International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 11, Issue 3, 2019, pp.-1518-1520.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

Research Article

COMPARATIVE STUDY BETWEEN BENZALKONIUM CHLORIDE TRI-SODIUM PHOSPHATE AND NALC-NAOH DECONTAMINATION METHODS FOR RECOVERY OF *MYCOBACTERIUM TUBERCULOSIS* FROM PULMONARY SAMPLE

BHIRANGE S., MISRA R.N., HATOLKAR S., GUPTA N. AND JADHAV S.*
Department of Microbiology, Dr D.Y. Patil Medical College Hospital and Research Centre, Dr D. Y. Patil University, Pimpri, Pune, 411018, India
*Corresponding Author: Email - patilsv78@gmail.com

Received: December 01, 2018; Revised: March 26, 2019; Accepted: March 27, 2019; Published: March 30, 2019

Abstract- Introduction: Tuberculosis [TB] is a major public health concern worldwide over the last decades. About 80% of the global TB burden is in low income countries. Globally Multi drug resistant Tuberculosis caused an estimated 6 lack new TB cases and 2.40000 deaths in 2016.MDR TB accounts for 4.1% of all new TB and 19% of previously treated cases. Most of them occur in South America, Southern Africa, India, China and the former Soviet Union. Microbial diagnosis of TB consists of conventional and molecular methods but decontamination plays major role for perfect staining and cultures. **Material and methods:** 60 pulmonary samples were treated with benzalkonium chloride Tri-sodium phosphate and NALC-NaOH methods. All processed for ZN staining and LJ cultures were incubated at 37°C for 8 weeks. **Results:** Out of 60 respiratory clinical samples, 36 (60%) clinical samples were Z-N smear positive and 24(40%) were Z-N smear negative. Culture positivity was observed as 33 (55%) by NALC-NAOH method and 35 (58.3%) by Benzalkonium chloride method.
Conclusion: Benalkonium chloride trisodium phosphate appears to be a reasonable incorporation in decontamination procedure and is fairly non-toxic to *mycobacterium* to improve growth in culture and a reasonably better mucolytic reagent.

Keywords- *Tuberculosis, Benzalkonium chloride method*

Citation: Bhirange S., *et al*., (2019) Comparative Study Between Benzalkonium Chloride Tri-Sodium Phosphate and NALC-NaOH Decontamination Methods for Recovery of *Mycobacterium Tuberculosis* from Pulmonary Sample. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 11, Issue 3, pp.-1518-1520.

Introduction

Tuberculosis (TB) is a major public health concern worldwide: despite a regular, although slow, decline in incidence over the last decade, as many as 8.6 million new cases and 1.3 million deaths were estimated to have occurred in 2012. About 80% of the global TB burden is in low-income countries, where pulmonary disease and transmission are serious public health problems [1,2]. Multi-drug-resistant tuberculosis (MDR-TB) is a form of tuberculosis (TB) infection caused by bacteria that are resistant to treatment with at least two of the most powerful first-line anti-TB medications (drugs), isoniazid and rifampin. Some forms of TB are also resistant to second-line medications, and are called extensively drug-resistant TB (XDR-TB) [3,4]. MDR-TB caused an estimated 600,000 new TB cases and 240,000 deaths in 2016 and MDR-TB accounts for 4.1% of all new TB cases and 19% of previously treated cases worldwide. Globally, most MDR-TB cases occur in South America, Southern Africa, India, China, and the former Soviet Union [5,6]. The microbiological diagnosis of TB is an important tool for disease control. It consists of both conventional methods (acid-fast microscopy, culture, biochemical identification, anti-tuberculosis drug-susceptibility testing; DST) and modern molecular techniques. The targets of microbiological testing include the detection and isolation of mycobacteria, species identification, detection of drug resistance, monitoring patient responses to therapy and epidemiological typing of *Mycobacterium* strains [7,8]. A culture is performed on either solid media, for example Lowenstein-Jensen (L-J) or Middlebrook 7H10/11 (Petroff's method, modified Petroff's, NAOH 2% N-acetyl-cystein), or liquid media. There are several types of decontamination methods for digestion and homogenization of pulmonary specimens for TB. In that we are selecting NALC–NAOH method and Benzalkonium Chloride tri-sodium phosphate method to evaluate for their independent efficacies and for culture recovery of *Mycobacterium tuberculosis*.

In developing countries, culture on Lowenstein-Jensen solid medium is the gold standard for microbiological diagnosis of TB and requires about 10 bacilli/ml of specimen for recovery of mycobacteria. The slow growth rate of the pathogen leads to a delay of 4-6 weeks in obtaining a definitive diagnosis [3,4, 9,10].

Material and Methods

The study was conducted at the Department of Microbiology, Dr. D. Y. Patil Medical College, Hospital and Research Center, Pimpri, Pune. 60 clinical samples were diagnosed for pulmonary tuberculosis. Each sample was processed with following procedures: Sample Preparation About 5 ml of BAL and sputum samples were collected. These were divided into two equal aliquots and processed for ZN Staining and decontamination. The amount of the samples was 2-2.5 ml approximately for both methods. BAL samples were centrifuged and sediment was processed for decontamination procedure. Work was done in Biosafety cabinet level II Ziehl-Neelsen (Spot and early morning samples of sputum were collected in 2 sterile wide mouth containers were processed and graded on the same day as per Revised National Tuberculosis Control Program (RNTCP) guidelines) (ZN) staining for each sample was done before and after decontamination [5].

Decontamination of sputum sample

1) NALC-NAOH method
2) Benzalkonium Chloride phosphate method

Culture: After decontamination with both methods. The samples were inoculated directly on Lowenstein Jensen (LJ) medium prepared in house.

Table-1 *Distribution of direct smear examination and culture results*

Results	Microscopy			Culture		
	Direct (%)	NALC-NAOH (%)	BCTSP (%)	Direct (%)	NALC-NaOH (%)	BCTSP (%)
Positive	36	36 (60 %)	36(60 %)	36	33 (55%)	35 (58.3%)
Negative	24	24 (40%)	24(40%)	24	24 (40%)	23 (38.3%)
contamination	NA	00	00	NA	3 (5%)	2 (3.3%)
Total		60			60	60

Table-2 *Week wise (Wk) growth observations on LJ Medium*

Methods	Total Samples	1st Wk	2nd Wk	3rd Wk	4th Wk	5th Wk	6th Wk	7th Wk	8th Wk	Total%
Nalc-NaOH method	60	0	4(6.7%)	9(15%)	10(16.7%)	3(5%)	2(3.3%)	4(6.7%)	1(1.6%)	33%
Benzalkonium trisodium phosphate Method	60	0	0	25(41.7%)	5(8.3%)	2(3.3%)	2(3.3%)	1(1.6%)	0	35%
Contamination NALC-NaOH method	0	0	0	0	1	2	0	0	0	
Contamination Benzalkonium chloride Tri-sodium Phosphate method	0	0	0	0	1	1	0	0	0	

Table-3 *Comparison of two decontamination methods for rate of contamination, negative culture and culture positives*

Concentration method	No. of contaminated slopes	Negative cultures (No of slopes with no growth upto 8 weeks	Positive cultures (No.of slopes with growth upto 8 weeks)
Nalc-NaOH method	3 (5%)	24 (40%)	33 (55%)
Benzalkonium tri-sodium phosphate Method	2 (3.3%)	23(38.3%)	35 (58.3%)

Table-4 *Sensitivity and specificity for culture of NALC-NaOH and BCTSP method*

NALC-NaOH/ BCTSP r	NALC-NaOH Method +ve	NALC-NaOH Method -ve
BCTSP Method +ve	32	3
BCTSP Method -ve	1	24

Note: NALC-NaOH method is Gold Standard and BCTSP method is a Test method

NALC-NAOH Method

The sample was treated with an equal volume of N-acetyl-L-cysteine (NALC) plus 2 percent NaOH, the mixture was vortexed for 20 sec and kept at room temperature for 15 min. To this was added phosphate buffer (pH- 6.8-7) and centrifuged at 3000 g for 15 min. The deposit was re-suspended in 1 ml of buffer, and 0.1 ml from this was used as an inoculum to LJ culture media [6].

Benzalkonium Chloride –Trisodium Phosphate Method

5 g analar tri-sodium phosphate was dissolved in 20 ml hot sterile distilled water, to which 0.35 ml of 17 per cent benzalkonium was added. Equal parts of sample and prepared solution were mixed in a mechanical shaker, and then allow it to stand for 30 min at room temperature; this solution was neutralized by adding phosphate buffer (pH 6.8-7) and centrifuged at 3000 g for 15 min [7, 8].The concentrated deposit was re-suspended in 1 ml of sterile normal saline, and then LJ culture media was then inoculated by 0.1 ml of sediment solution. The culture slants were incubated at 37ºC up to 8 weeks. All Slopes were observed for occurrence of growth daily for first week and then at weekly intervals for 8 weeks. Absence of growth at the end of 8th week was reported as negative culture. Contamination, if any, was recorded separately.

Results

Out of 60 samples, 36 (60%) were ZN smear positive and 24(40%) were smear negative. Culture positivity was observed as 33 (55%) by NALC-NAOH method and 35 (58.3%) by Benzalkonium chloride method [Table-1]. On LJ typically rough, tough and buff colored growth of *Mycobacteria* appeared frequently after 3 weeks; however, some specimens took a longer period for exhibiting typical growth (6-8 wk).

Discussion

In this study, we used the NALC-NAOH decontamination method of Kent and Kubica *el al*. as it is widely used and recommended by standard laboratory manuals by WHO, BCTSP concentration method resulted better than NALC-NAOH method [1]. Microscopy and culture are the two important tools for diagnosis of TB but as microscopy requires as few as 10-100 bacilli/ml to be detected and it also detects dead bacilli, culture is considered as Gold standard [9]. When researcher processed for Microscopy before and after decontamination with both methods did not show any difference in smear readings. Negative culture and contamination rate were minimum with BCTSP method. Study done by Kent and Kubica et. al. and Pathak, Deshmukh and Menon (1973) suggested to use NALC-NaOH method over modified Petroff's Method (NaOH-4%)[1,16]. We kept our concentration as suggested. Diagnosis cannot be established only with smear and it should be correlated with culture and also provides confirmation in smear doubtful cases. One sample which did not show growth after decontamination with NALC-NAOH method showed growth after decontamination with BCTSP method which increases its sensitivity. When growth rate was concern it has been observed that average growth rate is good *i.e.* generally in 3rd week with BCTSP method. Comparison of two methods after smear microscopy shown following results for sensitivity and specificities. In Smear positive samples culture of BCTSP Method showed 96.9% and 88.89% Sensitivity and specificity over culture of NALC-NaOH method while in smear negative cases it showed 100% Sensitivity and specificity over 95.65% method. In this study more positive culture were achieved by BCTSP method. BCTSP method is more efficient method shown by these results. Our results were also comparable by Chattergee M. and Zabel L. in their studies. [14-16] .It has been reported that decontamination rate of BCTSP is lesser due to its chemical combination. NaOH method may treat the sample harshly causing killing of Mycobacteria. Average growth time to be positive is considerably less *i.e.* two weeks for smear positive cases with BCTSP method is added advantage for early diagnosis however BCTSP processed sediments cannot be used for further processing like Molecular diagnostics could be one of the limitations.

Conclusion

The modified Petroff's method is applied in combination with NALC-NaOH method and is now widely used for decontamination of pulmonary samples from tuberculosis patients. Addition of benzalkonium to trisodium phosphate appears to be a reasonable digestion procedure since these are fairly non-toxic to mycobacteria and a reasonably better mucolytic reagent. As sample size is less in this study it could be a limitation to comment.

Application of research: Benzalkonium chloride trisodium phosphate appears to

be a reasonable incorporation in decontamination procedure and is fairly non-toxic to *mycobacterium* to improve growth in culture and a reasonably better mucolytic reagent.

Research Category: Medical Microbiology

Acknowledgement / Funding: Authors are thankful to Department of Microbiology, Dr D.Y. Patil Medical College Hospital and Research Centre, Dr D. Y. Patil University, Pimpri, Pune, 411018, India

***Principal Investigator or Chairperson of research: Dr Savita Jadhav**
University: Dr D. Y. Patil University, Pimpri, Pune, 411018
Research project name or number: Clinical case study

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Department of Microbiology, Dr D.Y. Patil Medical College Hospital and Research Centre, Pimpri, Pune, 411018, India

Conflict of Interest: None declared

Ethical approval: Ethical approval taken from Dr D.Y. Patil Medical College Hospital and Research Centre, Dr D. Y. Patil University, Pimpri, Pune, 411018, India.
Ethical Committee Approval Number: Nil

References

[1] Kent P.T., Kubica G.P.W. (1985) *Public Health Mycobacteriology. A guide for the level III Laboratory. Atlanta: Centers for Disease Control*, 21-44.
[2] [IUATLD] Tuberculosis Division, International Union Against Tuberculosis and lung Disease (2005) Tuberculosis bacteriology-priorities and indications in high prevalence countries: position of the technical staff of the Tuberculosis. Division of the International Union against Tuberculosis and Lung Disease. Int J Tuberc Lung Dis., 9(4), 355-61.
[3] Longo, Fausci, *et al*. (2012) *Harrison's Principles of Internal Medicine (18th ed.). New York: McGraw Hill. pp. Chapter 165: Tuberculosis. Retrieved* 7 Dec 2016.
[4] "Diagnosis and notification of multidrug-resistant TB" (PDF). WHO. Retrieved 7 December 2016.
[5] "Drug-resistant tuberculosis". World Health Organization. Retrieved 2018-10-02.
[6] "Multi-drug-resistant tuberculosis (MDR-TB) – 2015 Update". WHO. Retrieved 7 December 2016.
[7] Cruciani M., Scarparo C., Malena M., *et al*, (2004) *J Clin Microbiol* 42(5), 2321-2325.
[8] [WHO] World Health Organization. Laboratory services in tuberculosis control Part II: Microscopy. WHO/TB/98.258. Geneva: World Health Organization. 1998.
[9] (IUATLD) International Union Against Tuberculosis and Lung Disease. Sputum examination for tuberculosis by direct microscopy in low income countries.5th Edition. Technical Guide, Paris: International Union Against Tuberculosis and Lung Disease 2000.
[10] Manual of standard operating procedures (SOPs) - Revised National Tuberculosis Control Programme, Central Tuberculosis division Directorate General of Health services Ministry of health and family welfare, Nirman Bhavan, New Delhi.
[11] Buijtels P.C., Petit P.L., (2005) *J Microbiol Methods,* 62,83-8.
[12] Steingart K.R., Vivienne N.G., Megan H., Hopewell P.C., Ramsay A., Cunningham J., *et al*., (2006) *Lancet Infect Dis*., 6, 664-74.
[13] Kumar B., Vinay S., Abbas A., (2007) *W.B. Saunders Company*; pp. 516–22.
[14] Mitali Chattergee, Sushmita Bhattacharya, Kalapana Karak, Sujata G. Dastidar (2013) *J Med Res*.138(4), 541-548.
[15] Zabel L.T., Albus J., Eirinq P. (1998) *Clinical Microbiology and Infection*, 4,116-118.
[16] Pathak S.K., Deshmukh P.A., Menon C.R. (1973) *Ind J Tubercle* 20,85-7.