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COMPARISON OF MICROSCOPIC POSITIVITY IN SMEARS FROM SPUTA STAINED ACCORDING TO ZIEHL-NEELSEN IN DIFFERENT MODIFICATIONS

ŠLOSÁREK M.[1]), CEND DONDOV[2]), DĄBROWSKA L.[3]), FIEDLER E.[4]), JANKOVA E.[5]), KALFIN E.[6]), KAUSTOVÁ J.[7]), MEZENSKÝ L.[8]), MYŠÁK J.[9]), ŠAPKADŽIJEVA P.[6]), WOJCIECHOWSKA E.[10]) AND HYNČICA V.[1])

[1]) Institute of Hygiene and Epidemiology, Prague, Czechoslovakia; [2]) Gosudarstvennyi tuberkulosnyi dispanser, Ulan-Bator, Mongolia; [3]) Wojewódzka Przychodnia Przeciwgruzlicza, Koszalin, Poland; [4]) Forschungsinstitut für Tuberkulose und Lungenkrankheiten, Berlin-Buch, Deutsche Demokratische Republik; [5]) Tubbolnica Iskrec, Sofia, Bulgaria; [6]) Centrum dla pnevmologii i ftiziatrii, Sofia, Bulgaria; [7]) Regional Hygiene Station, Ostrava, Czechoslovakia, [8]) Regional Hygiene Station, Brno-Bohunice, Czechoslovakia; [9]) District Hygiene Station, Olomouc, Czechoslovakia; [10]) Wojewódzka Przychodnia Przeciwgruzlicza, Gdnask-Wrzeszcz, Poland.

Direct microscopy is still the basic method of examination in the diagnosis of tuberculosis. It reveals the epidemiologically most serious forms of disease the prevalence of which is an important epidemiological index (1). Unlike the culture finding, it yields an immediate result which makes it posible to start effective treatment and to take antiepidemic measures without delay.

For more than 80 years, microscopic proof of mycobacteria has been provided by the Ziehl-Neelsen method (2) using various modifications in which various influences in the preparation of slides, their fixation, staining, decoularization and counterstaining may play various roles. The object of the present study was to test the reproducibility of the method proposed by the International Union Against Tuberculosis (IUAT) and recommended for verification by the World Health Organization (WHO), to compare the results with those of the authors' own modification and to verify the applicability of cold staining. The results were evaluated quantitatively according to the number of the detected acidoresistant bacteria and simultaneously, the personal ability of the individual readers participating in the scanning to detect mycobacteria was assessed. The main goal of the study was the recommendation of a standard method suitable to be introduced into routine practice.

MATERIAL AND METHODS

A total of 9 laboratories from 5 countries participated in the study: 2 from Bulgaria, 3 from Czechoslovakia, 1 from the German Democratic Republic, 1 from Mongolia and 2 from Poland. On the basis of a previously obtained protocol, each of the laboratories prepared 200 coded slides from 50 suspect-positive sputa and without examination, despatched them to the next laboratory in the series. A total of 1 800 slides prepared from 450 sputa were thus successively evaluated and each smear was examined by a total of 8 different readers.

The following methods were used for the preparation and staining of slides:

Method A (according to IUAT and WHO)

A sputum sample was transferred by a loop onto the slide, spread over about 2/3 of its surface by means of another slide and, after drying in the air, fixed by flame and stained. Staining:

Solution 1		Solution 2	
Basic fuchsin	10 g	Sulphuric acid	25 %
Alcohol 96 %	100 ml	Solution 3	
5 % aqueous phenol solution ad	1 000 ml	Picric acid	0.75 %

Procedure: Fixed slides were covered with strips of filter paper, solution 1 was poured over them and heat was carefully applied until evaporation started. After 5 min, the strips of filter paper were removed by means of tweezers, the slides were rinsed with water and immersed into the first bath with solution 2 for 3 min, rinsed with water once again and immersed into the second bath with solution 2 for 1—2 min. After a thorough rinse with water, the slides were transferred into a bath with solution 3 for a period of 10 sec, rinsed again and dried in vertical position in the air.

Evaluation. The slides were examined in one line from the left to the right (one length comprising about 100 fields). When a small number of mycobacteria (up to 9 bacteria) were found, another 2 lengths (about 200 additional fields) were examined, but when the number of bacteria was large, only 1/4 of one length was examined. The results were always interpreted with respect to the number of fields (e.g., 25/100, 8/300, 40/25, and the like).

Before preparing further smears from the same sputum (B, C and D), each sputum was homogenized. In the odd laboratories the sputa were homogenized by shaking with the same volume of glass beads for 20 min, in the even laboratories, soda lye was used for homogenizing the sputa (2 ml 2N NaOH; after 20 min of action at 37 °C, 3.5 ml 1N HCl were added).

Method B

Homogenized sputum was applied onto the slide and spread across it by a pipette (over approximately the same area as in A), fixation and staining were the same as in method A, examination was carried out along a meander-like path up to a maximum of 100 fields and the result was recorded again with respect to the number of fields examined.

When evaluating the slides prepared by the methods A and B, the effect of the manner of examination on th eresult was simultaneously taken into consideration. For this reason, the readers from the odd laboratories examined all the A slides along the meander-like path only up to 100 fields and the B slides in one line (according to the IUAT and WHO schemes) while the readers from the even laboratories proceeded in the opposite manner.

Method C

Homogenized sputum was applied to the slide by means of a pipette in an amount of 0.05 ml and spread across an area 2 cm in diameter, circumscribed by a diamond. After drying, the slides were fixed in flame and stained with hot carbolfuchsin (solution 1) as in method A. After rinsing with water, the slides were decolorated with 25 % sulphuric acid (solution 2) in only one bath (1—3 min) and additionally stained with 1 % malachite green (30—60 sec). The slides were examined along the meander-like path up to 50 fields and the findings recorded as in methods A and B.

Table 1. Positive findings in sputum smears according to Ziehl-Neelsen using 4 different methods (A, B, C and D) and evaluated successively by 8 different persons

Slides prepared by laboratory No.:	Total of sputa	Positive in all examinations together (according to at least 1 reader)									
		slides								sputa	
		A		B		C		D			
1	50	37	74 %	34	68 %	33	66 %	37	74 %	47	94 %
2	50	50	100 %	50	100 %	50	100 %	50	100 %	50	100 %
3	50	17	34 %	34	69 %	33	66 %	24	48 %	41	82 %
4	50	38	76 %	43	86 %	43	86 %	40	80 %	49	98 %
5	50	40	80 %	43	86 %	41	82 %	42	84 %	48	96 %
6	50	42	84 %	46	92 %	43	86 %	41	82 %	50	100 %
7	50	35	70 %	42	84 %	43	86 %	41	82 %	50	100 %
8	50	28	56 %	35	70 %	34	68 %	31	62 %	48	96 %
9	50	43	86 %	43	86 %	48	96 %	46	92 %	50	100 %
Total	450	330	73.3 %	370*)	82.4 %	368	81.8 %	352	78.2 %	433	96.2 %

x) out of 449 — 1 slide (from lab. 3) was broken already in the first consignment

Method D

Homogenized sputum was spread over an area 2 cm in diameter as in C and, after drying, the slides were fixed with methanol (methanol was poured over them and allowed to evaporate). Staining with carbolfuchsin (solution 1) at room temperature in a tank for 2 hrs ,decolourization, counterstaining and examination were carried out in the same manner as in C. Before being despatched to the next laboratory, all smears were freed of immersion oil by rinsing in xylol.

All sputa were concurrently examined by culture. After homogenization, each sample was applied to 3 cotton wool swabs on a stainless wire and after 20 minutes of action of 1N HCl and neutralization with 2N NaOH, each swab was inoculated onto 2 media routinely used for the isolation of mycobacteria in the participating laboratories. The results of culture were read after 9 weeks of incubation at 37 °C.

The results were processed on a Hewlett Packard 2116 C computer. The percentage of positive smears or sputa was evaluated in the individual laboratories and according to the individual readers and the number of bacteria per 100 fields found by all readers together in all the 4 methods used was also assessed. This quantitative evaluation was carried out by the t-test with the values $t_{5\%} = 1.96$ and $t_{1\%} = 2.58$.

RESULTS

A survey of positivity of the sputa in the methods A, B, C and D using summarized results from all the 8 readers is presented in Table 1. To test the effect of homogenization on the detection of positivity, only the results yielded by

the methods A and B were compared, as the actual technique of staining was the same in these two methods. From the 450 sputa, a total (at least in one of the eight readers) of 330 (73.3 %) sputa were found to be positive in the method A (before homogenization of sputa) and 370 (82.4 %) sputa in the method B (after homogenization). This difference is highly significant in favour of B only in the case of homogenization using glass beads ($t = 4.79$) whereas after homogenization using soda lye, no significant difference between A and B was observed. No significant difference was found between the examinations in one line or along a meander-like path when the manner of reading the slides was considered.

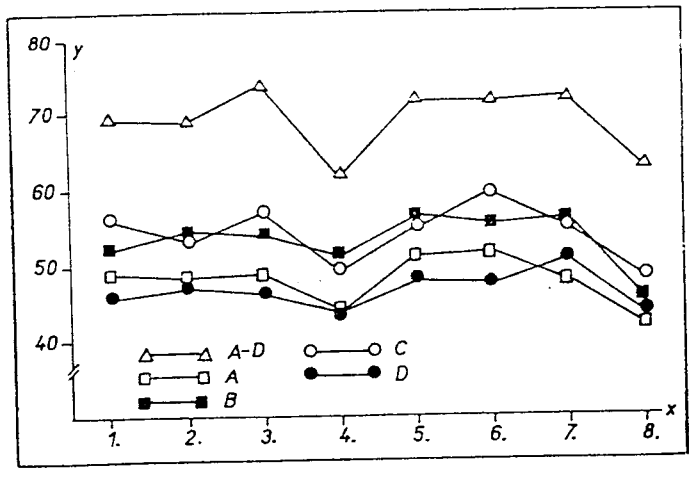

Fig. 1. Age of smears from sputa related to the percentage of positive findings in 8 successive examinations (Time between the preparation of smears and the 8th examination was 1.5—2 years). — y — % of positive findings, x — examination, A—D (sputa in total)

Method C (staining with hot carbolfuchsin) revealed a total of 368 positive sputa (81.3 %), method D (cold staining) 352 positive sputa (78.2 %). Although the detection of positivity in the individual groups of 50 sputa was substantially higher in some readers using method D than in other readers using method C, the difference between the two methods was highly significant in favour of C in quantitative evaluation of all results ($t = 16.97$).

As the study took approximately 2 years, we were also interested in the effect of the age of the slides on the detection of positivity. Summarized results assessed according to the percentage of positive findings in eight successional examinations of all slides (A—D) and in the sputa are comprehensively shown in Fig. 1. Although the number of slides gradually decreased because some of them were broken during transport (from the initial number of 1 799 in the first examination to 1 627 in the sixth examination and to 1 504 in the eighth scanning), the graphic representation of the percentage of positivity demonstrates that neither the age of the slides nor the repeated rinsing in xylol influenced the detection of positive findings.

The high coincidence of the percentages of positivity (in spite of slight differences in the preparation of the smears and in the technique of examination) in the methods B (82.4 %) and C (81.8 %) suggests that the manner of counterstaining (using picric acid or malachite green) has no substantial effect on the detection of mycobacteria in the smears from sputa.

Differences between the readers could not be statistically evaluated because the set of slides examined by each reader did not include those prepared by the particular reader himself. However, the correlation of positive results found by different readers in slides originating from the same laboratory, showed surprisingly great differences. In the extreme case, the "worst reader" (in the given case second in the sequence of circulation) detected in the material from one of the laboratories in the methods A—D together only 16 positive sputa out of a total of 50 (32 %) while the "best" (sixth in the sequence) recorded 48 positive sputa (96 %) in the same material. In the remaining sets, the variation range in the percentage of positive findings in different readers amounted to 34 % on an average. Out of 450 sputa, only 191 sputa (42.4 %) were defined as microscopically positive by all readers unequivocally (regardless of the method used) while the total number of microscopically positive sputa (according to at least one of the eight readers) was 433 (96.2 %). The above mentioned data demonstrate that the quality of the readers plays one of the absolutely essential roles in the detection of positivity in smears from sputa.

Only 233 (51.8 %) out of 450 sputa were found to be positive both by culture and microscopically while 196 sputa (43.5 %) were microscopically positive and culture-negative. Only 13 sputa (2.9 %) gave negative results in both methods, 4 sputa were found positive only by culture (0.9 %) while in 4 (microscopically positive) cases the cultures were overgrown (0.9 %). The unfavourable ratio between the culture positivity and microscopic positivity was obviously due to various shortcomings in the technique of culture in the first place and also because most of the sputa originated from patients under long-term treatment and the probability of detection in bacterioscopy was increased with respect to the large number of examinations of each sputum (32 in total, i.e., 4 smears scanned by 8 persons).

The number of sputa microscopically positive in one single examination only was 22 (4.9 %) including 5 cases of culture-positive sputa. As these isolated findings occurred sporadically in all methods (A—2, B—8, C—9, D—3) and a total of 7 different readers participated in them, we did not exclude them from statistical evaluation.

DISCUSSION

The results of direct microscopy of sputa from patients with tuberculosis of the lungs depend on a large number of various factors. The fundamental among them are as follows: Suitable selection of material, technique of processing the sputa before making smears, the preparation of smears itself, staining of smears and method of evaluation. These complex factors can be further divided into a number of partial operations each of which can to some extent, influence both

the quality and quantity of positive findings. As has been demonstrated by many previous studies and current experience of large laboratories, small differences in the preparation of slides and different modifications of the Ziehl-Neelsen method of staining do not substantially influence the determination of positivity, affecting only the quantity of the detected acidoresistant bacteria (ARB) in strongly positive sputa. On the other hand, in sputa with a low content of mycobacteria, correct selection of the most effective method is decisive even for the proof of positivity itself.

The present study was focussed mainly on the assessment of the influence of some basic operations in the preparation of slides and in the process of staining smears from suspect-positive sputa using various modifications of the Ziehl-Neelsen method. As we had expected, mechanical homogenization of the sputa by shaking them with glass beads, highly significantly increased the quantity of the detected mycobacteria. This technique can therefore be recommended to be used in all laboratories where the technical equipment and personnel are available. Homogenization using soda lye only induced dissolution of strongly viscous sputa, but dispersion of larger clumps of mycobacteria into smaller ones or into individual cells evidently did not occur. For this reason, the findings after homogenization of sputa using soda lye were both qualitatively and quantitatively identical with those obtained from the same sputa before their homogenization. The manner of examination the slides (in one line or meander-like on selected spots) had no effect on the amount of the detected ARB.

The 2-hour staining with carbolfuchsin at room temperature, chosen on the basis of preliminary orientative tests (3), was found less convenient in practice than the original technique with heating. Although the overall difference in the percentage of positive findings was relatively low on comparing these two techniques (3.6 %), the number of detected ARB was highly significantly greater when hot carbolfuchsin was used. This confirms the generally known phenomenon that different developmental forms of mycobacteria show different stainability and that some cells remain unstained even after using heated carbolfuchsin (4). The preparation of smears and some other factors in the course of the process of staining itself can have direct influence on the quality and quantity of the stained bacteria (5, 6). All these effects are more pronounced in cold staining, however, the convincing difference between the two methods under comparison was not revealed until quantitative machine-aided evaluation of summarized results from all readers was performed while even an opposite relationship (a larger number of positive findings in some readers after cold staining—method D than in hot staining—method C) was observed in some cases in the individual sets of 50 sputa (e. g., from the laboratories 1 and 5).

Surprisingly high differences were observed in the evaluation of the same preparations by different persons. In this respect, our results are essentially identical with those observed by Jørgen Nyboe*) in the years 1969—1971.

*) The IUAT Stained Smear Study (unpublished)

Although that study, in which 12 central tuberculosis laboratories from different countries participated, also demonstrated that repeated rinsing of the slides in xylol in the course of their circulation has only a negligible influence on the detected positivity, from the 500 slides under examination a total of 279 were found microscopically positive including 103 slides assessed unequivocally by all the 12 readers while a single reader recorded 92 positive findings. At the same time, the evaluation of the slides was carried out in both cases only by experts working for many years in the filed of tuberculosis microbiology. The considerable similarity of results obtained by two different readers evaluating the same set of slides (different results obtained only in 2 sputa) reported by Boulahbal and Larbaoui (7) can be considered a chance and also due to the fact that a small number of sputa was examined (20 positive sputa out of a total of 75). High differences between the observed positivity on evaluating the same preparations by different persons were manifest not only in smears from sputa stained according to Ziehl-Neelsen, but also in a comparative study of different staining methods for fluorescence microscopy (8). In our opinion, the most important factor influencing the quality of findings in the study of microscopic positivity mainly consists in the individual ability, attentiveness and momentary disposition of the workers who actually evaluate the slides.

The failures of the parallel examination by culture point out that this problem should be given increased atention. In the first place, a simple but sufficiently sensitive culture technique together with an optimum method of material decontamination should be developed.

SUMMARY

Some basic factors influencing the detection of microscopic positivity in the sputa of patienst with tuberculosis of the lungs, stained according to Ziehl-Neelsen in various modifications, were studied in 9 laboratories in 5 different countries (Bulgaria, Czechoslovakia, Mongolia, German Democratic Republic and Poland). Each laboratory prepared 200 coded slides from 50 suspect-positive sputa. The slides were stained using four different methods and despatched, without examination, to the next laboratory in the series. A total of 1 800 slides from 450 sputa were thus evaluated and each smear was successively examined by 8 different readers. The study has shown that the result of direct microscopy depends primarily on the individual qualities and attentiveness of the workers evaluating the slides while the remaining factors are less important. Mechanical homogenization of sputa before making the smear, carried out by shaking the sputum with glass beads, had a significant effect on the number of detected mycobacteria while homogenization using soda lye did not influence the positivity in any direction. The detection of mycobacteria after hot staining was significantly higher than after a 2-hour action of carbolfuchsin without heating. Additional staining with picric acid or malachite green had no effect on the amount of bacteria detected and the method of examination (in one line or meander-like on selected spots) was found to be completely unimportant.

RÉSUMÉ

Šlosárek, M., Cend Dondov. Dąbrowska L., Fiedler E., Jankova E., Kalfin E., Kaustová J., Mezenský L., Myšák J., Šapkadžijeva P., Wojciechowska E., Hynčica V.: **La comparaison de la positivé microscopique des frottis des crachats colorés d'après la méthode de Ziehl-Neelsen à des modifications différentes.**

Les auteurs ont étudié quelques influences de base sur le résultat de la positivité microscropique dans les frottis des cachats des malades souffrant de la tuberculose de poumons, colorés selon la méthode de Ziehl-Neelsen, à des modifications différentes. Au total il y avait 1800 préparations provenant de 450 crachats et chaque frottis a été successivement évakué par 8 lecteurs. Les résultats ont démontré que c'étaient des qualités individuelles des lecteurs qui avaient l'influence décisive sur l'isolment des mycobactéries, surtout dans les cas des sputums avec la positivité microscopique faible. Les autres facteurs (homogénisation, mode de la préparation des frottis et la colorisation) ont été mois significatifs ou ils se sont démontrés tout à fait sans importance (mode de la colorisation finale des préparations et les examinations).

ZUSAMMENFASSUNG

Šlosárek, M., Cend Dondov. Dąbrowska L., Fiedler E., Jankova E., Kalfin E., Kaustová J., Mezenský L., Myšák J., Šapkadžijeva P., Wojciechowska E., Hynčica V.: **Der Vergleich der mikroskopischen Positivität in Aufstrichen aus den gemäss Ziehl — Neelsen gefärbten Sputa in verschiedenen Modifikationen.**

Man hat einige grundlegende Einflüsse auf das Ergebnis der mikroskopischen Positivität in Aufstrichen aus den gemäss Ziehl — Neelsen gefärbten Sputa in verschiedenen Modifikationen der an Lungentuberkulose Erkrankten untersucht. Es wurden insbesamt 1800 Präparate von 450 Sputa bewertet und jeder Aufstrich wurde von 8 Lesern nacheinander ausgewertet. Die Ergebnisse zeigten, dass individuelle Qualitäten der Leser entscheidenden Einfluss auf das Auffangen von Mykobakterien, besonders im Falle schwach mikroskopisch positiver Sputa haben, die übrigen Faktoren (Homogenisierung, Methode der Vorbereitung von Aufstrichen und Färbung) weniger wichtig waren oder sich als ganz unwichtig zeigten (Methode der Nachfärbung von Präparaten und Untersuchungen).

RESUMEN

Šlosárek, M., Cend Dondov. Dąbrowska L., Fiedler E., Jankova E., Kalfin E., Kaustová J., Mezenský L., Myšák J., Šapkadžijeva P., Wojciechowska E., Hynčica V.: **La comparación de la positividad microscópica en escobillones de los esputos coloreados según el método Ziehl-Neelsen en varias modificaciones.**

Fueron examinados algunos influjos fundamentales en los resultados de la positividad microscópica en escobillones de los esputos de los tuberculosos coloreados según el método Ziehl-Neelsen en varias modificaciones. Fue evaluado en total 1800 preparados de los 450 esputos y cada escobillón fue evaluado por 8 lectores. Los resultados mostraron que el influjo decisivo en el aislamiento de la microbacterias, particularmente en casos de los esputos microscópicamente poco positivos, tienen las cualidades individuales

de los lectores; los demás factores (homogeneización método de preparar los escobillones y la coloración) fueron menos significantes o se mostraron completamente insignificantes (método de postcoloración de los preparados y testigos).

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M. Šlosárek, Institute of Hygiene and Epidemiology, 100 42 Prague 10, Šrobárova 48, Czechoslovakia