Rapid and sensitive detection of all members of the *M. tuberculosis* and *M. avium* complex by real-time PCR

by Dr. Sibylle Münz and Dr. Sven Cramer

Tuberculosis is still one of the most wide-spread and serious infectious diseases worldwide. In addition to the *Mycobacterium tuberculosis* complex, non-tuberculous mycobacteria, such as the *M. avium* complex, play a significant role as causative agents of opportunistic infections in immunocompromised patients. This article introduces a new diagnostic real-time PCR test for the rapid and reliable detection of the members of the *M. tuberculosis* complex as well as those of the *M. avium* complex.

Tuberculosis is the bacterial disease with the highest mortality rate worldwide [1]. It is estimated that one third of the world’s population is infected with *Mycobacterium tuberculosis*. About eight million new infections and two million deaths are reported each year [2]. The number of tuberculosis cases is rising [3], and in recent years tuberculosis has re-emerged as a serious global health problem due to the spread of drug-resistant strains [4, 5] and the increased number of HIV-infected patients [6, 7, 8] with opportunistic mycobacterial infections.

In humans the tuberculosis can be caused by all members of the *M. tuberculosis* complex except for *M. bovis* BCG, which is used for vaccination against tuberculosis. Members of the complex include *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. microti* and *M. canetti*. Transmission of the bacteria occurs via aerosols. Only persons with active tuberculosis are contagious; they spread the bacteria by coughing, sneezing or talking. In the primary stage tuberculosis mainly affects parts of the lung and the associated lymph nodes. Depending on the immune status of the patient, however, mycobacteria can colonise the entire lung and other organs [3]. Nearly 9% of patients infected with *M. tuberculosis* develop an acute tuberculosis in the first two years after infection [6]. In the remaining patients the immune system is able to encapsulate the bacteria. The result is a "latent" infection, which can be reactivated at any time causing active tuberculosis.

Non-tuberculous mycobacteria
Non-tuberculous mycobacteria (NTM) or Mycobacteria Other Than Tuberculosis (MOTTs) are all mycobacterial species, apart from *M. leprae*, which do not belong to the *M. tuberculosis* complex. Most of these species are ubiquitous organisms which can be found in soil, water, food and animals. In immunocompetent subjects infections with NTMs are usually asymptomatic, whereas in immunocompromised patients they can cause disease. Especially in patients with AIDS the bacteria can disseminate, leading to various clinical symptoms. The non-tuberculous mycobacterial species which are most frequently associated with an infection are the members of the *M. avium* complex (*M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominisuis* and *M. intracellulare*), which primarily cause lung disease with symptoms similar to pulmonary tuberculosis.

Diagnostics
Traditional methods for the detection of mycobacteria, such as the acid-fast stain and culture, have either a low sensitivity and/or specificity, or take weeks before a definite result is available. The acid-fast stain is a very rapid diagnostic method, but has a low sensitivity, with a detection limit of approximately 10⁴ mycobacteria/mL. It also has a limited specificity and detects not only mycobacteria but also other acid-fast bacteria like corynebacteria or nocardia. Culture, which, due to its high sensitivity, is still the gold standard in tuberculosis diagnostics, can require up to eight weeks before a final result is available. Therefore a rapid diagnostic method is required which combines high sensitivity and high specificity. This is especially needed for patients shedding low amounts of mycobacteria. The most promising diagnostic tools to approach this problem are tests based on the polymerase chain reaction (PCR). Real-time PCR in particular offers the speed, sensitivity, and specificity required for a fast and reliable detection of mycobacteria.

Early detection of mycobacteria by real-time PCR allows the immediate initiation of drug therapy and the isolation of contagious patients, thus allowing optimal patient management and reducing medical costs. In addition, fast and sensitive detection of tuberculosis is very important in order to disrupt the chain of infection.

RealArt Mycobac. diff. LC PCR Kit
Based on these requirements, a ready to use real-time PCR test has been developed for the detection of all members of the *M. tuberculosis* complex as well as all members of the *M. avium* complex. The commercially available RealArt Mycobac. diff. LC PCR Kit can not only detect members of the *M. tuberculosis* complex but also distinguish them from the different *M. avium* subspecies and from *M. intracellulare* by melting curve analysis. The kit is adapted for the detection of mycobacterial DNA from sputum, bronchial secretion, bronchoalveolar lavage, CSF, stomach fluid and peritoneal puncture using the LightCycler instrument.

Contents of kit
The kit contains, in one master mix, all reagents and enzymes for the specific amplification and detection of a 163 bp region of the mycobacterial genome. In addition, two

Figure 1. Detection of the quantitation standards (QS 1 - 4, ranging from 5 x 10¹ to 5 x 10⁴ copies/µL) and positive controls (*M. avium* Control, *M. intracellulare* Control) in the fluorimeter channel F2/Back-F1 of the LightCycler Instrument. NTC: non-template control.
Kit specificity
High specificity is one of the essential requirements for the diagnosis of mycobacterial infections. On one hand it is important to detect all members of the *M. tuberculosis* complex since, apart from *M. bovis* BCG, all of them can cause tuberculosis. On the other hand the increased incidence of infections with NTMs in immunocompromised patients necessitates the specific detection of the *M. avium* complex, members of which belong to the most prevalent non-tuberculous mycobacteria. Furthermore, since tuberculosis and NTM infections are treated differently, it is necessary that the members of the *M. tuberculosis* complex be distinguished from non-tuberculous mycobacteria like the *M. avium* complex. Non-tuberculous mycobacteria are often resistant to conventional antibiotics, so a rapid and reliable identification of the causative organism is essential for optimal treatment of the patient.

The specificity of the kit is ensured first and foremost by the selection of the primers and probes, as well as by the stringent reaction conditions. To avoid cross-reactivity, primers and probes have been checked for possible homologies to other known sequences by sequence alignments. In addition to the sequence alignments a control group listed in Table 1 has been tested for cross-reactivity. None of the members of this control group has tested positive with the kit.

Kit sensitivity
The high sensitivity of the kit has been verified by a probit analysis, which revealed an analytical sensitivity of 1.5 copies/µL for the *M. tuberculosis* complex, 3.8 copies/µL for *M. avium* subspecies and 2.8 copies/µL for *M. intracellulare*.

Summary
There are still more people dying from mycobacterial infections than from any other bacterial disease. Rapid and reliable diagnosis is essential both for effective drug therapy, and to stop the spread of the disease by promptly isolating contagious patients. Detection of mycobacteria by real-time PCR offers a fast, sensitive and specific diagnostic tool which fulfils all concensus requirements for the diagnosis of mycobacterial infections.

References

The authors
Sibylle Münz, Ph.D
Product Manager,
Sven Cramer, Ph.D
Research and Development,
artus GmbH
Königstraße 4a
22767 Hamburg
Germany
Tel +49 40 413647-00
Fax +49 40 413647-10

Table 1. List of potentially cross-reactive pathogens tested with the RealArt Mycobac. diff. LC PCR Kit. None of these pathogens has tested positive with the kit.