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COMPARATIVE EXPERIMENTAL STUDY OF THE PERFORMANCE OF BACTERIOSCOPIC METHODS IN DETECTING ACID-FAST BACILLI: ZIEHL-NEELSEN MICROSCOPY, CONVENTIONAL FLUORESCENCE, AND LED FLUORESCENCE MICROSCOPY

ABSTRACT

Performance of bacterioscopic methods was comparatively studied in Bacteriologic Laboratory of the Phthisiatry Research-Practice Center, to assess the detection of acid-fast bacilli (AFB) by Ziehl-Neelsen (ZN) microscopy, conventional fluorescence microscopy (FM), and LED fluorescence microscopy (LED-FM). A total of 400 positive smears detected by FM were re-observed by ZN microscopy; additionally, we analyzed diagnostic material from 648 patients of the Pulmonology Department of the Yakutsk City Clinical Hospital, in whom the hospital laboratory did not detect

presence of AFB in smears after triple ZN microscopy of direct sputum. To compare the performance of FM and LED-FM microscopy in detecting AFB, diagnostic material from 1082 new pulmonary tuberculosis cases was analyzed.

Keywords: tuberculosis, diagnosis, acid-fast bacilli, Ziehl-Neelsen microscopy, fluorescence microscopy, LED fluorescence microscopy, fluorochrome.

Introduction. Despite the introduction to clinical practice of advanced microbiological and molecular-genetic methods for diagnosis of tuberculosis (TB), microscopic methods are still the key methods used in primary examination of individuals with suspicion for TB. The value of these methods is in their availability, simplicity, and opportunities to possibly fast detection of those patients who present epidemiological risk [1, 6, 9].

Ziehl-Neelsen (ZN) stain microscopy is one of essential methods used to confirm the diagnosis of pulmonary TB, based on detection of acid-fast bacilli in patient's sputum [2, 6].

ZN microscopy is relatively simple to perform, but its sensitivity can be decreased due to a number of factors. It is obviously more so in laboratories with intense daily load of microscopic procedures performed, that can lead to reduced quality of specimen preparation and smear staining technique, while the lab staff might simply not have enough time to observe all incoming fields of view (FOV) due to high workload [7, 9, 10, 11].

Fluorescence microscopy (FM) is currently a method of choice, with significant advantages over ZN microscopy. The method's sensitivity is 10-15% higher than that of ZN microscopy, on average [10, 11, 12]. Compared to transmitted-light microscopy, FM has a number of advantages: high contrast of luminous objects against dark field; considerably larger visible area due to less magnification needed to observe the object; saving of time, and more. [3].

Use of LED (light emitting diode) technologies has been the latest advancement made in the field of FM, which dramatically increased performance of microscopy [8]. Estimation by WHO confirmed diagnostic accuracy of LED-microscopy, comparable to conventional FM, and superior performance of LED fluorescence microscopy (LED-FM) over ZN microscopy [4]. Despite the positive feedback on the utility of LED-FM, just one study on the use of LED-FM could be retrieved from available Russian-language literature [8].

Aim: Comparative assessment of the performance of different microscopy methods in detecting acid-fast mycobacteria.

Material and methods. In performing routine microbiological examinations were complying with the procedure descriptions provided in Appendices (10, 11) to the Order no.109 (issued 21.03.2003) "On improving anti-tuberculosis measures in the Russian Federation", and Order no.951 (issued 29.12.2014), of the Russian Federation Ministry of Health [5].

For ZN test, smears were treated with carbol fuchsin, decolorized with 3% hydrochloric acid-ethanol, and stained with 0.25% methylene blue.

ZN microscopy was performed using binocular microscope (Primo Star, Carl Zeiss, Germany) with 100x immersion lens, 10x eyepiece, and at 1000x magnification. Reading of results was done in minimums of 300 fields (negative results) and 100 fields (positive results).

For FM, sediment pH levels (sediments were obtained from the material prepared as described above) were adjusted to 6.8-7.0. Using a pipette, 1-2 drops of sediment were placed on the slide, and spread as a thin layer in the center of a slide, over an area of 2x1 sm. Then, smears were dried in biosafety hood for 15-20 min. at room temperature. Smears were fixed in dry-air sterilizer at 65-75°C for 2 hours. After staining with auramine OO and rhodamine C, specimens were examined using LED fluorescence microscope (Primo Star, Carl Zeiss, Germany) and conventional fluorescence microscope (Mikmed 2, Russia), at 400x magnification (40x lens, 10x/18 eyepiece).

Once the diagnostic material was processed with fluorescent dyes, and they started binding to waxy parts of microbial cell and penetrating to cytoplasm, followed by exposure to excitation light source, mycobacterial cells glowing orange or bright-yellow against black or dark-green background could be observed.

Statistical processing was performed using commonly employed software (Microsoft Excel, StatSoft Statistica 6), mean values (M±m), and significance values for statistical difference assessment (P).

Results and discussion. During the year 2016, we microscopically examined the sediments from various diagnostic samples, incoming to the lab, all collected from patients suspected for TB, or hospitalized patients on treatment. Overall, 9480 smears were subjected to FM, of them 1439 were AFB+. Detectability rate for AFB detected by FM method was 15.2%.

Of these 1439 AFB+ specimens, 400 smears were randomly selected for comparative examination by ZN microscopy, with the following categories of positivity: 100 smears (scanty); 100 "1+" smears (solitary AFB); 100 "2+" (moderate count of AFB); 100 "3+" (large count of AFB). All 400 positive smears were confirmed by culture tests for *M.tuberculosis* complex mycobacteria on liquid and solid media.

After observation by FM method, the smears were stained for ZN microscopy. Stained smears were then observed consecutively by 3 bacteriologists, who were unaware of the preceding results. In case of discrepancies in readings, the result received in most of the readings was considered final.

Results of comparative microscopic smear examinations are presented in Table 1.

As is seen in Table 1, out of 400 FM-positive smears, re-observation by ZN method showed the presence of AFB in 312 (78%) smears, while in 88 (22%) smears the presence of AFB could not be shown.

Among 100 smears classified as scanty, ZN microscopy was positive in 22 smears, and negative in 78 smears.

Out of 100 smears classified as "1+", 90 were proved positive by ZN, although only 34 were classified exactly as "1+", and 56 smears were shown to have scanty.

Smears classified as "2+" or "3+", were confirmed by ZN method in 100%. Positivity category "2+" matched in 52 smears; 44 smears were classified as "1+" and 4 smears as scanty. For category "3+", match was observed in 62 smears, while 28 smears were interpreted as "2+" and 11 smears as "1+".

Comparative study results supported better performance of FM over ZN microscopy, especially in scanty specimens, only 22% of which could be confirmed as positive by ZN method.

Hence, FM was shown to have a 22% higher sensitivity compared to ZN method, which is in agreement with literature data.

Our next step in comparative studies was to assess detectability of AFB by FM, using diagnostic material from 648 patients of the Pulmonology Department of the Yakutsk City Clinical Hospital, who had AFB-negative results of triple ZN microscopy of direct sputum performed at hospital laboratory. Based on indications, all patients were referred for further ex-

Table 1

Re-observation results for AFB+ fluorescence microscopic smears, by Ziehl-Neelsen microscopy (n, %)

Indicators:	Scores: FM+ smears			
	Scanty	1+	2+	3+
Total: AFB+ smears (FM) (n = 400)	100	100	100	100
Subjected to re-observation by Z-N microscopy (n = 400)	100	100	100	100
Of them, AFB+ n = 312 (78 %)	22	90	100	100
Of them, AFB- n = 88 (22 %)	78	10	-	-
Detection rate, %	22	90	100	100
Scoring AFB+ results (ZN): Scanty	22	56	4	-
1+	-	34	44	10
2+	-	-	52	28
3+	-	-	-	62

Table 2

Detection rates for direct sputum Ziehl-Neelsen test for AFB in clinical diagnostic laboratories of the Yakutsk City Clinical Hospital, 2014-2016

Years	Number of tests		Number of patients tested		Multiplicity, %
	Total	Of them, AFB+ n, %	Total	Of them, AFB+n, %	
2014	4378	19 (0.4)	2265	18 (0.8)	1.9
2015	4291	24 (0.5)	2216	23 (1.0)	1.9
2016	4971	25 (0.5)	2580	21 (0.8)	1.9

Table 3

Detection rates: FM for AFB in sediments from diagnostic materials collected from patients of Yakutsk City Clinical Hospital

Total tested	Of them, AFB+ n, %	Microscopy result scoring (n, %)			
		Scanty	1+	2+	3+
n= 648	22 (3.4)	10 (45.5)	3 (13.6)	7 (31.8)	2 (9.1)

Table 4

Results of FM and LED-M for sediments of diagnostic materials from newly identified patients with pulmonary tuberculosis

Microscopy method	Total new patients n= 1082	Of them, w/o cavities n, %	Of them, with cavities n, %	Test results		
				Positive sputum n, %	Positive sputum and cavities n, %	Positive sputum, no cavities n, %
FM	518	337 65.1	181 34.9	180 34.7	131 72.4	49 14.5
LED	564	392 69.5	172 30.5	251 44.5	143 83.1	108 27.5

amination to City Tuberculosis Treatment and Prevention Clinic.

As is seen from Table 2, over the period from 2014 to 2016, an annual number of ZN microscopies for TB performed at Yakutsk City Clinical Hospital ranged between 4291 (2015) and 4971 (2016), while the number of patients examined annually ranged from 2216 (2015) to 2580 (2016). AFB detectability rate (WHO standard is 1%) ranged between 0.8% (2014) and 1.0% (2015). Number of tests performed (WHO recommends 3.0) was stably 1.9 during the study period. Thus, reference values (detectability and number of tests) recommended by WHO were not met by primary level laboratory.

Examination of samples from 648 patients using FM method showed presence of AFB in 22 cases. AFB detectability rate was 3.4%, which was reliably higher (4.2% higher ($p<0.05$)) than the detectability demonstrated by ZN microscopy performed at Yakutsk City Clinical Hospital laboratory (0.8% in 2016). Furthermore, positive results were classified as scanty in 10 cases, as 1+ in 3 cases, as 2+ in 7 cases, and 3+ in 2 cases, i.e. 9 patients (40.9%) were shown to have excessive bacterial load, undetected by ZN microscopy (Table 3).

The next step was to comparatively analyze performance of FM and LED microscopy in detecting AFB in sediments from diagnostic material collected from newly diagnosed pulmonary TB patients. Material from a total of 1082 patients was examined, of them, 518 were subjected to FM, and 564 to LED-FM.

Results of comparative analysis are shown in Table 4.

As is seen, using FM method, 34.7% (180/518) of patients had AFB-positive results, while 65.3% (338/518) were negative. Using LED-FM, 44.5% (251/564) of patients were positive for AFB, and 55.5% (313/564) were negative. Detectability of AFB by LED-FM was 44.5%, which was reliably higher (9.8% higher ($p<0.001$)) than the detectability by FM (34.7%).

Using LED-FM, detection of smear-positive results (M+) among patients with lung destructions was 83.1% (143) and 27.5% (108) among patients without lung destructions, which was higher, compared to percent detected by FM (72.4% (131) and 14.5% (49), respectively).

Hence, performance of LED-FM was confirmed to be higher (by 9.8%, in our study), compared to conventional FM.

Conclusion. As the results of comparative study demonstrated, fluorescence

microscopes with mercury lamp or LED light source should be advised for use as more comprehensive compared to ZN microscopy, to achieve early detection of the presence of bacteria in diagnostic material and early diagnosis of TB infection at laboratories with high (>20 smears) daily workload.

As far as LED fluorescence microscopes are less demanding in terms of high-skilled maintenance and offer longer lamp life-span compared to conventional fluorescence microscopes, use of LED-technologies is economically reasonable and LED fluorescence microscopy should be recommended for extensive application in the diagnosis of TB.

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