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REVIEW ARTICLE

Acid-Fast Staining Revisited, a Dated but Versatile Means of Diagnosis

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Abstract:

Acid-fast staining has been utilized in the field of medicine since the late 1800s. It was, for a while, considered the gold standard for diagnosing leprosy and tuberculosis. Principally it uses 1 primary staining agent, carbol fuchsin; and methylene blue as a counter stain. The emergence of molecular-based diagnostic tests makes some of us question the relevance of acid-fast staining in daily practice. This literature review aimed to revisit the acid-fast staining method, its uses, and the modification that occurred throughout the years.

Keywords: Acid-fast staining, Ziehl-Neelsen, Kinyoun, Truant, Staining protocol, Bacteriological examination.

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1. INTRODUCTION

The discovery of tubercle bacillus of *Mycobacterium tuberculosis* was first described by Robert Koch in 1882. Even though Robert Koch was the first person to identify and isolate the *bacillus*, it was Paul Ehrlich who managed to demonstrate the acid-fast property of the said bacterium. Ehrlich did so by using aniline dyes to modify Koch's initial stain. Later in the 1890s, Franz Ziehl used carbolic acid (phenol) while Friedrich Neelsen changed the primary stain to basic fuchsin (later regarded as carbol-fuchsin), further modifying Ehrlich's method which was first used in 1882 [1]. This technique is what we now know as the Ziehl-Neelsen method. This method also utilized heat to help the primary stain to penetrate the waxy cell walls of the bacteria, hence also earning the "hot staining" nickname. It was later in 1915 that a "cold staining" technique was introduced, which uses a higher concentration of carbol-fuchsin in order to bypass the need to heat the slide while being stained.

Acid fastness is a property exhibited in some types of bacteria that gives them the ability to resist decolorization by acids during staining procedures. Bacteria that are known to have this property include *Mycobacterium sp.* and *Nocardia sp.* Upon microscopic examination, acid fast bacteria will appear red while non acid-fast bacteria will be stained blue or green due to the counterstains [2].

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Even though mostly acid fast staining is still primarily used in diagnosis, practice throughout the years has proven it to be useful in diagnosing other organisms exhibiting acid fastness, *i.e.* *Cryptosporidium sp.*, [3] *Isospora sp.*, [3] *Cyclospora sp.*, [4] *Taenia sp.*, [5, 6] Hydatid cysts [7] and bacterial endospores [8]. Therefore in this review, we aim to revisit the method, uses and modifications of acid-fast staining in current practice.

2. METHODS

Literature search was systematically carried out in the databases of National Library of Medicine (PUBMED), Medical Literature Analysis and Retrieval System Online (MEDLINE), SCOPUS and Web of Science. The search was done by entering the keywords: "acid-fast", "staining", "diagnosis", "method", "Ziehl-Neelsen", "Kinyoun", "Auramine-rhodamine", using the respective operators: "AND" and "OR". No specific timeframe was applied for the searches; we included articles that were published from inception until October 2021. We included case reports as well as reviews of various methods and usages of acid-fast staining.

Descriptive analysis was then performed to summarize the findings of each article regarding the following themes: "use of acid-fast staining in diagnosis" and "modification to the common staining technique".

3. RESULTS

3.1. The Staining Method

Acid-fast staining is a method that is widely used to detect *Mycobacterium sp.* as a means of screening, case finding, referral and treatment of tuberculosis in developing countries. The term acid-fast comes from the ability of the bacteria to retain the stain after being washed by acidic alcohol. The difference between acid-fast bacteria and non-acid-fast bacteria lies in the wax coating of their cell walls. This highly hydrophobic walls make them impermeable to other routine stains such as the gram stain. Heating the specimen, however, help dissolve the waxy coating hence enabling the stain to be absorbed by the cell wall.

Principally, acid-fast staining involves 3 main solutions: the primary stain, the decolorizing agent, and the counter stain. The primary stain used in ZN is mostly carbol-fuchsin. The decolorizing agent is usually acidic alcohol which is usually 3% HCl + 95% ethanol. And lastly, the counter stain, which is methylene blue.

The three common acid-fast staining methods are Ziehl-Neelsen (hot method), Kinyoun (cold), and Auramine-Rhodamine Fluorochrome (Truant method). Slides stained by both Ziehl-Neelsen and Kinyoun can be visualised using a standard bright-field microscope while the Truant method uses a fluorescent (ultraviolet) microscope.

The ZN method or otherwise known as the hot technique utilizes heat to help the primary stain penetrate the sample. For the ZN method, we need to prepare the carbol-fuchsin stain (0.3g of Basic Fuchsin, 10 ml of 95% ethanol, 5 ml of phenol, 95 ml of distilled water); the decolorizing agent (97ml of 95% ethanol, 3 ml of concentrated hydrochloric acid); and the Counterstain (0.3g of Methylene blue chloride, 100ml of distilled water).

The steps of the ZN method are as follows [9 - 11]:

1. An air dried smear is heat-fixed at 80°C for at least 15 minutes or for 2 hours on an electric hot plate at 65°C - 70°C.
2. A slide is placed with an air-dried and heat-fixed smear on a suitable staining device.
3. The slide is flooded with 0.3% Carbol-fuchsin.
4. The underside of the slide is carefully heated by passing a flame under the rack or by placing the slide on a hot plate until steam rises. We need to be careful not to let it boil as it may crack the slide and spatter the stain. The preparation is kept moist with stain and steaming for 5 minutes, and the heating is repeated as needed.
5. The slide is cooled down for 5 minutes before washing the slide in a gentle and indirect stream of tap water (or deionized water or distilled water) until no color appears in the effluent.
6. By holding the slide with forceps, it is washed with the decolorizing solvent followed by washing with tap water (or deionized water or distilled water), as above. The decolorizing and washing step is repeated until the stained smear appears faintly pink and the fluid washing off the slide runs clear.

7. The smear is flooded with the methylene blue counterstain for 20 to 30 seconds, and washed with tap water (or deionized water or distilled water), as above.

8. The smear is air-dried.
9. It is examined under oil immersion.

The Kinyoun method uses a more concentrated fuchsin and phenol for its primary stain, also known as the Kinyoun stain which is made by mixing 4 grams of basic fuchsin dissolved in 20 ml of ethyl alcohol, and 8 grams of phenol dissolved in 100 ml of distilled water. The decolorizing agent and counterstain are the same as those used for the ZN method.

The steps of the Kinyoun method are as follows [9 - 11]:

1. An air dried smear is heat-fixed at 80°C for at least 15 minutes or for 2 hours on an electric hot plate at 65°C - 70°C.
2. Slides are flooded with Kinyoun's carbol-fuchsin reagent and allowed to stain for 5 minutes at room temperature (without heating).
3. The slides are rinsed with deionized water and tilted to drain.
4. Decolorization is done with acid-alcohol for 3 minutes and the slides are rinsed again with deionized water.
5. They are redecolorized with acid-alcohol for 1-2 minutes or until no more red color runs from the smear.
6. The slide is rinsed with deionized water and the standing water is drained from the slide surface by tipping the slide.
7. The slide is flooded with methylene blue counterstain and allowed to stain for 4 minutes.
8. Then it is rinsed with distilled water and allowed to air dry.
9. It is then examined under high dry (400X) magnification, and acid-fast structures are confirmed under oil immersion (1000X).

Another method also known as Kinyoun - Gabbet or Tan Thiam Hok staining uses Gabbet stain (1 gram Methylene Blue, 20 ml of 96% [concentrated] H₂SO₄, 30 ml of 96% [absolute] alcohol and 30 ml of aquadest). The initial step is to flood the slide with Kinyoun stain for 3 minutes, then wash it by running water. Afterwards, Gabbett stain was poured onto the slide and left for 1 minute, and then washed by running water. Then the slide was left to dry in room air [10].

Other modifications of acid fast staining have also been reported. One example is a method performed by Zhao *et al* (2012) [9]. In Zhao's method, 2-3 drops of dioxogen (30%), a strong oxidant that induces lipid peroxidation in the cytoderm, were added into Carbol Fuchsin. After flooding the slide with carbol fuchsin + dioxogen, the slide was then left for 1-2 minutes without heating. Zhao *et al.* reported that this technique had similar detection rates as the traditional ZN staining and the Kinyoun technique. Zhao reported that this method takes approximately 5 minutes to complete which is considerably faster compared to the traditional ZN technique and the Kinyoun technique [9].

Khrisna & Gole in 2017 added bleaching agents (5% sodium hypochlorite [NaOCl]) before staining samples of tuberculous lymphadenitis using traditional ZN method. It is reported that the detection rate is far better than routine ZN method. AFB morphology was also preserved better in this method [12].

Truant method also known as the Auramine-Rhodamine method uses a different primary stain, decolorizing agent, and counterstain. For the primary stain, it utilizes a fluorescent reagent made from 1.5g of Auramine O, CI 41000; 0.75g of Rhodamine B, CI 749; 75ml of Glycerol; 10ml of Phenol; and 50 ml of distilled water. The decolorizing agent used is made from 99.5ml of 70% Ethanol and 0.5ml of concentrated hydrochloric acid. While the counterstain used is a mix of 0.5g of Potassium permanganate and 99.5g of distilled water [11].

The steps of performing this method are as follows [11]:

1. An air dried smear is heat-fixed at 80 °C for at least 15 minutes or for 2 hours on an electric hot plate at 65°C - 70°C.
2. The slide is then washed with a gentle and indirect stream of distilled water until no color appears in the effluent.
3. The smear is flooded with the decolorizing agent for 2 to 3 minutes, and then washed with distilled water as above.
4. The smear is flooded with the permanganate counterstain for 2 to 4 minutes.
5. The slide is washed with distilled water as above, blotted with an absorbent paper, and dried.
6. The slide was then examined with a fluorescence microscope equipped with a BG-12 exciter filter and an OG-1barrier filter. Acid-fast bacteria appear as brightly fluorescent, yellow-orange cells in a dark field while non-acid-fast cells appear dark.

3.2. Acid-fast Staining And Its Various Uses

As previously described, acid-fastness is the ability of an organism to retain the primary stain after being decolorized by an acidic decolorizing agent (hydrochloric acid + alcohol). Organisms that are acid fast will appear red when examined using bright-field microscopy due to the carbolfuchsin. Non-acid-fast organisms will release the primary stain when decolorized and will appear blue due to the counterstain methylene blue.

3.3. Taeniasis

In 2019, Amer *et al.* [6], wrote a report using ZN staining to screen for taeniasis among housemaids in Hail, Saudi Arabia. It is said that ZN could be used to detect *Taenia sp.* eggs in the faeces specimens of infected patients. For the ZN staining, after preparing the sample on the slides, the samples were then stained with carbol-fuchsin 3%, decolorized with acidic alcohol (70% ethanol + 1% HCl), and was counterstained using 3% methylene blue. The excess stain was washed, and the slide was let to dry at room temperature [6]. ZN provided the ability differentiate between the eggs of *Taenia solium* and *Taenia saginata*. The embryophore of *Taenia saginata's* eggs was entirely coloured magenta. *Taenia*

solium's embryophores on the other hand were blue from methylene blue. The possible explanation was that there was different acid-fast resistance produced by the vitellogenin in the embryophore blocks between the two species [6].

Another study was done by Jimenez *et al.* [5] Using the similar staining method, Jimenez *et al.* used preserved parasite materials, comprising mature and gravid proglottids. The staining of taenia eggs in the proglottid material showed that the oncospheres always stain in blue in both species with magenta hooks. As the embryophores mature and become thicker, coloration gets more intense. A blue to mixed magenta was found in those of *T. solium* and a more marked magenta color in *T. saginata* [5, 6].

These studies demonstrate the use of ZN staining as a means of screening and early detection of taeniasis. To add, it is possible that ZN can fully distinguish fully mature *T. solium* from *T. saginata* eggs. However due to the low percentage of this finding (35% of the cases), Jimenez *et al.* stated that this method is not well suited for practical use [5].

3.4. Echinococcosis (Hydatid Cyst of the Liver)

Hydatid cyst disease is due to *Echinococcus granulosus*, endemic in kettle and sheep-raising regions of the world. Hydatid cyst disease most commonly affects the liver (55-70%) followed by the lung (18-35%). Hydatid cysts is also known to cause obstructive jaundice and abdominal pain.

Pan and Hunter [13] reported a case of a man presenting with a sharp pain in the right side of his abdomen which radiated to his chest. A CT scan showed two cystic lesions in the liver. Gross examination showed 0.3 cm walls densely fibrous with focal calcification, containing soft yellow material without any specific organisms. Haematoxylin and Eosin (HE) stain very faintly revealed hooklet-like structures. ZN and trichrome staining was performed, highlighting similar hooklet-like structures that are stained as purple-blue and pink-red, respectively [12, 13].

A similar report was written by Mohanty *et al.* [7], on a male patient with dull aching intermittent pain and a slow growing mass in the epigastric region. Ultrasonography revealed an enlarged liver with a lesion in the left lobe with cysts in multiple sizes inside. While appearing as an undiscernible gram-negative organism, ZN stains revealed pink-red coloured hooklets which were semitranslucent, refractile and sickle-shaped with an inner semi-translucent core of a similar shape [7].

These studies show the advantages of ZN staining in identifying hydatid cysts.

3.5. Nocardiosis

Nocardia is a genus of nonmotile, non-spore forming, aerobic-rod-shaped bacteria ubiquitous in soil and water. Some members of this genus are known to cause nocardiosis [14].

Bagali *et al.* [15], reported a case of a patient with dyspnea, cough with expectoration and haemoptysis persisting for 1 month, accompanied with chest pain, general weakness, anorexia, with a history of rheumatic heart disease. The patient

had bilateral pedal oedema and diminished breath sounds with dullness of percussion over the right lower lobe. Chest x-ray revealed right sided pleural effusion. Thoracentesis revealed straw colored pleural fluid. Gram smear revealed filamentous bacteria and conventional ZN of AFB was negative [15]. Bagali *et al.* performed a modified ZN staining using 1% sulphuric acid revealing acid fast branching filaments belonging to *Nocardia sp.* Incubation of the pleural fluid sample in the blood and Sabouraud's dextrose agar for 48 revealed dry whitish to tan colonies which further developed into chalky white dry colonies. After clearing tyrosine and hypoxanthine crystal, further observation revealed *Nocardia brasiliensis* [15].

Another case of the use of ZN for pulmonary nocardiosis was reported by Shariff *et al.* [16]. Five (5) cases of pulmonary nocardiosis in immunocompromised patients were reported. The patients had similar presentations to the case reported by Bagali *et al.* Gram staining from the sputum samples, broncho-alveolar lavage (BAL) and bronchial aspirate showed gram-positive filamentous branching rods with beaded appearance. ZN staining showed acid-fast branching filamentous rods with beaded appearance suggestive of *Nocardia* [16].

Huang *et al.* [17], utilized ZN staining in combination with metagenomics next generation sequencing (mNGS) to diagnose a case of brain abscess. A patient with a history of left breast cancer resection and diabetes mellitus came with a sudden loss of consciousness after experiencing chronic intermittent cough and fever for 7 months. The MRI examination revealed a ring enhancing mass lesion at the right anterior temporal lobe. A sample of the mass, which was then found to be an abscess, was stained using ZN and found to be acid-fast. Afterwards, mNGS was performed to accurately pinpoint the pathogen of *Nocardia asiatica* [17].

Wu *et al.*, (2018) [14] reported the discovery of *Nocardia cyriacigeorgica* in a patient with a history of pulmonary tuberculosis presenting with chronic recurrent expectorating cough. Chest CT scan revealed enlarged right hilar and mediastinal lymph nodes, patchy shadows and central bronchiectatic changes and multiple infarctions. ZN staining of the BAL did not reveal acid-fast bacilli. After being initially treated for allergic bronchopulmonary aspergillosis (ABPA), the symptoms reappeared 6 weeks later. Modified ZN revealed a weakly acid-fast organism. Fungal culture showed negligible growth, while bacterial culture revealed *Nocardia cyriacigeorgica* which was then confirmed using gene sequencing and a 100% sequence identity was acquired [14].

3.6. Microsporidia

Microsporidia are obligate spore-forming microorganisms which can affect almost every organ system in immunocompetent or immunocompromised individuals. Microsporidia share many resemblances with fungi and may have wide morphological variations. Several stains have been used and described as sensitive and ideal for detection of microsporidial spores, including Gram's chromotrope stain, modified ZN stain (1% sulphuric acid or 1% acid alcohol; cold method), potassium hydroxide-calcofluor white stain (KOH+CFW), Gomori's methenamine silver stain, Giemsa and

toluidine blue stain.

Mittal *et al.*, in 2019 [8] aimed to evaluate the acid and heat fastness of microsporidia from corneal tissue specimens. In the study, Mittal *et al.* [8], performed ZN staining on the biopsy sections of 9 cases of corneal stromal microsporidiosis for their acid fastness. It was found that modified ZN staining (1% acid alcohol or 1% sulphuric acid) without heating demonstrated spores varying from bright red, bluish red, to pale red, leaving spores unstained, giving them debris like appearances. In contrast, microsporidial spores-stained bright red using conventional ZN stain. Staining was also found to be significantly better using 3% hydrochloric acid alcohol with heating. Brighter staining was observed in 5% to 30% sulphuric acid concentration. It was concluded, therefore, that microsporidia spores are strongly acid fast and heating allowed better penetration of the modified ZN staining method, showing more microsporidia spores in bright red color [8].

3.7. Paragonimiasis

Lung flukes (*Paragonimus*) infections are food-borne trematodiasis and zoonosis present in tropical Asia. Paragonimiasis is known to have similar symptoms and is often misdiagnosed and treated as sputum-negative tuberculosis. The diagnostic approach for paragonimiasis is using wet slides direct examination, but one of the limitations of wet slide direct examination is that the samples cannot be re-examined after it's dried up. ZN staining was previously believed to destroy paragonimus eggs during the 1960s. However, in their research, Slesak *et al.* [18] have shown that similar to wet slides and (formalin-ether concentration technique) FECT, ZN staining had a higher sensitivity in diagnosing paragonimiasis compared to wet slides at lower costs. Continuous heating of the slides did cause reduced quantity of the eggs. Slesak *et al.* also stated that for paragonimiasis, ZN stained slides should be observed in 10x lens for paragonimus eggs in addition to 100x for tuberculosis [18].

In 2011, Slesak *et al.*, evaluated the ZN staining and its modification to diagnose paragonimiasis. Slesak *et al.* stated that *Paragonimus* eggs were clearly visible in direct examination and in ZN staining. *Paragonimus* eggs appeared brownish reddish in ZN staining [18]. Moreover, ZN staining has the potential to aid in epidemiological research regarding paragonimiasis.

3.8. Cryptosporidiosis and Isosporiasis

ZN staining has also been reported to be able to detect *Cryptosporidium sp.* and *Isospora belli*, which are the most common opportunistic entero-parasites in patients with AIDS that cause diarrhoea. Pacheco *et al.* [3], in 2013 analysed stool samples for *Cryptosporidium* and *Isospora belli* using modified ZN, safranin (SF) and auramine (AR). Sample concentration was done to eliminate debris for a clearer examination and to increase the chance of protozoa cysts, especially in asymptomatic individuals with low parasite discharge [3].

The sample concentration performed using the sedimentation by centrifugation technique yielded more *Isospora belli* parasites. Pacheco *et al.*, also stated that a significantly higher proportion of *Cryptosporidium sp.* oocytes

was also observed in faecal smears stained by ZN ($p = 0,01$) and AR ($p=0,05$) when compared to those stained using SF [3].

3.9. Cyclospora Cayetanensis

Cyclospora cayetanensis is a coccidian parasite known to cause diarrhoea. The diagnosis of Cyclosporiasis is usually made by finding the spherical oocysts in stool samples which are stained with the ZN method or by auto fluorescence under UV illumination.

Di Gliullo *et al.* [4], in 2000 reported that they found *Cyclospora cayetanensis* in a sputum sample of a patient with weight loss, purulent expectoration and a history of pulmonary tuberculosis. Upon modified Ziehl-Neelsen (Kinyoun technique) examination of the sputum sample, large spherical acid-fast *Cyclospora cayetanensis* oocysts were found [4].

CONCLUSION

Despite the current advances in medicine, acid-fast staining still plays an important role in terms of diagnostics till this day. Even though in general it is still more popularly used in the diagnosis of *Mycobacterium sp.*, this review has shown that it is also beneficial to incorporate acid fast staining for screenings. This review also showed that the ZN method and the Kinyoun technique has been modified to a certain extent, but still resembles the original technique first introduced by Ziehl and Neelsen back in the late 1890s.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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