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Performance of the Ogawa-Kudoh method for isolation of mycobacteria in a laboratory with large-scale workload

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ABSTRACT

In Uruguay (population 3,323,906; notified tuberculosis incidence 18.4/100,000), virtually all 30,000 samples yearly collected for mycobacterial culture countrywide are processed in a central laboratory. An average of 110 samples are routinely shipped daily and maintained 48-96 hours at room temperature until cultured on Löwenstein-Jensen slants using the standard NALC-NaOH decontamination procedure. The much simpler Kudoh decontamination/culture method -swab and Ogawa (acidified) medium- was compared with NALC-NaOH/Löwenstein-Jensen for isolation of mycobacteria from sputa under routine conditions. To this aim, 784 sputum samples were cultured by both methods in the summertime. Gross agreement was 0.99, kappa: 1. Kudoh performance was as follows: sensitivity 100% and accuracy 98.9%. Assays using a modified culture medium, different decontamination times and NaOH concentrations showed the versatility of this procedure. Thus, the Kudoh method is suitable for culturing mycobacteria from naturally contaminated samples even when processing is deferred two to four days after collection.

Key words: Kudoh, *Mycobacterium tuberculosis***,** culture, sputum samples

RESUMEN

Rendimiento del método de Ogawa - Kudoh para el aislamiento de micobacterias en un laboratorio con trabajo a gran escala. En Uruguay (3 323 906 habitantes; incidencia notificada de tuberculosis 18,4/100 000), más del 95% de los cultivos para micobacterias de todo el país (30 000 por año) son procesados en un laboratorio central. Un promedio de 110 muestras de expectoración diarias son enviadas y mantenidas a temperatura ambiente durante 48-96 horas hasta ser procesadas por el método de descontaminación con NALC-NaOH y cultivadas en medio Löwenstein-Jensen. Con el objeto de evaluar el método de Kudoh (hisopo y medio de Ogawa acidificado) como una alternativa más sencilla para el cultivo de micobacterias en este tipo de muestras, se procesaron por ambos métodos 784 esputos durante los meses de verano, a fin de comparar los resultados obtenidos. La concordancia bruta fue de 0.99; el coeficiente kappa fue 1. El método de Kudoh mostró una sensibilidad del 100% y una exactitud del 98,9%. Algunos ensayos en los que se empleó un medio modificado y diferentes concentraciones de NaOH y tiempos de descontaminación demostraron la gran versatilidad del método. Se concluye que el método de Kudoh puede ser aplicado para el cultivo de muestras naturalmente contaminadas, aun después de haber permanecido 2 a 4 días a temperatura ambiente antes de ser cultivadas.

Palabras claves: Kudoh, *Mycobacterium tuberculosis***,** cultivo, muestras de expectoración

Uruguay –a country with 3,323,906 inhabitants– has a notified tuberculosis (TB) incidence of 18.4/ 100,000 (13). Pulmonary forms constitute up to 85% of the cases; of which 80 to 85% are bacteriologically confirmed by microscopy and/or culture (13). More than 95% of the sputum samples collected countrywide for TB diagnosis and treatment control are processed in one laboratory: the National Reference Center for Mycobacteria (CERNAMY). The average number of clinical specimens cultured for *M. tuberculosis* is 30,000 per year (range: 28,000 - 32,000); the total cost per year is US$ 92,000. Sputum samples are shipped at room temperature without the addition of any antiseptic and processed between 2 and 4 days after collection. After decontamination, digestion and concentration by the standard N-acetyl-L-Cysteine-NaOH (NALC-NaOH) method (8, 9), the specimens are cultured on an egg-based Löwenstein-Jensen medium according to IUATLD recommendation. Culture contamination varies within a range of 5-9%, depending on the season of the year, the highest rates being registered in the summer months; the rate of culture-positive samples is 5.5% (5). Different methods for culturing mycobacteria are used worldwide. Some of them were developed aiming at simplicity, low cost and a better chance of accomplishment at network level. A simple and inexpensive method for the culture of just-emitted sputum samples was reported by Kudoh and Kudoh in 1974 (10). Briefly, each of two sterile swabs impregnated with a useful particle of sputum sample is submerged into a tube containing 2 ml of 4% NaOH during two minutes for de-

contamination and immediately inoculated onto a slant of Ogawa culture medium (acidified egg-based medium). Recently, a multicentric study for evaluation of this technique was performed in Latin America with very promising results (6). Still, there is insufficient information about the accuracy of the Kudoh method under routine conditions, i.e., in non sterile samples that remain unrefrigerated during 2 or more days before being processed. The aim of this study was to evaluate the performance of the Kudoh method for the culture of mycobacteria in sputum samples, after being maintained and shipped at room temperature without the addition of any substances to prevent the overgrowth of contaminating flora.

The study was prospective and blind, with clinical sputum samples shipped from all over the country and assays performed by different technicians under routine conditions. All macroscopically purulent or muco-purulent sputum samples received in the TB Central Laboratory during the study period were included. All culture media were prepared in-house, and quality controlled by WHO/PAHO standard procedures (14). Performance of Kudoh was evaluated using the NALC-NaOH method as gold standard.

A total of 784 clinical samples were cultured by both methods, performed according to Kudoh *et al.* (10) and the NALC-NaOH method, respectively (8, 9). After decontamination, each specimen was inoculated onto 2 slants of each Ogawa and Löwenstein-Jensen (IUATLD modification) media. The study was performed during the summer at an average temperature of 25 °C (minimum 22 °C - maximum 32 ºC); cultures were performed by technicians under routine conditions, incubated at 37 °C (normal atmosphere) and read after 7, 21 and 56 days of incubation.

The acidified IUATLD medium was compared with the Ogawa medium for culture after the Kudoh decontamination procedure. One hundred smear - positive samples were inoculated onto 2 slants of Ogawa medium and 2 slants of IUATLD acidified Löwenstein-Jensen medium; this latter medium was acidified to pH 6.4 with the addition of citric acid (1.35 g/l of medium). The composition of both egg-based media differs slightly: the Ogawa formula omits magnesium salts and asparagine, and includes higher concentrations of glycerol (2% *vs* 0.75%) and malachite green (2% *vs* 1.25%). Mycobacterial growth was evaluated as described above.

The effect of variations in decontamination time and NaOH concentration on mycobacterial recovery rates was assessed. In other set of experiments, a total of 25 isolated smear-positive sputum samples were comparatively cultured with the Kudoh method and increasing NaOH concentration of up to 6% and decontamination time up to 4 minutes. The original description of the Kudoh method (2-minute decontamination and 4% of NaOH) was considered the gold standard. Mycobacterial growth was quantified according to the WHO/PAHO semi-quantitative scale (14). Colony size, growth rate and aspect of colonies (smooth or rough) were also recorded.

Cultures were considered contaminated when microorganisms other than mycobacteria grew on the slants with or without media liquefaction. The contamination of only one tube was recorded as partial contamination. Mycobacterial growth was quantified according to the semi-quantitative scale proposed by the WHO/PAHO (14).

Kudoh and NALC-NaOH methods performed similarly regarding contamination and mycobacterial growth (Table 1). All positive cultures were identified as *Mycobacterium tuberculosis* and there was a very good correlation between size, growth rate, number of colonies and direct microscopy results. Similar percentages of contamination were observed after combining partial contamination with total contamination. The diagnostic performance of Kudoh (*vs.* NALC-NaOH as gold standard) for positive and non-positive (negative + contaminated) categories was: sensitivity 100% and diagnostic accuracy 98.9%. Agreement was 0.99; kappa: 1. Both methods showed identical results improving the diagnostic sensitivity by 22% over microscopic examination alone.

Results of assays with different decontamination times and NaOH concentrations are shown in Table 2. Mycobacterial growth was slightly affected when increasing decontamination times and NaOH concentrations. However, independently of the number of colonies, positive cultures were obtained in all time/NaOH concentration combinations. Different growth rates could be partially explained by the difficulty to standardize the inocula with this method.

A better morphology (rough and dry) and size of the colonies and more luxurious growth was observed when the acidified IUATLD culture medium was used instead of the Ogawa one.

Mycobacterial culture is still essential for TB diagnosis, being more specific and sensitive than microscopy. In Uruguay 14 - 20% of TB cases are confirmed by this method (5). In addition, culture provides strains for drug susceptibility testing and epidemiological surveillance. The main disadvantages of conventional cultures are:

Table 1. Comparative results of cultures from 784 sputum samples, processed by the Kudoh and NALC-NaOH methods.

Result(*)	Kudoh	%	NALC-NaOH	%
N(2t)	710	90.5	710	90.5
N(1t) – C(1t)	20	2.6	22	2.8
C(2t)	36	4.6	35	4.5
P(2t)	18 (**)	2.3	17 (**)	2.2

(*) N: negative, C: contaminated, P: positive, 1t: only one culture tube, 2t: two culture tubes. Data for the combinations N(1t)-P(1t) and C(1t)-P(1t) were null with both methods.
(**)Fourteen samples were positive to the direct microscopic examination

Table 2. Degree of positivity of 25 positive cultures according to different decontamination procedures applied to the Ogawa-Kudoh method.

(*) Nº of positive samples/(Nº col)	Modification of Ogawa-Kudoh (minutes - NaOH concentration)					
	4-4%		2-6%		4-6%	
7 (+++)	**4** (+++)	1 (++) 2(+)	**6** (+++)	1 (++)	**3** (+++)	4 (++)
8 (++)	**2** (++)	6 (+)	**5** (++)	1 (+++) 2(+)	**1** (++)	1 (+++) 6 (+)
6 (+)	**4** (+)	2 (1-19 col)	**4** (+)	2 (1-19 col)	**4** (+)	**1** (+++) 1 (1-19 col)
4 (1-19 col)	**3** (1-19 col)	1 (++)	**2** (1-19 col)	2 (+)	**2** (+)	2 (1-19 col)

(*) Results obtained with the original Ogawa-Kudoh method (2 minute-decontamination - 4% of NaOH). All samples had positive microscopy for mycobacteria. Colony counts were done according with the WHO/PAHO semiquantitative scale (12). Col: colonies.

delay in obtaining mycobacterial growth, high costs, complexity, and biosafety requirements. The Kudoh method is extensively used in several Asian countries, including Japan (8). Several reports in Latin America about the use of the Ogawa medium (12) and the swab Ogawa-Kudoh method documented good results (2, 3, 11). Jaspe *et al*., working in field conditions, reported similar performances when this method was compared with the standard Petroff digestion-decontamination procedure (7). In certain states of Brazil, the Ogawa-Kudoh swab method is routinely applied for primary culture of sputum samples (1, 4).

The centralization of bacteriological TB diagnosis in Uruguay generates some problems inherent to large-scale workload, namely the need of a high budget, an increased risk of intra-laboratory cross-contamination and culture contamination with overgrowing flora. This last issue is especially crucial in Uruguay, since our laboratory receives samples that have been obtained at least 48 hours earlier and transported at room temperature. Delays and lack of refrigeration can be the cause of culture contamination reaching 9% in the warmest months. Although the NALC-NaOH method is the reference procedure for decontamination and digestion, the adoption of a simpler technique with acceptable levels of contamination and recovery of mycobacteria may be an alternative for the routine culture of large numbers of respiratory samples.

The original Kudoh method has shown to be very useful for peripheral laboratories which process small numbers of samples daily, but decontamination time (2 minutes) may be difficult to maintain when 80 samples are serially processed by one technician; thus we considered of relevance to know how long it is possible to extend decontamination times without affecting mycobacteria growth. Our results showed that the decontamination time of the procedure duration is not critical and acceptable outcomes can still be obtained by increasing it up to 4 minutes. Furthermore, if a stronger decontaminant action is needed (i.e. deferred processing of naturally contaminated samples), it is also possible to increase the NaOH concentration with acceptable recovery of mycobacteria in culture.

Other issues to be considered are colony morphology and growth time, since these primary cultures are further used for phenotypic identification of mycobacterial species, drug-susceptibility testing and molecular identification techniques. In our hands, the substitution of Ogawa for the acidified IUATLD medium in primary Kudoh culture resulted in a more luxurious growth that granted a better handling of further bacteriological procedures.

The advantages of the Kudoh method using the Ogawa medium or another acidified egg-base medium are evident: it is inexpensive (its use would save 30,000 US dollars per year to the National TB Program in Uruguay), it is very simple to perform and does not require agitation and centrifugation steps. Therefore, we can also expect a reduction of equipment, intra-laboratory cross contamination rates and fewer biosafety requirements for laboratory personnel.

In conclusion, the Kudoh method for primary isolation of mycobacteria may be implemented in laboratories that work with large scale workload and turned out to be applicable for naturally contaminated respiratory samples that are maintained at room temperature during 2 to 4 days before being cultured, even in the summer months. The Kudoh method is simpler and cheaper than the NALC-NaOH one and performs similarly.

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