World J Microbiol Biotechnol (2010) 26:523–526
DOI 10.1007/s11274-009-0201-5

ORIGINAL PAPER

An alternative sputum processing method using chitin for the isolation of *Mycobacterium tuberculosis*

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Received: 26 August 2009 / Accepted: 28 September 2009 / Published online: 13 October 2009

Abstract An alternative bio-friendly sputum processing method is the need of the hour to augment the rate of detection of TB cases and to improve the sensitivity of rapid growth based diagnostic methods. Chitin, mucolytic in nature and present ubiquitously in animal kingdom, was found to have decontaminating activity when used for processing sputum specimens. The aim of the present study is to develop an alternative bio friendly sputum processing method using chitin. Smear microscopy was done on direct sputum samples and on the deposits obtained after processing with modified Petroff's method as well as Chitin method. Two direct smears were made from each of the sputum samples and stained by Ziehl Neelsen and Auramine phenol (AP) method. The samples were divided in to two aliquots and processed by chitin and modified Petroff's method. Smears were made from each of the deposits and stained by both methods. The deposits were inoculated on to two Lowenstein Jensen slopes. AP method showed a sensitivity of 95% in direct smear. Samples processed by chitin and the deposit smears stained by AP method showed a sensitivity of 80% and a specificity of 89% compared to that of modified Petroff's method. The sensitivity of chitin culture is 87% and the specificity is 85%. Chitin–H_2So_4 solution took less time compared to 4% NaOH to homogenize the mucopurulent sputum specimens. Chitin–H_2So_4 can be used as an alternative method of sputum processing for the detection of *M. tuberculosis*.

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Keywords Chitin · Sputum processing · *M. tuberculosis* · Petroff's method

Introduction

Processing of sputum samples is the key step for the primary isolation and drug susceptibility testing of mycobacteria. The presence of normal flora and opportunistic microorganisms in these samples necessitate the decontaminating procedure as these organisms overgrow and mask the growth of tubercle bacilli otherwise. Sputum specimens have mucous strands, enzymes and other inhibitory factors that hamper the detection of viable tubercle bacilli in rapid broth based detection systems (Park et al. 2003) though not by conventional diagnostic procedures. The compromise in the detection of tubercle bacilli is greatly influenced both by the nature of samples and chemicals used for processing specimens (Thornton et al. 1998). An ideal processing method should be capable of efficient mucolysis of sputum specimens and control of normal flora without compromising the viability of *Mycobacterium tuberculosis*.

Several methods of sputum processing exist for the recovery of *M. tuberculosis*, which exhibit varying degrees of efficiency (Steingart et al. 2006). Processing of sputum samples by modified Petroff's method (Kent and Kubica 1985) involves the use of strong alkali, which is deleterious to tubercle bacilli to some extent warranting stringent adherence to the time of exposure to 4% NaOH. The traces of chemicals used for sputum processing may affect the host cell receptors, disrupting phage adsorption and expression of genes especially in phage based assays (Kumar et al. 2007). Therefore, an alternate novel, bio-friendly and non-chemical method of sputum processing is

the need of the hour to improve the detection of TB cases by rapid methods.

Chitin is a natural polymer found ubiquitously, forming the major portion of exoskeletons in multitude of organisms (Merzendorfer and Zimoch 2003). Chitin dissolved in hexa-fluroisopropanol was found to exhibit both mucolytic and decontaminating activity suitable for processing sputum specimens for smear microscopy (Farnia et al. 2002). Sulfuric acid at 5% concentration has been used conventionally as a decontaminating agent in paucibacillary extrapulmonary specimens (Mitchison and Aber 1974). In the present study we explored the efficacy of chitin–H_2SO_4 in mucolysis, decontaminating and in retrieving *M. tuberculosis* on solid media in comparison with modified Petroff's method.

Materials and methods

Standardization

Preliminary standardization experiments were carried out to identify the ideal solvent among H_2SO_4, HCl, and NaOH to dissolve chitin. Further evaluation was done to determine the ideal concentration of chitin that could lyse the mucous completely and retain the decontaminating ability without affecting the retrieval of *M. tuberculosis*.

Preparation of chitin–H_2SO_4

About 2 g of chitin (Hi-Media) was dissolved in 10 ml of concentrated sulphuric acid (Qualigens) and left overnight in shaker incubator at 37°C for complete dissolution. The solution was made up with 190 ml of distilled water to get a final concentration of 1% chitin in 5% H_2SO_4 and sterilized by Seitz filtration.

Comparison of smears

A total of 154 sputum samples were collected from patients attending Tuberculosis Clinics, Chennai. Two direct smears were made from each specimen; one was stained by Ziehl-Neelsen (RNTCP 1998) (ZN) technique and the other by fluorescent staining technique using auramine phenol (Selvakumar et al. 2004) (AP), coded and examined. The samples were aliquoted into two; one was processed by modified Petroff's method and the other by chitin–H_2SO_4. Briefly, each sample was mixed with double the volume of chitin–H_2SO_4, shaken for 15 min allowing complete homogenization and made up to 20 ml with sterile distilled water. The samples were centrifuged at 2,500×g for 15 min and the pellets were washed again with sterile distilled water. Two smears each were made from deposits obtained by both methods and stained by ZN and AP, coded, examined and compared.

Comparison of culture

A total of 248 samples were collected in sterile containers, aliquoted, randomized and processed by both methods as above. From the deposits thus obtained, two Lowenstein Jensen (LJ) slopes were inoculated, randomized and incubated at 37°C. The slopes were examined every week up to 8 weeks and observed for the appearance of growth. Decontaminating ability of both processing methods was assessed by inoculating one loopful of the processed deposits on to blood agar and incubating at 37°C for 18–24 h.

Results

Standardization

Among the different solvents tested, H_2SO_4 at 5% concentration was found to be most effective in dissolving chitin completely resulting in a clear solution. Chitin at 1% concentration in 5% H_2SO_4 was found to completely lyse the sputum in 30 min. The decontaminating ability and the capacity to retrieve *M. tuberculosis* were found to be comparable with that of modified Petroff's method.

Comparison of smears

Direct smears stained by AP and ZN methods were positive in 104 and 96 samples, respectively among 109 total positives, exhibiting a sensitivity of 95 and 88%. Deposit smears stained by auramine-phenol from chitin method compared to that from modified Petroff's method showed a sensitivity of 80% and a specificity of 89%. The agreement between the methods was 83% with kappa value of 0.637 (Table 1a). A similar comparison of smears from both methods stained by ZN showed a sensitivity and specificity of 85 and 94%, respectively. The agreement was 88% with kappa value of 0.755 (Table 1b).

Comparison of culture results

Cultures from sputum specimens processed by chitin in comparison with those processed by modified Petroff's method showed a sensitivity of 87% and specificity of 85%. The agreement between the methods was 86% with kappa value of 0.674 (Table 2). Chitin method was found to be more efficient when compared to modified Petroff's method in controlling the growth of normal flora. The

Table 1 Comparison of deposit smears from Petroff's and chitin methods

		Petroff's deposit		
		Pos	Neg	Total
a. AP staining				
Chitin deposit	Pos	86	5	91
	Neg	21	42	63
	Total	107	47	154
b. ZN staining				
Chitin deposit	Pos	84	3	87
	Neg	15	51	66
	Total	99	54	153

Note for a sensitivity 80%; specificity 89%; agreement 83%; kappa = 0.637

Note for b sensitivity 85%; specificity 94%; agreement 88%; kappa = 0.755

former method showed growth of normal flora in 5.8% of samples and the latter in 14.4% of samples.

Discussion

Chitin is one of many naturally occurring polymers resembling cellulose. Chitin dissolved in hexa fluroisopropanol (HFP) was found to improve the yield of smear microscopy (Farnia et al. 2002). Due to the higher molecular weight of chitin, a maximum of 30 min was only required to achieve complete sedimentation of bacilli with no need for centrifugation while other liquefaction methods needed more time. In 1998, Thornton et al. found that C18-Carboxypropyl betaine could homogenize sputum samples in 90 min and the sensitivity of smear microscopy was 93%. Bobadilla-del-Valle et al. (2003) used Cetyl Pyridinium Chloride (CPC), sodium carbonate and sodium borate to transport sputum and found all of them to be good mucolytic and decontaminating agents as well. *M. tuberculosis* was isolated from 86% of samples with sodium carbonate and sodium borate while the other samples showed overgrowth of bacteria and fungi. On the other hand, CPC showed a better decontaminating ability improving the positivity rate

Table 2 Comparison of Petroff's culture results with chitin culture results

	Petroff's culture results			
		Positive	Negative	Total
Chitin	Positive	157	10	167
Culture results	Negative	24	57	81
	Total	181	67	248

Sensitivity 87%; specificity 85%; agreement 86%; kappa = 0.674

up to 98%. In the present study, 1% chitin dissolved in 5% H_2SO_4 homogenized the sputum samples by 30 min. It controlled the growth of normal flora more efficiently without hampering the retrieval of tubercle bacilli. This is the first report on chitin in H_2SO_4 for processing sputum samples for the primary isolation of mycobacteria on solid media.

Solid or liquid media are being used for primary isolation of *M. tuberculosis* from clinical samples. This primary isolation is affected by the overgrowth of normal flora escaping the action of the processing chemical. Such surviving normal flora is controlled by the action of malachite green or antibiotics added to the medium (McClatchy et al. 1976). Chitin–H_2SO_4 controls the growth of normal flora more efficiently alleviating the use of any supplement thus making it more suitable and bio-friendly for such use.

The rapid diagnostic methods using liquid media to isolate *M. tuberculosis* include BACTEC 460, MGIT, MB/BACT, ESP Culture System II etc., which employ modified 7H9 medium supplemented with antibiotics in the form of PACT or PANTA to inhibit the growth of non-mycobacterial contaminants (Middlebrook et al. 1977). Phage based assays have been developed to detect viable *M. tuberculosis* and to do rapid drug susceptibility testing (Carriere et al. 1997). The incubation of the processed sputum samples overnight in broth media is mandatory in such a venture to allow the host cells to multiply and form one or two generations so that phage infection and expression will be optimal. However, the remnants of chemicals used for processing and overgrowth of normal flora influence the growth of tubercle bacilli during overnight incubation (Park et al. 2003). The problem of overgrowing normal flora in sputum deposits and probable interference in the adsorption of phage to bacteria by the remaining traces of processing chemical warrants newer sputum decontaminating methodologies especially in rapid phage based assays. As the above problems can be effectively handled by the use of chitin, it forms an ideal bio-friendly alternative to the existing sputum processing procedures.

Staining by AP method exhibited a high sensitivity compared to that of ZN corroborating with the earlier findings when the AP stain was applied to liquefied, concentrated samples (Murray et al. 2003), CPC treated samples (RNTCP 1998) and histological sections (Greenwood and Fox 1973).

The sputum samples processed by chitin and deposits thus obtained were found to be acidic in nature as sulfuric acid was used to dissolve chitin. When comparing the culture results of both methods, 24 out of 181 samples were negative by chitin method while 10 positives are not detected by Petroff's culture method. Neutralization of the pH of the chitin–H_2SO_4 processed deposit must lead to

help to use the method for the improved detection of tubercle bacilli. The adjustment in the pH to allow the normal growth of tubercle bacilli in chitin–H_2SO_4 processed samples will greatly promote the rapid TB diagnostic methods involving the incubation of processed samples in liquid media.

Conclusion

One percent chitin in 5% H_2SO_4 effectively controlled normal flora in sputum specimens. The retrieval of *M. tuberculosis* from chitin-processed samples and the control of normal flora were comparable with modified Petroff's method. Neutralization of sputum deposits processed by chitin can be ideal for the broth based rapid detection of *M. tuberculosis*.

Acknowledgments The study was supported in part by the WHO, with funds from USAID, through SEARO, New Delhi. One of the authors BS wishes to acknowledge the support from Indian Council of Medical Research, New Delhi, India.

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