A New and Convenient Method to Get Paraffin-Embedded Sections of Tuberculous Bacteria as Positive Quality Controls for Acid-Fast Staining

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Abstract

Background: Acid-fast staining for the detection of *Mycobacterium tuberculosis* has a high false negative-rate, partly due to it is hard to get a good positive control tissue of acid-fast staining. We aimed to design a simple and convenient method for making positive quality controls for acid-fast staining in paraffin-embedded sections.

Methods: Three methods were used to get more tuberculous bacteria, which involving centrifugation, mixing, and culture of tubercle bacilli in pleural fluid, prior to the preparation of paraffin-embedded sections.

Results: Culturing tubercle bacilli in pleural fluid proved to be, by far, the best of the three methods, with sufficient bacteria, convenient observation, clear staining, and potential for upscaled production.

Conclusions: The application of this method should improve the detection rate of tuberculous bacteria, thus facilitating clinical treatment.

Background

Tuberculosis (TB) is one of the top 10 causes of death worldwide^[1]. The global burden of TB remains strong even after years of efforts to reduce its incidence and mortality. According to the most recent Global Tuberculosis Report (2019) edited by the World Health Organization (WHO), TB is considered the ninth cause of death worldwide and the leading cause of mortality by a single infectious agent, with the highest rate of infections and death toll rate mostly concentrated in developing and low-income countries^[2]. In 2017, 10.1 million people presented with TB resulting in 1.6 million deaths (including 0.3 million people with HIV). About 87% of global TB incidence is from 30 high-burden countries. China is one of these 30 countries, reporting third largest number of cases^[1]. There are various diagnostic modalities available for the diagnosis of TB lymphadenitis which include fine-needle aspiration cytology (FNAC), histopathological examination of excised lymph nodes, culture, Ziehl-Neelsen (ZN) staining for acid-fast bacilli (AFB), imaging studies, and molecular tests^[3]. Even though culture is considered as gold standard for the diagnosis, formalin-fixed paraffin-embedded tissue with AFB staining was used as the initial diagnostic test in suspected cases of TB as it is a simple and less expensive outpatient diagnostic procedure. AFB staining of sputum smear samples has a long history in the diagnosis of TB, most commonly employing ZN stain, but the quality control of AFB in the tissue section is not perfect^[4].

Special staining is a technique developed based on hematoxylin and eosin (HE) staining. It is an important auxiliary technique used in pathology diagnosis. It primarily helps identify special structures or components in tissues. ZN method is a type of special staining, which is usually used to detect the presence of tuberculosis bacteria in sputum or tissue. In pathological examination, the rate of positive acid-fast staining tests is very low. To ensure quality control, a positive control must be set up for special staining to guarantee the reliability of staining results. At present, the conventional method is to consider

pathological wax as a positive control, slice the wax and then place the tissue section of the strip under examination on a slide for staining. However, this method has certain limitations: first, such a tissue to be used as a positive control is difficult to obtain; second, in case a positive control is identified, the bacterial content is often low and is distributed unevenly; therefore, it does not guarantee the presence of bacteria in each section, even if the section is continuous. A section not having bacteria will therefore lead to misjudgment and miscalculation.

As it is difficult to obtain positive tissues containing adequate amount of bacteria for successful acid-fast staining, we aimed to make use of cultured tubercle bacillus as the control tissue. Direct smear, as a method, is inconvenient to handle and cannot be used repeatedly over a long period; therefore, we considered using cell wax to make tuberculosis bacterial wax block. To our knowledge, there is no relevant literature regarding the same; hence, we attempted three methods of preparing paraffin-embedded tissue sections for experimental generation of positive controls: centrifugation, mixing, and pleural fluid culture.

Materials And Methods

Clinical data

The tubercle bacillus we used was first cultured for 10 days in a 37°C CO₂ incubator in our hospital laboratory. Its identity was confirmed by an acid-resistant cold-staining method. The patients provided written informed consent for the collection and publication of their medical information at the first visit. Ethics approval was not required for this study. Three pathologists reviewed the slides independently and the diagnoses were confirmed by histological and special stain studies.

Preparation of bacteria

After culturing, we employed the following three methods to prepare bacteria:

(1) *Centrifugation method*

In a biosafety cabinet, 15 ml of 75% alcohol was added to a 50 ml centrifuge tube (marked A). Cultured tubercle bacteria were picked into 75% alcohol with an inoculation ring, and the tube was covered tightly. The bacteria were then allowed to soak and disinfect for 30 min, followed by centrifugation at 2000 rpm for 10 min and discarding of the supernatant.

(2) *Mixing method*

In a biosafety cabinet, 0.5 ml of 75% alcohol was poured into each of two 50 ml centrifuge tubes (marked B1, 2). Cultured tubercle bacteria were picked into each tube with an inoculation ring, the tubes were covered tightly, and the bacteria were allowed to soak and disinfect for 30 min. The tubes were then shaken and their contents were mixed in a third tube (marked B). This tube was centrifuged at 2000 rpm for 10 min and the supernatant was discarded.

(3) *Pleural fluid culture method*

In a biosafety cabinet, 5 ml of fresh pleural fluid was added to a 50 ml centrifuge tube (marked C). Cultured tuberculous bacteria were added to the pleural fluid with an inoculation ring. The tubes were covered and placed in a CO₂ incubator for 48 h, followed by the addition of 40 ml of 75% alcohol to soak and disinfect for 30 min. Centrifugation was then performed at 2000 rpm for 10 min and the supernatant was discarded.

Preparation of wax blocks

The three centrifuge tubes prepared above (A, B, and C) were placed in a 65 °C water bath for 1 min. An equal volume of 2% agar solution was added, and after a quick, gentle mix, the tubes were placed in ice water for solidification. After the agar was completely solidified, one end of the tip of a long bamboo stick was inserted along the side of the test tube in order to clamp out the agar block. This was then cut into a suitable size of about 3 × 5 mm, placed in an embedding box, and dehydrated for paraffin-embedded sectioning.

ZN stain for AFB

For evaluation of the number and dispersion of bacteria, we randomly selected six bacterial wax blocks prepared using each of the three methods and cut five pieces of each wax block (to determine the depth of bacteria distribution in the wax blocks, five sections were sliced every 100 µm). Wax blocks were sliced, each of 4µm thickness, and five slices cut for each wax block. The staining was performed according to a modified ZN (Wade-Fite) method^[5]. In summary, the slides were baked at 70 °C for 30 min and dewaxed twice for 10 min each in gasoline turpentine mixture. The residual liquid around the slice was soaked by an absorbent paper keeping the slice slightly moist. Further, the tissue was outlined with Dako immunohistochemical pen and rinsed with water for 1 min. Next, the slide was completely immersed in phenol basic fuchsin solution(Maxin Biotech,Fuzhou, China) at room temperature for 20-30 min, following which the excess dye is washed off with running water. Next, 20% sulfuric acid was used for differentiation, followed by rinsing with running water for 5 min. The slides were further stained with Mayer's hematoxylin which would help stain the nuclei. The slides were further rinsed with running water for 10 min, oven-dried at 50-60 °C, immersed in fresh xylene, and finally sealed with neutral gum.

Evaluation of sections

The sections were randomly submitted to five pathologists for evaluation after staining, with each pathologist examining six slides. The slides were observed under a low power objective and then examined under a high power objective (x600 magnification).

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 16.0 software (SPSS, Inc., Chicago, IL, USA) was employed for statistical analysis of the data. Statistical evaluation was performed by χ^2 test. $P <$

0.05 was considered statistically significant.

Results

Comparison of the number of bacteria generated by each method

To the best of our knowledge, there are few relevant reports defining the standard number of bacteria required for an effective control, and therefore, we aimed to investigate this. The number of layers of bacterial colonies was first evaluated to represent the density of bacterial colony distribution. In a high-power field of vision, it could be observed that the colonies from the centrifugation and mixing methods were "large and thick", while those from culture methods were "small and thin". In order to facilitate counting of the bacteria, we stipulated a number of accumulated bacteria ≥ 30 as a bacterial group. At least 20 high-power fields were studied. Five pathologists (marked A-E) counted six slices and calculated the average number of bacterial colonies (Table 1, Fig. 1).

As seen in Table 1, there was no significant difference between the number of colonies generated by the centrifugation (average 6.72) and mixing (average 6.32) methods ($P = 0.698 > 0.05$), while the number of colonies from the pleural fluid culture method (average 69.00) was much higher and the difference was statistically significant ($P < 0.001$).

Comparison of bacterial community thickness among different groups

Since there are no relevant reports defining standards of bacterial stratification within colonies, we defined 4 μm as the standard colony thickness, which was the thickness of one normal section. Observed levels of stratification are shown in Table 2 and Fig. 1, which demonstrate no significant difference in the number of bacterial layers(4 μm) between the centrifugation (average 6.8) and mixing methods (average 6.02) ($P = 0.115 > 0.05$), while the number of bacterial layers from the pleural fluid culture method (average 1.24) was significantly smaller ($P < 0.001$).

Discussion

As a chronic infectious disease, TB continues to remain a worldwide health problem, especially in developing countries^[5]. *Mycobacterium tuberculosis* are classified as acid resistant bacteria. They have variable lipids in their cell wall that can combine with phenol basic fuchsin dye to form a complex, which can resist the decolorization of acid, and hence can be stained as an acid-fast organism. The common acid-resistant bacteria include *M. tuberculosis* and *Mycobacterium leprae*, which causes leprosy^[6].

ZN staining is the traditional dyeing method for acid-fast bacilli, but the stain needs to be heated and can be potentially harmful if spilled. At present, the phenol basic fuchsin method (modified Wade-Fit) is more commonly used for dyeing; the method does not require heating and produces relatively stable results. The "cold staining method", which was used in this study to verify the identity of cultured tuberculosis

bacteria, is an improved ZN method. This two-step cold staining method has been shown to have advantages over the conventional ZN method for microscopic examination of tuberculosis bacteria^[7].

The quality of TB diagnosis has an important impact on TB control programs. If TB patients cannot be diagnosed in time due to inadequate examination results, the rates of misdiagnosis and missed diagnosis will be increased. Moreover, unnecessary treatment for non-TB patients can promote the development of multi-drug-resistant (MDR)- TB^[8]. Therefore, a reliable method for staining paraffin-embedded sections plays a key role in the diagnosis of TB^[9].

To ensure reliable staining, a correct and appropriate positive control is of great importance. However, the rate of positive acid-fast staining tests is rather low for both pulmonary TB and extrapulmonary TB. In one study, a total of 62456 samples; 60923 (97.5%) were pulmonary and 1533 (2.5%) were non-pulmonary samples, especially pleura. 2853 (4.6%) Acid-resistant bacilli (ARB) positivity was detected and mycobacterial culture positivity was in total 12.2%. In 356 specimens the cultures were negative in despite the positive ARB results^[10]. In another Indian study, a 13.3% positive rate was found among 8850 cases evaluated by light-emitting diode fluorescence microscopy^[11]. Ghaleb et al. reported incidence of urinary tract tuberculosis in renal patients. Three hundred urine samples were processed for the detection of *M. tuberculosis* by ZN smear examination, of which two were positive by both ZN smears and Lowenstein Jensen medium, with an incidence rate of 0.66%^[12]. Jain et al. indicated that spinal TB is an example of deep *M. tuberculosis* infection in which only 10–30% of cases can be detected by ZN staining^[13]. What's more, even when samples are true positives, the rate of false negative results is quite high.

Despite the importance of reliable positive control tissue, obtaining such a simple and stable control tissue of TB bacteria is a challenge. Positive paraffin sections can be used but often the low bacterial concentration cannot guarantee the detectability of TB bacteria after re-section. In cooperation with a medical laboratory department, we cultivated TB bacteria to make paraffin-embedded sections for use as positive quality controls. Based on our previous experience with cell wax block preparation, we initially prepared wax blocks following centrifugation of cultured bacteria. Bacteria could be easily found in the sections but formed large and thick clumps. As an ideal control tissue, the blocks should not only have a large number of tuberculous bacteria to ensure reliable quality control, but the bacteria should also be evenly distributed in each section. However, our mixing method was not effective. We speculated that it might be the small size of the tubercle bacillus combined with the strong adhesion of the culture medium that inhibited the action of the vibrator used for mixing.

In search of a novel method, we attempted fresh pleural fluid as a culture medium. Bacteria were freely grown by natural invasion and growth and successful results were obtained. The large bacterial group was effectively separated and the bacteria were distributed evenly and were rarely in more than two layers. Additionally, high concentration of bacterial growth made it convenient for observation. This method was therefore able to generate ideal positive quality control material.

It needs to be emphasized that biosafety is of utmost importance. Stringent security measures must be taken and the operation must be standardized in a biosafety cabinet before fixing bacteria in 75% alcohol, as the bacterium can be killed in 75% alcohol only after two minutes.

Conclusions

In conclusion, the preparation of bacterial wax blocks based on pleural fluid culture is a reliable and practical method. To our knowledge, we are the first group to report this method, which will be of great benefit for positive quality control of TB special staining, improving the reliability of the assay.

Abbreviations

TB:Tuberculosis ; ZN:Ziehl-Neelsen; AFB:acid-fast bacilli; ARB:Acid-resistant bacilli.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article

Competing interests: The authors declare that they have no competing interests

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Authors' contributions: Lili Tao is mainly responsible for design, data collection, literature review and paper writing. Guanyin Yu mainly polishes the paper and contacts with the magazine. Chuqiang Huang is mainly responsible for data statistics, analysis and image processing. Junliang Tan is responsible for bacterial culture and preparing bacterial wax. Xiaomin Yin participated in the discussion of the results and the adjustment of the experimental scheme. Yu Hao provides cultured tuberculous bacteria and continuing the culture after adding pleural effusion. All authors read and approved the final manuscript.

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Tables

Table 1
Comparison of the number of bacteria generated by each method

Number of bacteria						
Methods	A	B	C	D	E	average
Centrifugation	6	6.8	6.8	6.2	7.8	6.72
Mixing	7.2	7.8	4.4	6.8	5.4	6.32
Pleural fluid culture	68.4	63	72.4	69.8	71.4	69

Table 2
Comparison of bacterial community thickness by each method

bacterial community thickness						
Methods	A	B	C	D	E	average
Centrifugation	6.6	6.5	7	7.3	6.6	6.8
Mixing	5.8	6.3	5.8	5.6	6.6	6.02
Pleural fluid culture	1.2	1.1	1.1	1.3	1.5	1.24

Figures

Figure 1

tuberculous bacteria by ZN stain in each method (A,D: centrifugation;B,E: mixing; C,F: pleural fluid culture.
ABC: x200 magnification; DEF: x600 magnification