mazon-Suastegui JM. Probiotic effect of Streptomyces strains alone or in combination with Bacillus and Lactobacillus in juveniles of the white shrimp *Litopenaeus vannamei*. Aquaculture International. 2017; **25**:927-939

[38] Dharmaraj S, Dhevendaran K. Evaluation of Streptomyces as a probiotic feed for the growth of ornamental fish *Xiphophorus helleri*. Food Technology and Biotechnology. 2010;**48**:497-504

[39] Garcia-Bernall M, Medina-Marrerol R, Rodriguez-Jaramillo C, Marrero-Chang O, Campa-Cordova AI, Medina-Garcia R, et al. Probiotic effect of *Streptomyces* spp. on shrimp (*Litopenaeus vannamei*) post larvae challenged with *Vibrio parahaemolyticus*. Aquaculture Nutrition. 2017:1-7

[40] Yagi Y, Kessler RE, Shaw JH, Lopatin DE, An F, Clewell DB. Plasmid content of *Streptococcus faecaloid* strain 39-5 and identification of a pheromone (cPD1)-induced surface antigen. Journal of General Microbiology. 1983;**129**(4):1207-1215

[41] Schachtsiek M, Hammes WP, Hertel C. Characterization of *Lactobacillus coryniformis* DSM 20001T surface protein Cpl mediating coaggregation with and aggregation among pathogens. Applied and Environmental Microbiology. 2004;**70**(12):7078-7085

[42] Henkel M, Muller MM, Kugler JH, Lovaglio RB, Contiero J, Syldatk C, et al. Rhamnolipids as bio surfactants from renewable resources: Concepts for next-generation rhamnolipid production. Process Biochem. 2012;**47**(8):1207-1219

[43] Marchant R, Banat IM. Microbial bio surfactants: Challenges and opportunities for future exploitation. Trends in Biotechnology. 2012;**30**(11):558-565

[44] Rickes EL, Brink NG, Koniuszy FR, Wood TR, Folkers K. Crystalline vitamin B12. Science. 1948;**107**(2781):396-397

[45] Lichtman H, Watson J, Ginsberg V, Pierce JV, Stokstad EL, Jukes TH. Vitamin B12b: Some properties and its therapeutic use. Experimental Biology and Medicine. 1949;**72**(3):643-645

[46] Sanscartier D, Zeeb B, Koch I, Reimer K. Bioremediation of diesel-contaminated soil by heated and humidified bio pile system in cold climates. Cold Regions Science and Technology. 2009;**55**(1):167-173

[47] Radwan SS, Barabás G, Sorkhoh NA, Damjanovich S, Szabo I, Szollosi J, et al. Hydrocarbon uptake by Streptomyces. FEMS Microbiology Letters. 1998;**169**(1):87-94

[48] Barabas G, Vargha G, Szabo IM, Penyige A, Damjanovich S, Szollosi J, et al. n-Alkane uptake and utilisation by Streptomyces strains. Antonie van Leeuwenhoek. 2001;**79**(3-4):269-276

[49] Mason MG, Ball AS, Reeder BJ, Silkstone G, Nicholls P, Wilson MT. Extracellular heme peroxidases in actinomycetes: A case of mistaken identity. Applied and Environmental Microbiology. 2001;**67**(10):4512-4519

[50] Schutze E, Klose M, Merten D, Nietzsche S, Senftleben D, Roth M, et al. Growth of streptomycetes in soil and their impact on bioremediation. Journal of Hazardous Materials. 2014;**267**:128-135

[51] Polti MA, Garcia RO, Amoroso MJ, Abate CM. Bioremediation of chromium (VI) contaminated soil by *Streptomyces* sp. MCI. Journal of Basic Microbiology. 2009;**49**(3):285-292

[52] Attwa AI, EI Awady ME. Bioremediation of zinc by Streptomyces aureofacienes. Journal of Applied Sciences. 2011;**11**(5):87

[53] Dimkpa CO et al. Involvement of siderophores in the reduction of metal-included of auxin synthesis in *Streptomyces* spp. Chemosphere. 2008;**74**(1):19-25

[54] Gilis A et al. Effect of the siderophore alcaligin E on the bioavailability of Cd to Alcaligenes eutrophus CH34. Journal of Industrial Microbiology. 1998;**20**(1):61-68

[55] Fuentes MS, Benimeli CS, Cuozzo SA, Amoroso MJ. Isolation of pesticide-degrading actinomycetes from a contaminated site: Bacterial growth, removal and dechlorinating of organochlorine pesticides. International Biodeterioration & Biodegradation. 2010; **64**:434-441

[56] Behal V. Bioactive products from Streptomyces. Advances in Applied Microbiology. 2000; **47**:113-156

[57] Tanaka Y, Omura S. Agro active compounds of microbial origin. Annual Review of Micro biology. 1993;**47**(1):57-87

[58] Umezawa H, Okami Y, Hashimoto T, Suhara Y, Hamada M, Takeuchi T. A new antibiotic, kasugsmycin. The Journal of Antibiotics. 1965;**18**:101-103

[59] Ikeda H, Omura S. Control of avermectin biosynthesis in *Streptomyces avermitilis* for the selective production of a useful component. The Journal of Antibiotics. 1995;**48**(7):549-562

[60] Merriman PR, Price RD, Kollmorgen JF, Piggott T, Ridge EH. Effect of seed inoculation with *Bacillus subtilis* and *Streptomyces griseus* on the growth of cereals and carrots. Crop & Pasture Science. 1974;**25**(2):219-226

[61] Manulis S, Shafrir H, Epstein E, Lichter A, Barash I. Biosynthesis of indole-3-acetic acid via the indole-3-acetamide pathway in *Streptomyces* spp. Microbiology. 1994;**140**(5):1045-1050

[62] Aldesuquy HS, Mansour FA, Abo-Hamed SA. Effect of the culture filtrates of Streptomyces on growth and productivity of wheat plants. Folia Microbiologica. 1998;**43**(5):465-470

[63] Dhanasekaran D, Sakthi V, Thajuddin N, Panneerselvam A. Preliminary evaluation of Anopheles mosquito larvicidal efficacy of mangrove Actinobacteria. International Journal of Applied Biology and Pharmaceutical Technology. 2010;**1**(2):374-381

[64] Rajesh K, Dhanasekaran D, Tyagi BK. Mosquito survey and larvicidal activity of Actin bacterial isolates against Culex larvae (Diptera: Culicidae). Journal of the Saudi Society of Agricultural Sciences. 2013;**14**(2):116-122

[65] Ando K. How to discover new antibiotics for insecticidal use. In: Takahashi et al., editors. Natural Products: Proceedings of the 5th International Congress of Pesticide Chemistry. Kyoto, Japan: Elsevier; 1982. p. 253

[66] Pampiglione S, Majori G, Petrangeli G, Romi R. Avermectins, MK-933 and MK-936, for mosquito control. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1985;**79**(6):797-799

[67] Zizka Z, Weiser J, Blumauerova M, Jizba J. Ultrastructural effects of macrotetrolides of *Streptomyces griseus* LKS-1 in tissues of *Culex pipiens* larvae. Cytobios. 1988;**233**:85-91

[68] Rao KV, Chattopadhyay SK, Reddy GC. Flavonoids with mosquito larval toxicity. Journal of Agricultural and Food Chemistry. 1990;**38**(6):1427-1430

[69] Gaines HD, Collins RP. Volatile substances produced by Streptomyces odoriferous. Lloydia. 1963;**26**(4):247

[70] Schwarzer D, Marahiel MA. Multimodular biocatalysts for natural product assembly. Die Naturwissenschaften. 2001;**88**:93-101

[71] Bacha N. Caractérisation des polycetones synthases intervenant dans la biosynthese d' ochratoxine A, d'acide penicillium, d'aspe lactone et d'isoasperlactone chez Aspergillus westerdijkiae [Thèse de doctorat]. France: Institut National Polytechnique de Toulouse; 2009. p. 236

[72] McDaniel R, Thamchaipenet A, Gustafson C, Fu H, Betlach M, Betlach M, et al. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. Proceedings of the National Academy of Sciences. 1999;**96**(5):1846-1851

[73] Davis NK, Chater KF. Spore colour in *Streptomyces coelicolor* A3 (2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. Molecular Microbiology. 1990;**4**(10):1679-1691

[74] Austin MB, Noel JP. The chaconne synthase superfamily of type III polyketide synthases. Natural Product Reports. 2003;**20**(1):79-110

[75] Khosla C, Gokhale RS, Jacobsen JR, Cane DE. Tolerance and specificity of polyketide synthases. Annual Review of Biochemistry. 1999;**68**(1):219-253

[76] Gokulan K, Khare S, Cerniglia C. Metabolic pathways: Production of secondary metabolites of bacteria. In: Batt CA, Tortorello ML, editors. Encyclopedia of Food Microbiology. Vol 2. Elsevier Ltd, Academic Press; 2014. pp. 561-569. ISBN: 9780123847300

Edited by Shymaa Enany

Actinobacteria have an extensive bioactive secondary metabolism and produce a huge amount of naturally derived antibiotics, as well as many anticancer, anthelmintic, and antifungal compounds. These bacteria are of major importance for biotechnology, medicine, and agriculture. In this book, we present the experience of worldwide specialists in the field of Actinobacteria, exploring their current knowledge and future prospects.

Published in London, UK

IntechOpen

ISBN 978-1-78984-615-7