# Decontamination methods for samples preserved in cetylpyridinium chloride and cultured on thin-layer agar.

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# Decontamination methods for samples preserved in cetylpyridinium chloride and cultured on thin-layer agar

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SUMMARY

**SETTING: Long transportation times of samples to culture laboratories can lead to higher contamination rates and significant loss of viability, resulting in lower culture positivity rates. Thin-layer agar (TLA) is a sensitive culture method for the isolation of *Mycobacterium tuberculosis* that has been optimised with *N*-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) decontaminated samples. The combination of the TLA culture method and other decontamination procedures has not been extensively validated.**

**DESIGN: Among 390 smear-positive samples, we compared the culture positivity of samples decontaminated using the Petroff method vs. NALC-NaOH neutralised with phosphate buffer (PBS), applied to samples preserved with cetylpyridinium chloride (CPC) or CPC-free, and then of CPC-preserved samples decontaminated with NALC-NaOH neutralised using Difco neutralising buffer. The sediments were inoculated on TLA, and then on MGIT 960 or Löwenstein-Jensen (LJ) as gold standards.**

**RESULTS: Decontamination with NALC-NaOH yielded higher culture positivity in TLA than in the Petroff method, which was further enhanced by neutralising CPC with the Difco buffer. Surprisingly, culture positivity on LJ also increased after using Difco buffer, suggesting that CPC may not be completely neutralised in egg-based medium.**

**CONCLUSIONS: After transportation in CPC, decontamination using NALC-NaOH followed by neutralisation using Difco buffer resulted in the best recovery rates for samples inoculated on TLA and on LJ.**

**KEY WORDS**: tuberculosis; neutralising buffer; thin-layer agar; cetylpyridinium chloride

USE OF AUTOMATED nucleic acid amplification techniques can speed up the diagnosis of tuberculosis (TB) and the detection of multidrug-resistant TB, but does not eliminate the need for conventional culture, both for phenotypic drug susceptibility testing (DST) against drugs other than isoniazid (INH) and rifampicin (RMP) and for patient monitoring during treatment.[1] However, in most countries, samples need to be transported to central laboratories for comprehensive diagnostic services, which can take several days, resulting in increasing sample contamination and loss of mycobacterial viability, with subsequent lower recovery rates.[2] *N*-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) is commonly used to reduce contamination in specimens before inoculation on selective media. Alternative decontamination techniques include the Petroff method[3] and cetylpyridinium chloride (CPC), an ammonium compound added to samples during storage or transportation for partial digestion and decontamination.[4]

Thin-layer agar (TLA) is an inexpensive, sensitive culture method for *Mycobacterium tuberculosis* based on microcolony detection.[5] When used for smear-positive samples, the median time required for mycobacterial detection is 14 days, vs. 9.6 days with the liquid culture BACTEC™ MGIT™ 960 system (BD, Sparks, MD, USA) and 23 days using solid Löwenstein-Jensen (LJ) medium.[6–8] However, TLA culture can yield high contamination rates when inoculated with samples previously treated with Petroff or NALC-NaOH alone.[6,7] The combined application of NALC-NaOH and CPC has rarely been described, and combination of CPC with the Petroff method is discouraged.[9] The use of CPC is incompatible with agar-based media unless CPC is neutralised, limiting its application with TLA. Sediment-containing CPC can be inoculated on LJ, as phospholipids present in egg-based medium can neutralise the residual of this ammonium compound.[10,11] The Difco neutralising buffer (BD) contains monopotassium phosphate with buffering capability, and sodium thiosulfate and aryl sulfonate,

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which inactivate the effect of chlorine and quaternary ammonium compounds, respectively. Its effect on CPC has been described elsewhere,[10,11] although its applicability to agar medium needs further investigation.[12]

The objective of the present prospective study was to identify an improved method to recover mycobacteria in TLA from samples that need to undergo prolonged transportation. To our knowledge, no studies have described decontamination with NALC-NaOH of samples preserved in CPC and combined with Difco buffer before inoculation onto TLA plates.

We report the application of TLA to samples decontaminated using four different methods: 1) NaOH neutralised with hydrochloric acid (HCl) (i.e., the Petroff method); 2) NALC-NaOH alone neutralised with 6.8 pH phosphate buffer solution (PBS) (NALC-NaOH/PBS); 3) NALC-NaOH in combination with CPC neutralised with PBS (NALC-NaOH-CPC/PBS); and 4) NALC-NaOH with CPC neutralised using Difco buffer (NALC-NaOH-CPC/Difco). Decontamination with CPC alone was not used for routine testing, as previous experience has suggested that this results in a high contamination rate (data not presented). The performance of TLA is analysed in terms of positivity rate, contamination rate and time to positive results, expressed as turnaround time (TAT), compared to culture results using the MGIT 960 or LJ medium gold standards.

## MATERIALS AND METHODS

### *Study design*

The Mycobacteriology Laboratory at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, receives diagnostic samples from various Médecins Sans Frontières TB projects for culture and DST. In the field, each patient submitted three samples for microscopy investigation, from which two were selected based on higher microscopy grade; these were stored at 2–8°C for a maximum of 4 days. Before shipment at room temperature to the ITM, one of the two samples was added to an equal volume of 1% CPC and 2% NaCl.

At reception, CPC-free samples were randomly decontaminated using NALC-NaOH or the Petroff method, while samples containing CPC were treated only with NALC-NaOH. Sediments obtained from samples containing CPC, which is incompatible with liquid medium,[13] were inoculated onto LJ, while sediments from samples without CPC were inoculated in MGIT 960. All portions of sediments from smear-positive mucopurulent samples to be discarded were inoculated in parallel onto TLA plates.

We first compared three decontamination methods: Petroff, NALC-NaOH/PBS and NALC-NaOH-CPC/PBS. We then substituted PBS with the Difco buffer to compare NALC-NaOH-CPC/PBS with NALC-NaOH-CPC/Difco. Results recorded in the study database were de-identified from patient data.

The study protocol was approved by the Ethical Review Board of the ITM, Antwerp, Belgium.

### *Decontamination methods*

#### *Petroff method*

For the Petroff method, reagents were prepared as described by Kent.[11] Samples were added to 3% NaOH in equal volume, incubated at room temperature for 20 min, then neutralised with HCl. After centrifugation at 3000 x *g* for 20 min, the sediment was added to 1 ml sodium chloride (NaCl) 0.85% solution, then inoculated onto MGIT 960 according to the manufacturer's instructions.[13] The remaining resuspended sediment was diluted with 2.5 ml of NaCl solution; 0.1 ml of the 1:6 dilution was inoculated into the TLA medium on each half of the plate.

#### *NALC-NaOH/PBS method*

For the NALC-NaOH/PBS method, NALC-3% sodium hydroxide citrate (NALC-NaOH) was prepared as described by Kent,[11] then added to an equal volume of the sample. After 20 min incubation, the sample was neutralised with PBS and then centrifuged. The sediment was resuspended with 1 ml NaCl solution, and then inoculated into MGIT 960. The remaining resuspended sediment was treated and inoculated onto TLA as described above.

#### *NALC-NaOH-CPC/PBS method*

The samples were processed as in the NALC-NaOH/PBS method, except for the use of distilled water instead of NaCl solution to resuspend the sediment and a 10-min incubation time to minimise killing of mycobacteria by the combined decontamination methods.

#### *NALC-NaOH-CPC/Difco method*

For the NALC-NaOH-CPC/Difco method, the decontamination procedure was as described above for samples with CPC, except that PBS was substituted by Difco buffer during the neutralisation step.

### *Inoculation onto TLA plates*

Plates were prepared using a plastic Petri dish (BD) divided into two: one half for growth control and the other half for mycobacterial identification. The growth control contained 5 ml Middlebrook 7H11 enriched with 10% oleic acid, albumin, dextrose and catalase (OADC) (BD), followed by piperacillin, trimethoprim and amphotericin (Sigma, St Louis, MO, USA), all at 4 μg/ml concentrations.[14] The other half of the plate contained the same medium enriched

with *p*-nitrobenzoic acid (PNB) (500 μg/ml) (Sigma). After inoculation, the TLA plates were left to dry for 30 min in the biosafety cabinet, sealed with parafilm, leaving 1 cm uncovered to allow ventilation, and then incubated at 37°C in a 7.5% carbon dioxide incubator.

The plates were examined using microscopy at x100 magnification at days 5, 7, 9, 13, 15, 20, 25, 30, 35 and 40. Detection of *M. tuberculosis* was based on the presence of growth only on the growth control, confirmed by the appearance of typical cording. Plates were reported as contaminated when the contaminant growth hampered interpretation of the plate, and negative if no growth was detected within 40 days.

### *Inoculation on BACTEC MGIT 960 and LJ*

Cultures in MGIT 960 were performed following the manufacturer's instructions. Positive tubes were checked using microscopy and blood agar medium, and incubated for 48 h at 37°C. LJ medium was prepared according to international standards, then inoculated and incubated at 37°C and examined weekly for up to 8 weeks. Mycobacterial growth detected either in MGIT 960 or on LJ was identified using LJ medium containing PNB (500 μg/ml), while a PNB-free LJ tube was used as control; both were inoculated with 0.1 ml of the suspension from positive cultures.

### *Analysis of the data*

Positivity and contamination rates were calculated using the total number of samples inoculated as denominator. We used McNemar's tests to measure the agreement between TLA and the gold standard methods, and Fisher's exact test to compare the positivity rate of the different decontamination methods. TATs were compared using the Wilcoxon signed rank-sum test or the Wilcoxon Mann-Whitney test for paired and independent samples, respectively. Pairwise comparisons of transportation time were made using the Mann-Whitney test, adjusting the level of significance to $<0.01$ using the Bonferroni correction for multiple pairwise comparisons. TATs were calculated from day of inoculation to day of positive cultures. In the case of MGIT 960, this included the time required for preliminary identification using smear microscopy and blood agar medium.

## RESULTS

Between February 2010 and March 2011, we received 390 smear-positive samples: 173 containing CPC and 217 without CPC. The 173 CPC-containing samples were further decontaminated with NALC-NaOH, of which 94 were neutralised using PBS and 79 with Difco. Of the 217 specimens without CPC, 56 were decontaminated using the Petroff method and 161 with NALC-NaOH/PBS. The positivity rate was equally distributed among the decontamination groups. The median (interquartile range) transportation time of samples decontaminated with Petroff was 13 days, significantly longer than for samples decontaminated with NALC-NaOH/PBS (10 days, $P < 0.01$), NALC-NaOH–CPC/Difco (10 days, $P = 0.03$) or NALC-NaOH-CPC/PBS (9 days, $P < 0.01$). All culture-positive samples were identified as *M. tuberculosis*. Table 1 shows the performance of the tests when samples were decontaminated using Petroff, NALC-NaOH/PBS or NALC-NaOH-CPC/PBS. The overall positivity rate for TLA combined with the gold standard was 69.6% using Petroff, 90% using NALC-NaOH/PBS and 81.9% using NALC-NaOH-CPC/PBS.

Culture on TLA yielded higher positivity rates for samples decontaminated with NALC-NaOH/PBS ($P = 0.005$) and NALC-NaOH-CPC/PBS ($P = 0.608$) than Petroff. However, the positivity rate was

**Table 1** Performance of TLA, BACTEC™ MGIT™ 960 and LJ inoculated after decontamination using Petroff or NALC-NaOH neutralised with PBS

| Method | TLA *n* (%) | MGIT 960 *n* (%) | LJ *n* (%) | McNemar's test *P* value |
|---|---|---|---|---|
| Petroff ($n = 56$) | | | | |
| Positivity rate | 32 (57.1) | 33 (58.9) | | 0.7815 |
| Contamination rate | 9 (16.1) | 10 (17.9) | | 0.7630 |
| TAT, days, median [IQR] | 7 [5–28] | 16 [9–49] | | 0.0059 |
| NALC-NaOH/PBS ($n = 161$) | | | | |
| Positivity rate | 125 (77.6) | 142 (88.2) | | 0.0011 |
| Contamination rate | 6 (3.7) | 8 (5.0) | | 0.5271 |
| TAT, days, median [IQR] | 8 [5–32] | 15 [8–69] | | 0.001 |
| NALC-NaOH-CPC/PBS ($n = 94$) | | | | |
| Positivity rate | 58 (61.7) | | 74 (78.7) | 0.0006 |
| Contamination rate | 0 (0.0) | | 0 (0.0) | 1 |
| TAT, days, median [IQR] | 12 [5–40] | | 28 [16–79] | <0.001 |

TLA = thin-layer agar; MGIT = Mycobacteria Growth Indicator Tube; LJ = Löwenstein-Jensen; NALC-NaOH = *N*-acetyl-L-cysteine-sodium hydroxide; PBS = phosphate buffer solution; TAT = turnaround time; IQR = interquartile range; CPC = cetylpyridinium chloride.

significantly reduced for NALC-NaOH-CPC/PBS compared to NALC-NaOH/PBS (77.6% vs. 61.7%, $P = 0.009$), while no contamination was detected either on TLA or on LJ. Four TLA plates inoculated with sediment from decontamination with NALC-NaOH/PBS and one with NALC-NaOH-CPC/PBS showed partial contamination, which did not prevent interpretation. LJ and MGIT 960 showed better positivity rates than TLA when using NALC-NaOH. TAT for TLA increased slightly for samples containing CPC.

All plates inoculated after decontamination with NALC-NaOH-CPC/PBS presented debris that interfered with microscopy reading. To assess whether Difco would better neutralise the effect of CPC, we substituted PBS for Difco buffer (Table 2). The overall positivity rate for combined TLA and LJ using NALC-NaOH-CPC/Difco was 93.7%. The positivity rate significantly increased for both media compared to samples treated with NALC-NaOH-CPC/PBS for TLA (61.7% vs. 86.1%, $P < 0.001$) and for LJ (78.7% vs. 93.7%, $P = 0.008$); no plates were contaminated. The median TAT was reduced on both TLA and LJ. All plates could be clearly readable, as the Difco buffer completely eliminated debris (Figure).

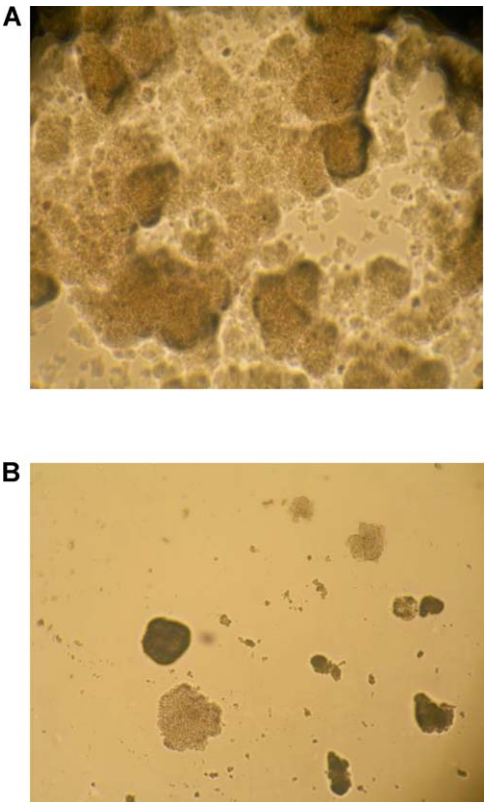


**Figure** TLA appearance by microscopy reading at x10 magnification with and without Difco neutralisation: **A**) sediment with CPC inoculated onto TLA and neutralised with PBS; **B)** sediment with CPC inoculated onto TLA and neutralised with Difco buffer. TLA = thin-layer agar; CPC = cetylpyridinium chloride; PBS = phosphate buffer solution. This image can be viewed online in colour at http://www.ingentaconnect.com/content/iuatld/ijtld//2014/00000018/00000008/art00017

## DISCUSSION

Although TLA has been optimised for use with NALC-NaOH decontaminated samples, use of other decontamination procedures had not yet been extensively validated. In the first phase of our study, samples processed with NALC-NaOH/PBS showed higher positivity rates than NALC-NaOH-CPC and Petroff, with contamination rates below the recommended threshold of 5–10% for samples processed after long transportation.[15] The Petroff method gave lower positivity rates for TLA and higher contamination rates, in line with contamination rates on TLA and in MGIT 960 (17% and 21.1%, respectively) found by Martin et al.[7] However, in contrast to our results, the positivity rates reported by Martin et al. were very high (97.3% on TLA and 97% on MGIT 960) for smear-positive samples. In our study, Petroff also adversely affected the performance of MGIT 960, which was not significantly different from TLA.

**Table 2** Performance of TLA and LJ inoculated after decontamination with NALC-NaOH-CPC/Difco ($n = 79$)

| | TLA *n* (%) | LJ *n* (%) | McNemar's test *P* value |
|---|---|---|---|
| Positivity rate | 68 (86.1) | 74 (93.7) | 0.0143 |
| Contamination rate | 0 (0.0) | 0 (0.0) | 1 |
| TAT, days, median [IQR] | 8 [5–27] | 26 [13–48] | <0.001 |

TLA = thin-layer agar; LJ = Löwenstein-Jensen; NALC-NaOH = *N*-acetyl-L-cysteine-sodium hydroxide; CPC = cetylpyridinium chloride.

These results for Petroff could be partially explained by the delay in processing the samples, which was longer than for samples decontaminated with other methods.

In our study, the contamination rate for NALC-NaOH/PBS was lower than that reported by Mejia et al. (16.5%)[8] and Martin et al. (26%).[6] Robledo et al., in a multicenter study, found rates of between 2.7% and 9.5%.[16] These higher rates may be due to the lower concentration of antibiotics added to the medium (0.02 µl/ml instead of the 4.0 µl/ml used in our study). Smithwick et al. reported attempting to find an alternative procedure to NALC-NaOH, but failing to find a concentration of CPC that would allow growth of mycobacteria on agar while controlling contamination.[17] The authors then concluded that CPC could be considered a valuable method if combined with LJ to recover mycobacteria. Selvakumar et al. reported similar results, finding comparable positivity rates for samples treated with NaOH on the day of sampling and samples to which CPC had been added and processed after 7 days, while the perfor-

mance of NaOH for CPC-free samples processed after 1 week was significantly lower.[4]

Our study demonstrated that CPC reduced contamination rates but increased TAT and significantly affected positivity, possibly due to the higher NaOH concentration used here compared to the study by Smithwick et al.[17] (2% vs. 1% final concentration) and to the use of CPC in combination with NALC-NaOH, which is more harsh than CPC alone. The use of Difco buffer to neutralise the effect of CPC greatly improved the performance of TLA in terms of positivity rates. The combination of TLA and Difco buffer resulted in recovery rates comparable to MGIT 960 combined with NALC-NaOH/PBS, considered the gold standard method for decontaminating and culturing samples for mycobacterial investigation.

These results are similar to the findings by Pardini et al., who reported higher positivity rates in samples containing CPC and neutralised with Difco buffer compared to using NaOH alone (63.8% and 47.1%, respectively) before inoculation onto agar and LJ medium.[10] The positivity rate increased to 78.3% after retreatment of sediments obtained from CPC-containing samples with NaOH alone. However, in this study decontaminants were not applied simultaneously, the results for this second retreatment were combined with the results of using CPC alone, and the authors did not report the performance for agar and LJ separately.

In our experience, Difco buffer also improved the reading of the plates by eliminating debris from CPC-containing samples, as colonies were clearly visible by microscope at an earlier stage of growth, shortening the time to detection. Recovery of mycobacteria on LJ from samples treated with NALC-NaOH-CPC/PBS was lower than the rate reported in another study.[18] Interestingly, the Difco buffer also improved the recovery rate on LJ from 78.8% to 93.7% ($P = 0.005$), suggesting that CPC may not be completely neutralised by the egg-based medium during incubation.

However, except for when the Petroff method was used, the TLA positivity rate was significantly lower than the two gold standards. This could be due to the greater dilution of the inoculum on TLA than in MGIT 960 and LJ required for microscopic interpretation, which could be reduced to improve positivity. Even if CPC increased the TAT for TLA plates, the time to positivity for TLA was significantly shorter than in MGIT 960 and LJ, regardless of the decontaminant used. In addition, NALC-NaOH-CPC/Difco reduced the time to detection to 8 days, equal to that of samples without CPC.

In conclusion, in our experience, NALC-NaOH-CPC/Difco is a preferable method for decontamination of samples inoculated onto TLA, with improved recovery of mycobacteria from LJ when applied to samples processed after transportation in CPC. TLA showed a significant improvement over MGIT 960 in performance with this decontamination method, and had a significantly shorter isolation time than LJ. The disadvantage of TLA is the lower positivity rate than with LJ and the additional effort required to read the plates using a microscope. However, TLA provides simultaneous detection and identification of culture growth without opening the plate, and reduces the need for safety requirements in the laboratory compared to MGIT 960 and LJ, which require manipulation of colonies to allow identification. As the recovery rate on LJ for samples treated with Difco buffer proved the best method, the combination of TLA and LJ for samples treated with this reagent would represent the optimal strategy for both fast and sensitive mycobacterial recovery. Contamination was absent from CPC-containing samples with both the TLA and LJ methods, which suggests that it may be possible to reduce the time of decontamination or NaOH concentration to improve positivity. Future studies are required to test whether Difco buffer on samples containing CPC can make these compatible with inoculation in liquid media to reduce the contamination rate while maintaining good recovery rates.

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## RESUME

**CONTEXTE : Des temps de transport longs des échantillons jusqu'aux laboratoires de culture peuvent entrainer un taux de contamination plus élevé et une perte significative de viabilité, d'où un taux de positivité réduit des cultures.**

**OBJECTIFS : L'agar en fine couche (TLA) est une méthode de culture sensible pour l'isolation de *Mycobacterium tuberculosis* préalablement optimisé avec des échantillons décontaminés au *N*-acetyl-L-cysteine-sodium hydroxyde (NALC-NaOH). Sa combinaison avec d'autres procédures de décontamination n'a pas été largement validée.**

**SCHÉMA : Dans cette étude sur 390 échantillons à frottis positifs, nous avons comparé la positivité des cultures d'échantillons décontaminés avec la méthode Petroff contre NALC-NaOH neutralisée avec un tampon phosphate (PBS), appliquée aux échantillons soit préservés avec du chlorure de cetylpyridinium (CPC) ou sans CPC, puis aux échantillons conservés sur CPC décontaminés par NALC-NaOH neutralisé avec du tampon neutralisant Difco. Les sédiments ont été inoculés sur TLA, puis les standards de référence MGIT 960 ou Löwenstein-Jensen (LJ).**

**RÉSULTATS : La décontamination par NALC-NaOH a abouti à un taux plus élevé de positivité des cultures sur TLA que sur Petroff, qui a encore été améliorée ensuite par neutralisation du CPC avec le tampon Difco. Etonnamment, la positivité des cultures sur LJ a également augmenté après tampon Difco, suggérant que le CPC ne serait pas complètement neutralisé en milieu à base d'œuf.**

**CONCLUSIONS : Après transport sur CPC, une décontamination par NALC-NaOH suivie par une neutralisation par tampon Difco a abouti aux meilleurs résultats avec les échantillons inoculés sur TLA et sur LJ.**

## RESUMEN

**MARCO DE REFERENCIA: El prolongado lapso de transporte de las muestras a los laboratorios de cultivo puede ocasionar mayores tasas de contaminación y una pérdida considerable de la viabilidad de las micobacterias, con la consecutiva disminución de la tasa de positividad de los cultivos.**

**OBJETIVOS: El cultivo en agar de capa delgada (TLA) es un método sensible de aislamiento de *Mycobacterium tuberculosis* que se ha optimizado gracias a la descontaminación de las muestras con *N*-acetil-L-cisteína en una disolución de hidróxido de sodio (NALC-NaOH). No se ha validado ampliamente la asociación de este método con otros procedimientos de descontaminación.**

**MÉTODO: En el presente estudio se analizaron 390 muestras con baciloscopia positiva y se comparó la positividad del cultivo de las muestras descontaminadas con el método de Petroff y de las muestras descontaminadas mediante NALC-NaOH, que se neutralizaban con solución salina amortiguada por fosfatos cuando las muestras se habían conservado en cloruro de cetilpiridinio (CCP). Luego, en las muestras conservadas en CCP y descontaminadas con NALC-NaOH, se sustituyó la solución salina por un amortiguador neutralizante de Difco. Los sedimentos se sembraron en TLA y en los medios de referencia, a saber el sistema MGIT 960 o el medio de Löwenstein Jensen (LJ).**

**RESULTADOS: La descontaminación con NALC-NaOH aportó una mayor proporción de cultivos positivos en TLA que el método de Petroff y esta ventaja se aumentó al neutralizar el CCP con el amortiguador Difco. Inesperadamente, la positividad de los cultivos en medio de LJ también aumentó con el uso del amortiguador Difco, lo cual parece indicar que el CCP no se neutraliza totalmente en el medio a base de huevos.**

**CONCLUSIÓN: Después de transportar las muestras en CCP, la descontaminación con NALC-NaOH seguida de neutralización con el amortiguador Difco mejora las tasas de aislamiento de micobacterias de las muestras sembradas en TLA y en medio de LJ.**