VIEWPOINT

# A revaluation of the use of conventional Ziehl-Neelsen stain for detection of non-tuberculous mycobacteria

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## ABSTRACT

Detection of non-tuberculous mycobacteria is difficult and not always consistent. These are emerging as opportunistic pathogens in a number of clinical conditions, in part due to their remarkable stress intolerance. The Ziehl-Neelsen stain is optimal for the detection *of Mycobacterium tuberculosis* complex but is less reliable for the detection of non-tuberculous mycobacteria when using acid-alcohol as a decolouriser. Acid alcohol decolourisation should not be relied on where non-tuberculous mycobacteria are suspected, or the possibility of dormant forms of mycobacteria exists. In those instances other decolourisation methods should be considered, such 20-25% sulphuric acid or Gabbett's methylene blue.

**Keywords:** mycobacteria, Ziehl-Neelsen stain, non-tuberculous mycobacteria, mycobacteria staining methods, basic fuchsin.

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## PERSPECTIVE

The Ziehl-Neelsen stain has been used since 1883 for the detection of *Mycobacterium tuberculosis* complex. The stain in the present form consists of carbol fuchsin as the primary stain, acid-alcohol as the decolourising agent, and methylene blue as the counter-stain. For non-tuberculous mycobacteria it has been recommended that the decolourising agent should be a strong acid in water without addition of alcohol (1). *Mycobacterium tuberculosis* complex are considered to be acid-alcohol fast while non-tuberculous mycobacteria are considered to be acid-fast. The acid alcohol decolouriser is now the common Ziehl-Neelsen decolouriser in routine use in medical and research laboratories.

A clinical distinction is also made between *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria. The former is considered to be invariably obligate pathogenic when detected in humans, while non-tuberculous mycobacteria may be present without causing disease, and can be described as saprophytes, commensals, and symbionts (2).

### History of staining for Mycobacterium species

In 1882, Robert Koch announced the discovery of the causative organism of tuberculosis by application of a novel staining method (3). The procedure outlined in Koch's original publication was time consuming and difficult to reproduce. Within a year, Koch's original method had been supplanted by more rapid methods, the most notable example is the Ziehl-Neelsen stain.

As well as staining *Mycobacterium tuberculosis* complex, Koch found that his original method also stained *Mycobacterium leprae.* Koch used his stain on sectioned tissue, and it was left to Erlich to use his modification for the diagnosis of pulmonary TB through sputum examination. Erlich's modification, following soon after Koch's announcement, relied on basic fuchsin and aniline oil as the primary stain and the use of nitric acid as the decolourising agent (4). Erlich's modification was quickly adopted by Koch and other researchers (3).

In 1883 Ziehl described a modification that substituted Erlich's aniline oil and basic fuchsin with a primary stain containing basic fuchsin and phenol (carbolic acid) (4). Supplementing this, in 1883 Neelsen advocated the substitution of nitric acid as the decolouriser, with sulphuric acid. The concentration of phenol was increased, and the decolourising agent was improved with the addition of ethanol to the sulphuric acid (5). Thus the Ziehl-Neelson stain was born. Neelsen published the method for the Ziehl-Neelsen stain in a small book he compiled for anatomical pathologists (6) and records of the modifications exist in a paper written by Johne, (discoverer of Johne's disease, an inflammatory bowel disease in ruminants) recalling his discussions with Neelsen (7,8).

By 1892 the Ziehl-Neelsen stain was widely used and accepted as the "gold standard" for the diagnosis of tuberculosis. Since then there have been numerous modifications of the Ziehl-Neelsen stain, mainly involving the concentration of carbol-fuchsin and the composition of the decolouriser. Henry Gabbett proposed the use of Gabbett's methylene blue, which contained acid, ethanol and methylene blue, resulting in a two step stain which was used for many years with success and is still used in some parts of the world (9) In 1915 Kinyoun described the "cold stain" for the detection of *Mycobacterium tuberculosis* complex. (10). By 1972 it was common knowledge that if non-tuberculous mycobacteria were to be reliably excluded, an oxidising decolouriser was required (2).

## DISCUSSION

For over 130 years, the Ziehl-Neelsen stain has been the primary method for the laboratory detection of the genus *Mycobacterium*. Over that time, the use of the Ziehl-Neelsen stain has been refined to suit cost-effectiveness, reliability and convenience in the medical laboratory. The Ziehl-Neelsen method used today is optimised for the detection of *Mycobacterium tuberculosis* complex, the primary human mycobacterial pathogen.

The decolouriser, using acid-alcohol is optimal for this purpose, delivering stained smears that are simple to examine and interpret, but is not reliable for the detection of non-tuberculous mycobacteria. Several other methods, using strongly acidic decolourising methods are recommended for that purpose. The recommended decolouriser when looking for dormant forms of mycobacteria and non-tuberculous mycobacteria is 20-25% sulphuric acid, which is a moderate oxidising agent. When mycobacteria are present in a dormant/

latent state, there is a reduced atmosphere. The reduced state will also occur in walled cavities containing *Mycobacterium tuberculosis* complex. Cell-wall-deficient mycobacteria in the dormant state will often also produce a positive Ziehl-Neelsen stain when the preparation is oxidised prior or during staining.

Medical laboratories are encountering increasing numbers of non-tuberculous mycobacteria in pathogenic roles including in opportunistic clinical infections, Latent, or dormant variants of the genus *Mycobacterium* are also thought to be responsible for recurrence of tuberculosis in treated patients and are increasingly linked to sarcoidosis, inflammatory bowel disease and similar 'autoimmune' diseases (11-13). Some researchers, using the acid-fast decolouriser, have reported identifying presumptive cell-wall-deficient mycobacteria present in blood cultures from patients presenting with these diseases(14).

We believe that, where non-tuberculous mycobacteria are expected, it is not sufficient to use the acid-alcohol decolourising step on the Ziehl-Neelsen stain. This may be particularly relevant where automated liquid culture-based detection methods of culture are used and cultures signaling positive growths are discarded based on the Ziehl-Neelsen stain alone.

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