ACTA
AMAZONICA

PKO – Alternative method for isolating mycobacteria from sputum

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ABSTRACT

We elaborated an alternative culture method, which we denominated PKO (initials in tribute of respect to Petroff, Kudoh and Ogawa), for isolating *Mycobacterium tuberculosis* from sputum for diagnosis of pulmonary tuberculosis (TB), and to compare its performance with the Swab and Petroff methods. For the technique validation, sputum samples from patients suspected of pulmonary TB cases were examined by acid-fast microscopy (direct and concentrated smear), PKO, Swab and Petroff methods. We found that Petroff and PKO methods have parity in the effectiveness of *M. tuberculosis* isolation. However, by the PKO method, 65% of isolated strains were detected in a period of ≤15 days, while by the Petroff method the best detection was in an interval of 16-29 days (71%). In positive smear samples, the average time of PKO isolation is only superior to the one related for Bactec 460TB. In conclusion, the exclusion of the neutralization stage of pH in the PKO reduces the manipulation of the samples, diminishes the execution time of the culture according to the Petroff method and facilitates the qualification of professionals involved in the laboratorial diagnosis of Tuberculosis.

KEYWORDS

Diagnostic method, *Mycobacterium tuberculosis*, PKO, Tuberculosis

PKO – Um método alternativo para o isolamento de micobactérias em amostras de escarro

RESUMO

Foi elaborado um método de cultivo alternativo, denominado por nós PKO (iniciais referentes à Petroff, Kudoh e Ogawa), para o isolamento do *Mycobacterium tuberculosis* em amostras de escarro para o diagnóstico da tuberculose pulmonar (TB). Para validação da técnica, amostras de escarro de pacientes suspeitos de TB foram submetidas aos métodos de baciloscopia (direta e pós-concentração), PKO, Swab e Petroff. A análise comparativa entre o método de Petroff e o PKO mostrou paridade de resultados em relação ao isolamento e número de colônias de *M. tuberculosis*. Porém, pelo método PKO, 65% das cepas isoladas foi detectada em um período ≤ 15 dias, enquanto que pelo método de Petroff a melhor detecção ocorreu em um intervalo de 16-29 dias (71%). O tempo médio de isolamento pelo PKO é somente superior ao sistema comercial Bactec 460TB em amostras positivas na baciloscopia. A exclusão da etapa de neutralização de pH no método PKO reduz a manipulação das amostras, diminui o tempo de execução do cultivo em relação ao de Petroff e facilita o treinamento de profissionais que realizam o diagnóstico laboratorial da TB.

PALAVRAS-CHAVE

Método diagnóstico, *Mycobacterium tuberculosis*, PKO, Tuberculose

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INTRODUCTION

The automatized bacteriological methods are rapid and reliable in isolating and identifying *Mycobacterium tuberculosis*. However, in the public health network of developing countries, most laboratories working in Tuberculosis (TB) control still use the classical methods of isolation, because of the high cost of equipment and maintenance of the automatized bacteriological methods. Thus, the classical methods should be improved for optimized use in laboratories with limited resources. This becomes more important because in developing countries such as Brazil, about 40% of the population with pulmonary TB is not diagnosed by the direct microscopy method (Who, 2004) recommended by Tuberculosis Control Program of the Brazilian Health Department. Despite the intense care and directions on procedures for obtaining secretions, a large part of these patients have successive negative microscopy tests for acid-fast bacilli (AFB). They are patients who present paucibacillary secretions (David *et al.*, 1989), that is, clinical samples with less than 10.000 AFB. ml^{-1}. In such cases, the isolation of *M. tuberculosis* in culture mediums is still the only method indicated and accepted for the verification of diagnostic suspicion. To carry it out, four distinct stages are essential: decontamination, centrifugation, neutralization and inoculation in culture mediums. The different forms used generated different classical methods which are used in the routine diagnosis of TB.

Standing out among these are the Petroff method, and the Swab method, described by Kudoh & Kudoh (1974), which was initially formulated by Nassau (1958). In the Petroff method, the sample of sputum is submitted to a process of digestion and decontamination with 4% NaOH, centrifuged so that the concentration of the bacilli occurs and the sediment is neutralized (with 4% HCl) for subsequent inoculation in egg-based medium and neutral pH (Löwenstein-Jensen). In the Swab method, one sterilized swab is introduced in the sputum sample, submitted to rotatory movements to impregnate it with purulent particles, immersed in solution of 4% NaOH, and introduced in a tube containing the egg-based culture medium with pH 6.4 (Ogawa modified medium), while smearing and squeezing the swab over its medium surface.

The Petroff method has the disadvantage of being harmful to the bacilli due to the exposure time to NaOH and the necessity of a neutralization stage, whereas the Swab method has less exposure time to NaOH and few stages to perform. However, the Swab method is not recommended for samples with negative acid-fast microscopy (Vasanthakumari, 1990), because of the absence of centrifugation that increases the number of bacilli in the aliquot to be inoculated in culture mediums.

Using the beddings of the Petroff decontamination method with a shorter exposure period to NaOH as established by Kudoh and the modified culture medium of Ogawa, the alternative PKO method was elaborated (initial references to the Petroff, Kudoh and Ogawa) and the results according to the Swab and Petroff methods were analyzed. The objective was to eliminate the neutralization stage of the sample, to use a decontaminant agent that was easily acquired and prepared, mainly to diminish the expense of culture medium production, and to use in paucibacillary samples of patients suspected of pulmonary TB.

MATERIAL AND METHODS

SAMPLING

This study was carried out in 108 sputum samples from patients suspected of pulmonary TB, who was cared for in the public health network in Manaus/Amazonas/Brazil. No patient suffered from imunodeficiency illness or used imunossupressors drugs.

MICROSCOPY EXAMINATION

These samples had previously undergone acid-fast microscopy (direct smear) by laboratories of the public health network. However, it was repeated to confirm the results. This was done according to the Zielh-Neelsen technique recommended by the Brazilian Health Department (Campelo *et al.*, 2001) and the World Health Organization (1998). Acid-fast microscopy was also carried out after decontamination and centrifugation of the samples (concentrated smear), as established by Salem *et al.* (1990)

CULTURE ASSAY

After completing the direct smear, the samples were processed by the Swab method described by Kudoh and Kudoh (1974). They were then divided into two equal parts. One part was processed by the Petroff method with a 15 minute exposure to 4% NaOH (Salem *et al.*, 1990), and the other by the present proposed method – PKO. The flow of activities and procedures of carrying out each method for primary isolation of *M. tuberculosis* are demonstrated in Figure 1.

The modified mediums of Ogawa (pH 6.4) and Löwenstein-Jensen were produced as described by the World Health Organization (1998) and David *et al.* (1989), respectively.

GROWTH AND IDENTIFICATION

The inoculated mediums were incubated at 37ºC, with a contamination verification reading after 24 hours. Lately, the verification of mycobacterial growth was made daily. The cultures without mycobacterial growth were considered

negative after a period of two months, and the positive cultures for acid-fast bacilli were submitted to subcultures for making phenotypic tests.

Mycobacterial strains were first submitted to tests of growth speed and pigment production. All non-pigmented and slow growing strains, which in the biochemical tests produced niacin and strongly reduced nitrate to nitrite, were identified as *M. tuberculosis* strains. The others were considered as nontuberculous mycobacteria (NTM) and were not included in the analyses of the results.

All isolated strains were deposited in the "Bacteria of Medical Interest Collection" of the National Research Institute of Amazonia.

RESULTS ANALYSIS

The accuracy of each method was calculated by sensitivity and specificity (vertical table analysis of type contingency 2 x 2). The agreement between the methods was analyzed by the Kappa index whose interpretation was that used by Pereira (2002). The rapidity of the diagnosis in the different methods was evaluated by the growth speed of *M. tuberculosis* strains in the primary isolation.

RESULTS

Two of the 108 samples were excluded from analysis due to contamination of inoculated culture tubes. In 34 of the 106 analyzed, *M. tuberculosis* was isolated by one or more of the studied methods (Table 1).

The results of acid-fast microscopy of the 106 analyzed samples permitted the formation of two study groups: one contained 16 positive direct or concentrated smear samples for AFB, and the other contained with 90 negative direct and concentrated smear samples for AFB. Of these, 22 (64.7%) were isolated by the Swab method, 31 (91.2%) by the Petroff method, and 31 (91.2%) by the PKO method (Table 2).

From all the positive smear samples, *M. tuberculosis* was isolated in the three studied methods, while in the negative smear; *M. tuberculosis* was isolated in only 18, offering 20% of diagnostic enlargement of TB. Of these, 6 (33.3%) were isolated by the Swab method, 15 (83.3%) by the Petroff, and 15 (83.3%) by the PKO. The number of isolated colonies varied from 1 to >200 in 3 inoculated culture tubes.

The results of sensitivity, specificity and agreement among the methods, and the results according to the acid-fast microscopy are presented in Table 3.

For the evaluation of the growth speed of *M. tuberculosis* strains, 3 analysis periods were used: ≤15 days, 16 at 29 days and ≥ 30 days. Only in the positive smear samples did not the visible growth exceed 29 days (Figure 2).

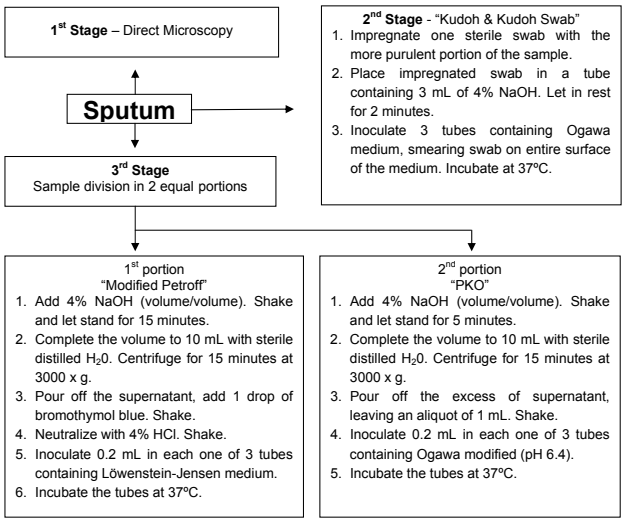

Figure 1 - Activity protocol and procedures for verification of the accuracy of the PKO method.

Table 1 - Number of samples containing *M. tuberculosis* strains in conformity with isolation in one or more of the studied methods.

Methods	Samples with *M. tuberculosis*	
	Nº	%
Swab alone	0	-
Petroff alone	3	8.8
PKO alone	2	5.9
Swab and Petroff	0	-
Swab and PKO	1	2.9
Petroff and PKO	7	20.6
Three methods	21	61.8
TOTALS	34	100.0

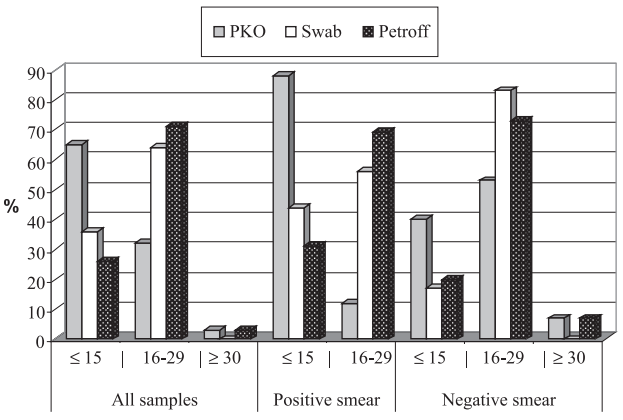

Figure 2 - Isolation frequency of *M. tuberculosis* strains in accordance with different time intervals and microscopy result.

Table 2 - Isolation frequency of *M. tuberculosis* strains according to the methods and the microscopy results.

Method/ Culture Result		Petroff				TOTAL		PKO				TOTAL	
		Culture +[a]		Culture -[b]				Culture +[a]		Culture -[b]			
		Smear		Smear		Smear		Smear		Smear		Smear	
		P[c]	N[d]	P	N	P	N	P	N	P	N	P	N
PKO	+	16	12	0	3	16	15	-	-	-	-	-	-
	-	0	3	0	72	0	75	-	-	-	-	-	-
TOTAL		16	15	0	75	16	90	-	-	-	-	-	-
SWAB	+	16	5	0	1	16	6	16	6	0	0	16	6
	-	0	10	0	74	0	84	0	9	0	75	0	84
TOTAL		16	15	0	75	16	90	16	15	0	75	16	90

a = positive culture for *M. tuberculosis*; b = negative culture for *M. tuberculosis*; c = positive for acid-fast bacilli; d = negative for acid-fast bacilli

Table 3 - Accuracy and agreement among the Swab, Petroff and PKO methods in the isolation of *M. tuberculosis*, in accordance with microscopy result.

Accuracy	Sample with smear					
	Positive and Negative			Negative		
	Petroff/ PKO	Petroff/ Swab	PKO/ Swab	Petroff/ PKO	Petroff/ Swab	PKO/ Swab
Sensitivity	90.3%	67.7%	70.9%	80.0%	33.3%	40.0%
Specificity	96.0%	98.6%	100.0%	96.0%	98.6%	100.0%
Agreements						
Observed	94.3%	89.6%	91.5%	93.3%	87.7%	90.0%
Kappa	0.86	0.73	0.76	0.76	0.42	0.53
Interpretation	Excellent	Good	Good	Good	Regular	Regular

The average time of growth detection of *M. tuberculosis* in different systems and culture methods are presented in Table 4.

Table 4 - Average time of growth detection of *M. tuberculosis* in different systems and culture methods.

Smear	Average time of detection (in days) of micobacterial growth						
	Literature Data				This study		
	BACTEC 460TB[a]	MB/BacT ALERT 3D[a]	BACTEC MGIT 960[b]	ESP Culture System II[b]	Swab	Petroff L-J	PKO
Positive	8.3	11.5	11.1	15.1	16.4	17.7	11.3
Negative	16.8	19.9	14.1	24.4	18.5	23.5	18.8

a = Piersimoni *et al.* (2001); b = Williams-Bouyer *et al.* (2000).

DISCUSSION

In the analysis of 106 samples, the culture by the Swab method provided isolation of *M. tuberculosis* in a lower percentage (20.7%) than obtained by Kudoh and Kudoh (1974) and by Susemihl *et al.* (1993), 37,5% and 32,2%, respectively. But in a higher percentage than that of Vasanthakumari (1990), 9.7%, Coelho *et al.* (1999), 16.2% and Farache Filho *et. al.* (1999), 7.11%. These percentile differences suggest that they are dependent on the quantitative negative and positive smear samples for acid-fast bacilli in the composition of the study groups. This is confirmed in the present study, since the isolation percentage only in the negative smear samples decreased to 6.7%. This is reinforced by the accuracy of the results of the Swab in relation to Petroff or PKO in the analysis of all the samples which offered sensitivity values of 67.7% and 70.9%, respectively. In the analysis of negative smear samples, the sensitivity values decreased to 33.3% according to the Petroff and 40.0% according to the PKO. These results and those obtained by agreement values (Table 3) confirm the reduced sensitivity and low agreement of the method in negative smear samples. However, the simplicity of the technique, the shorter bacilli exposition period to NaOH solution, the 100% isolation and development of *M. tuberculosis* in a period of ≤ 15 days in 44% in positive smear samples (Table 4) indicate its preferential use in laboratories of less complexity, like the ones existing in public health units that have to isolate *M. tuberculosis* in samples of patients suspected of drug resistance and in inquiries of drug resistance.

The results from the Petroff and PKO methods demonstrated that both supplied *M. tuberculosis* isolation in equal numbers. Despite the same quantity, three samples presented positivity only in Petroff and 3 only in PKO (Table 2). All were negative smear samples inducing the hypothesis of a non-homogeneous division of the sample in the protocol established for the present work (Figure 1). Thus, it is possible that one fraction contained bacilli and another did not, since digestion and homogenization did not occur previously to the division of the samples. This premise was backed by the results of the number of isolated colonies in these samples, where the minimum was one colony and the maximum 14 in the three culture tubes inoculated. Consequently, it is possible to deduce that

submitting the sample to NaOH (digestion) in a previous stage of division could diminish the absence of colonies in one of the methods.

The accuracy values as well as those of observed agreement show that the Petroff and PKO methods have parity in the effectiveness of *M. tuberculosis* isolation, whether in the analysis of all samples or only in the negative smear (Table 3). However, it was evident that by the PKO method, 65.0% of the strains isolated were detected in a ≤15 day period (Figure 2), while the major detection by the Petroff method, was in an interval of 16-29 days (71.0%). When these results are compared to semi and automatized methods (Table 4), it shows that the average time of isolation by PKO is superior only to that of BACTEC 460TB in a positive smear. In a negative smear, it loses out only to the BACTEC systems. In this sense, the PKO is the most adequate for use in the public health network, for it does not require equipment and its daily cost is much lower than that of related systems.

Besides all that has been said, the exclusion of the neutralization stage in the PKO reduces the manipulation of the samples, minimizing the aerosol production and the contamination probability of its work environment. Beyond this fact, the time of execution in relation to the Petroff method has been reduced, which facilitates the qualification of professionals involved in the laboratorial diagnosis of TB.

Thus, the simplicity of executing of the PKO method makes it easy to introduce in the public health units, thus extending the laboratory network for the diagnosis of TB by the culture method, and consequently, contributes to the control of TB.

ACKNOWLEDGEMENTS

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Ministério da Ciência e Tecnologia (MCT) and the staff of Laboratório de Micobacteriologia do Instituto Nacional de Pesquisas da Amazônia – INPA.

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Recebido em 16/03/2007
Aceito em 10/07/2007