Isolation and identification of etiological agent of pulmonary tuberculosis in patients visiting National Tuberculosis Center, Thimi, Bhaktapur

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Abstract

Introduction Pulmonary tuberculosis usually occurs in the apex of the lungs. The diagnosis can only be made reliably on demonstrating the presence of tubercle bacilli in the sputum by means of microscopy and/or culture in the laboratory. If it is desirable to identify *M. tuberculosis* complex prior to processing for drug susceptibility testing, the culture, if affluently growing, can be subjected to biochemical tests, i.e., Niacin, Nitrate reduction, and 68°C labile catalase test.

Objectives This study was performed in National Tuberculosis Center, Thimi, Bhaktapur, with an objective to isolate and identify the etiological agent of pulmonary tuberculosis by ZN staining, culture and biochemical testing of the subcultures. It is suggested that biochemical test is one of the best method to diagnose pulmonary tuberculosis.

Methods The study was cross-sectional study. All the samples were processed by modified Petroff's method. ZN staining of each sample was done and cultured. Subculture of each sample was performed and Niacin, 68^0C heat labile catalase, Nitrate reduction test and growth on para-nitrobenzoic acid (PNB) containing medium was performed from it.

Results A total of 200 clinically suspected sputum samples were examined by ZN staining and cultured in Ogawa medium. Primary culture samples were subcultured in the LJ medium. The subcultures were observed for their cultural characters for 4 weeks and then subjected for biochemical tests for their confirmation as *M. tuberculosis.* From the 200 subcultures Niacin, Nitrate reduction, 68ºC labile catalase test and growth on PNB containing medium was performed. Out of 200 samples, 190 (95%), 189 (94.5%), 6 (3%) and 7 (3.5%) were positive for Niacin, Nitrate reduction, 68ºC labile catalase and growth on PNB containing medium and 10 (5%), 11 (5.5%), 194 (97%) and 193 (96.5%) were negative for the respective tests and were Mycobacterium other than tuberculosis (MOTT).

Conclusion Identification of *Mycobacterium tuberculosis* by biochemical test is best alternative to modern techniques like Polymerase Chain Reaction (PCR) and Nucleic acid Amplification (NAA) when they are unavailable.

Keywords M. tuberculosis, Niacin, Nitrate, Catalase, PNB.

Introduction

Tuberculosis is a disease of global importance. One-third of the world population is estimated to have been infected with *Mycobacterium tuberculosis* and eight million new cases of tuberculosis arise each year. The tuberculosis crisis is likely to escalate since the human immunodeficiency (HIV) epidemic has triggered an even greater increase in the number of tuberculosis cases. The majority of tuberculosis patients are 15 to 45 years of age, persons in their most productive years of life. Tuberculosis kills over two million people world-wide each year, more than any other single infectious disease, including AIDS and malaria.[1]

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Pulmonary tuberculosis usually occurs in the apex of the lungs. These develop cavities which contain large populations of tubercle bacilli that can be detected in a sputum specimen. Pulmonary tuberculosis is suggested by persistent productive cough for three weeks or longer, weight loss, night sweats and chest pain. "The diagnosis can only be made reliably on demonstrating the presence of tubercle bacilli in the sputum by means of microscopy and/or culture in the laboratory."[1]

Smear examination of sputum specimens obtained from the patients representing with respiratory symptoms suggestive of TB remains the cornerstone of diagnosis of TB in most of locations with a high burden TB. The sensitivity of smear microscopy to identify all cases of TB, even in good centers, is only about 60 percent and patients whose smears are negative for acid fast bacilli (AFB) represent a diagnostic dilemma.[2]

The definitive diagnosis of tuberculosis depends on the isolation and identification of *M. tuberculosis*. The inoculation of concentrated bacilli from processed clinical specimens on solid media is a standard approach for confirmation of tuberculosis. Culture methods are more sensitive than microscopy as it can detect 10-100 mycobacteria per ml of sample and give positive result. Therefore culture is deemed to be gold standard for diagnosis of TB. Despite its enhanced sensitivity and specificity, culture is of impractical clinical use, because it is costly time consuming and requires specialized safety laboratories, which is not performed in most low income countries.[3,4,5]

Culture increases the number of tuberculosis cases found, often by 30-50%, and detects cases earlier, often before they become infectious. Since culture techniques can detect few bacilli, the efficiency of diagnosing failures at the end of treatment can be improved considerably. Culture also provides the necessary material for drug susceptibility testing. Culture of specimens is, however, much more costly than microscopy and requires facilities for media preparation as well as skilled staff.[1]

Identification of mycobacteria can be rather complex and needs a multitude of biochemical tests ascertain to which species a mycobacterium belongs. In the context of surveillance of drug resistance, the process can be simplified to a considerable degree as it will only be necessary to decide whether the *Mycobacterium* is a pathogenic species of the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis,* or *M. africanum*) or not.[6]

Usually, in the context of surveillance of drug resistance, it is only necessary to separate with reasonable certainty *Mycobacterium tuberculosis* complex from environmental mycobacteria, and there is no need to be certain about exact speciation within the complex. In this case a very simple identification procedure suffices.[6]

If it is desirable to identify *M. tuberculosis* complex prior to processing for drug susceptibility testing, the culture, if affluently growing, can be subjected to biochemical tests, i.e., niacin, nitrate reduction, and 68°C labile catalase tests that permit identification of *M. tuberculosis* and *M. bovis.* It should be noted, however, that some strains of *M. tuberculosis* and *M. bovis* are niacin negative. Cultures with too scanty growth for biochemical tests are tested against the anti-tuberculosis drugs isoniazid, rifampicin, streptomycin, and ethambutol plus para-nitrobenzoic acid and thiophene-2 carboxylic acid hydrazide. Almost all strains of *M. tuberculosis* complex are susceptible to para-nitrobenzoic acid and *M. bovis* is susceptible to both para-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide. However, identification of *M. bovis* must be ensured by niacin and nitrate reduction tests. In areas of the world where *M. africanum* is prevalent, its proper identification is difficult and not recommended if pyrazinamide susceptibility is not being tested.[6]

If it is considered to be more economical to perform identification and drug susceptibility testing simultaneously, the cultures grown on para-nitrobenzoic acid have to be submitted to biochemical tests.[6]

Methods

Settings

This study was conducted in National Tuberculosis Center, Thimi, Bhaktapur, Nepal from June 2005 to December 2005.

Sputum samples

Random samples were collected from the patients visiting the National Tuberculosis Center during the study period. Sputum is the sample of choice in this study. Among triplicate sputum samples (first, on the spot; second, early morning sample; and third, on the spot) collected at NTC only early morning sample per patient was included in this study. All samples were collected in leak proof, wide mouth, transparent, sterile and stopper plastic container. The patients were given clear instruction about the quality and quantity of samples. Adequate safety

precautions were taken during the specimen collection to prevent the spread of infectious organism.

Study process

For staining and culture, sputum samples were first decontaminated and concentrated by centrifugation using 4% NaOH, according to Modified Petroff's method. Smear on a slide was prepared for ZN staining. Remaining sediments were inoculated in the Ogawa medium. After growth on Ogawa medium, it was subcultured on Lowstein-Jensen (LJ) media. Biochemical tests were performed from subcultures.

Smear preparation

Concentrated Sputum smear was prepared on a clean, new and unscratched slide at one end with the relevant patient's number. An appropriate portion of the sample was transferred to the slide with the help of the broken end of a wooden stick. The sample was smeared on the slide over an area of approximately 2.0 by 1.0 cm and made it thin enough to be able to read through it. The smear was allowed to air dry for 15 minutes without heating. Thereafter, the smear was heat fixed passing the slide through a flame 3 to 4 times with the smear uppermost and allowed to cool before staining. These entire steps were performed inside a safety cabinet.

Staining procedure

Ziehl-Neelsen stain

1% carbol fuchsin was poured to cover the entire surface of the slides. The slides were heated underneath until vapour start rising. The slides were to stand for 5 minutes. The slides were then rinsed with tap water and excess water was drained off. The slides were decolorized with 3 % acid alcohol for 3 minutes. The slides were rinsed thoroughly with tap water and excess water was drained off. The slides were flooded with 0.3% methylene blue and let to stand for 1 minute. The slides were gently rinsed with tap water and excess water was drained off from the slides. The slides were allowed to air dry. The slides were examined under microscope in 1000x oil immersion.

Biochemical test

Niacin test

1ml of sterile water was added to the culture slant. If the growth was confluent, the medium was punctured with pasture pipette to allow contact of water with the medium. The tubes were placed horizontally so that the fluid covers the entire surface of the medium. 30 minutes was allowed for the extraction of niacin. The extraction time may be longer if the culture has few colonies. The slants were raised for 5 minutes to allow the fluid to drain to the bottom. 0.5ml of the fluid extract was removed to a clean screwcap tube. Sequentially added 0.5ml of the 4% aniline solution and 0.5ml of 10% cyanogen bromide solution. The tubes was closed and the solution was observed for the formation of a yellow colour (=positive result) within 5 minutes. The yellow colour appears as a ring at the interface of the two reagents, or if the is shaken, as a yellow column of liquid. 2-3ml of 4% NaOH was added to each tube and discarded.

Nitratre Reduction test

0.2ml of sterile saline was added to a screw-cap tube. A sterile loop was used to emulsify 2 loopfulls of a 4week old culture in the saline. 2ml of $NaNO_3$ substrate was added to it. Shaked well and incubated in a 37ºC water bath for 3 hours and removed. The reagents were added in following order: 1 drop diluted HCL, 2 drops .02% sulfanilide, 2 drops .01% N-naphthyethylene-diamine. Examined immediately for the formation of pink to red colour and compared to the standard.

Catalase test

With a sterile pipette, aseptically 0.5ml of 0.067/M phosphate buffer, pH 7.0 to 16X125 mm screw cap tubes. Several loopfulls of test cultures was suspended in the buffer solution using sterile loops. The tubes containing the emulsified cultures were placed in a previously heated water bath at 68ºC for 20 minutes. Time and temperature was critical. The tubes were removed from the heat and allowed to cool to room temperature. 0.5ml of freshly prepared Tween-peroxide was added to each tube and caps were replaced loosely. Then it was observed for the formation of bubbles appearing on the surface of the liquid. The tubes were not shaken as Tween 80 may form bubbles when shaken, resulting in false positive results. The negative tubes were held for 20 minutes before discarding.

Growth on medium containing para-nitrobenzoic acid (PNB)

Two slopes of LJ medium containing glycerol and one tube containing p-nitrobenzoic acid was inoculated at a concentration of 500mg/litre. One LJ slope and the PNB slope was incubated at 37ºC in an internally illuminated incubator and examined at 3, 7, 14 and 21 days. When growth was evident on the LJ slope it was examined for pigment. If an internally illuminated incubator was not available, the slopes were removed from the dark incubator as soon as growth was evident, loosened the caps to admit some oxygen and exposed them to daylight (but not direct

sunlight) or placed them 1m from the laboratory bench lamp and examined for pigment the following day. The other slope was incubated at 25ºC and examined at 3, 7, 14, and 21 days.

Microscopy reports

In recording and reporting of microscopic results, the following reporting scale was used for ZN staining.

Number of Bacilli Seen in Smear	Results Reported
No AFB per 300 oil immersion fields	*negative*
1-9 AFB per 100 oil immersion fields	*record the exact number*
10-99 AFB per 100 oil immersion fields	1+
1-10 AFB per 10 oil immersion fields	2+
> 10 AFB per oil immersion fields	3+

The number of AFB found is an indication of the degree of infectivity of the patient as well as the severity of tuberculosis.

Culture and identification

Single slope slant per specimen were inoculated each with one 4mm loopful of the centrifuged sediment, distributed over the surface. All the cultures were incubated at 37^0 C until growth was observed and those tubes in which growth was not observed after 8 weeks were regarded as negative were discarded.

All cultures were examined after 48-72 hours after inoculation to detect any contaminants. Thereafter cultures were examined on 7th day for rapid growers once weekly thereafter, up to 8 weeks, for slow growers after which they were subcultured.

Typical colonies of *M. tuberculosis* were rough, tough, crumbly, waxy, buff- colored and slow growers (growth appeared after 2-3 weeks after inoculation). Growth of mycobacteria was confirmed by typical colony morphology and microscopy of AFB. The definite diagnosis of *M. tuberculosis* was done by performing biochemical test. *M. tuberculosis* is Niacin and Nitrate reduction positive, catalase negative (at 68^0 C) and no growth on PNB containing medium.

A patient was defined as a "TB-positive case" if one of the three sputum specimens had a positive culture and as a "non-TB case" if none of the three sputum specimens showed growth.

Statistical analysis

Statistical analysis was performed using statistical product and service solution (SPSS) software version 11.5.

Results

A total of 200 culture positive samples were analyzed and 189 of the patient were diagnosed as having PTB by performing Niacin, Nitrate Reduction, 68 degree Heat labile catalase test and growth on PNB containing medium. Out of 200 samples 162 (81%) were sputum smear positive by ZN staining.

In 200 samples, 142 (71%) were male and 58 (29%) were female. 189 out of 200 samples were confirmed as being infected with *M. tuberculosis* by the above mentioned tests. Among 189 cases 132 (66%) were male and 57 (28.5%) were female. Maximum no. of confirmed TB cases was observed in age group of 21 to 30 (58, 29%). On the basis of age wise distribution no significant difference was seen in TB cases.

Considering the distribution of TB among genders, more males have been found to be infected than females, in all age groups. Most number of cases occurred in the age group of 21-30 (31.5%) and least in the age group 0-10 (2.5%). Except in age group 0-10 female (1%) and male (1.5%) and in the age group above 60 where male and female cases are equal i.e. 5 percent in all other age group the number of male exceeds that of female (table 1).

Table 1: Distribution of total cases by age and sex.

Age group(years)	Male		Female		Total	
	No.	%	No.	%	No.	%
0-10	3	1.5	2	1	5	2.5
11-20	9	4.5	3	1.5	12	6
21-30	48	24	15	7.5	63	31.5
31-40	28	14	14	7	40	20
41-50	32	16	10	5	42	21
51-60	14	7	4	2	15	7.5
Above 60	10	5	10	5	23	11.5
Total	**142**	**71**	**58**	**29**	**200**	**100**

Out of 162 smear positive samples 118 (72.84%) were male and 44 (27.16%) were female. The maximum number of smear positive samples lies in the age group of 21-30 (46, 28.4%) and minimum in the age group of 0-10 (2 female and 1 male). In all age groups except 0-10 the number of male smear positive sample is greater than females (table 2).

Table 2: Distribution of total smear positive cases by age and sex

Age group(years)	Female		Male		Total	
	No.	%	No.	%	No.	%
0 - 10	2	1.23	1	0.62	3	1.85
11 - 20	2	1.23	9	5.56	11	6.79
21 - 30	11	6.79	35	21.60	46	28.40
31 - 40	12	7.41	23	14.20	35	21.60
41 - 50	7	4.32	28	17.28	35	21.60
51 - 60	4	2.47	14	8.64	18	11.11
Above 60	6	3.70	8	4.94	14	8.64
Total	**44**	**27.16**	**118**	**72.84**	**162**	**100.00**

The present study conducted different biochemical tests for the diagnosis of PTB. The different tests were Niacin test, Nitrate reduction test, Heat labile catalase test and growth on PNB containing medium. 95 percent were positive for Niacin test, 94.5 percent were positive for Nitrate reduction test, 97 percent were negative for heat labile catalase test and no growth was seen on 93.5 percent samples (table 3).

Table 3: Comparative results of biochemical tests of total samples.

Niacin test	Nitrate Reduction test	Heat labile Catalase test	Growth on PNB medium	Sample Number	Percent
Negative	Negative	Negative	Growth	2	1.00%
Negative	Negative	Negative	No Growth	2	1.00%
Negative	Negative	Positive	Growth	5	2.50%
Negative	Negative	Positive	No Growth	1	0.50%
Positive	Negative	Negative	No Growth	1	0.50%
Positive	Positive	Negative	No Growth	189	94.50%
			Total	**200**	**100.00%**

Out of 200 samples, 189 (94.4%) samples tested positive for Niacin test and Nitrate reduction test, negative for heat labile catalase test and no growth on PNB containing medium. Thus 189 samples can be confirmed as *M. tuberculosis*. The rest are MOTT and their specific identification requires different biochemical tests and if possible PCR.

Discussion

According to the WHO guidelines for tuberculosis control, patient with more than three weeks history of cough should be screened for PTB with smear microscopy for AFB. Because the clinical signs and symptoms of PTB are not specific, the accurate performance of acid-fast microscopy is vital for the early recognition of PTB patients for the adequate treatment, respiratory isolation, and contact. Although acid-fast microscopy is more than 100 years old, it still remains the initial and most rapid step in the diagnosis of tuberculosis. Acid-fast microscopy is simple to perform and therefore applied to any laboratory.[7]

The usual staining laboratory technique for staining AFB used worldwide has been the ZN method, which has also accepted as the conventional method. However the method requires controlled heating for success, and there are certain disadvantages, e.g. multistage staining, a cumbersome heating procedure and the discomfort caused by aerosols of phenol.[7]

Although a presumptive diagnosis of tuberculosis may be made by an experienced laboratory technologist on the basis of the characteristics of tubercle bacilli described before, it is best to do confirmatory tests. Unfortunately there is no completely reliable single test that will differentiate *M. tuberculosis* from other mycobacteria. Nevertheless, the following tests (Niacin, Nitrate reduction, 68^0C heat labile catalase test and growth on PNB containing medium)when used in combination with the characteristics described before will enable the precise identification of >95% of *M. tuberculosis* strains.[1]

Niacin (nicotinic acid) plays a vital role in the oxidation-reduction reactions that occur during metabolic processes in all mycobacteria. Although all mycobacteria produce niacin, comparative studies have shown that, because of a blocked metabolic pathway, *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its definitive diagnosis. Niacin negative *M. tuberculosis* strains are very rare, while very few other mycobacterial species yield positive niacin tests.[1 8]

Cultures grown on egg medium yield the most consistent results in the niacin test and LJ medium is therefore recommended. A culture must be at least three to four weeks old and must have sufficient growth of more than 50 colonies. Because *M. tuberculosis* excretes niacin into the growth medium, cultures with confluent growth may give a false-negative niacin reaction because the extracting fluid cannot come in contact with the culture medium. When this occurs, expose the underlying medium surface by either scraping away or puncturing through some of the culture growth.[1 8]

Aeration of cultures intended for niacin testing is very important. Caps should be loose on slants throughout the entire incubation period and special Cap-o-Test stoppers are recommended.[1]

M. tuberculosis is one of the strongest reducers of nitrate among the mycobacteria, which allows for this test to be used in combination with the niacin test in differentiating *M. tuberculosis* from the other mycobacteria.

Cultures to be tested for nitrate reduction should be four weeks old and have abundant growth Löwenstein Jensen egg medium are recommended.[1]

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen, ie. $2H_2O_2 \rightarrow 2H_2O + O_2$. The oxygen bubbles into the reaction mixture to indicate catalase activity. Virtually all mycobacteria posses catalase enzymes, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis*.[1]

In laboratories where facilities and reagents for niacin and nitrate testing are not available, identification of tubercle bacilli may be done by a combination of one or more of the catalase tests described previously together with growth at 25°C on LJ medium and growth on LJ medium containing p-nitrobenzoic acid at 37°C. Problems with incubation at 25°C may be encountered in tropical regions. A refrigerated incubator should be used where available; as an alternative, a water bath within a refrigerator or cold room should be used.[1]

In this study the total number of males 142 (71%) were greater than females 58 (29%). This is due to the large number of suspected patients visiting hospital and is included in the study. This does not mean that males are more susceptile than females. The most number of TB patients have been found in the age group of 21-30 (30.5%) which justifies that it occurs in the productive age group.

Out of 200 samples, 162 (81%) were positive by ZN staining and rest 19% grew in primary culture. Thus culture is the only definitive diagnosis of tuberculosis that depends on the isolation and identification of *M. tuberculosis*. Culture remains the gold standard method for tuberculosis. Although the primary culture assumes that it is *M. tuberculosis* the definite diagnosis requires biochemical tests which can detect more than 95% of M. tuberculosis strains.

Modern techniques like PCR and NAA can be performed but they are expensive and not available in all parts of the country. In this study biochemical tests diagnosed 189 (94.5%) of total samples as *M. tuberculosis* and rest was MOTT. Thus it is very important to perform biochemical tests for the definitive diagnosis of *M. tuberculosis*.

Conclusion

Isolation and identification of etiological agent of Pulmonary Tuberculosis is very important. Identification of isolates by biochemical tests is a traditional and reliable method as around 95% of isolates have been identified as *M. tuberculosis* and rest MOTT the treatment regime for which varies. Biochemical tests are suitable when other modern methods are unavailable and should be conducted.

Acknowledgement

I wish to express most sincere gratitude towards Dr. Anjana Singh, Head of Central Department of Microbiology and Mr. Binod Lekhak, Assistant Professor, Central Deparment of Microbiology (CDM) and Dr. Keshab Bhakta Shrestha, Senior Consultant Chest Physician and Senior Medical Epidemiologist. The Author would like to thank all staff members of CDM and National Tuberculosis Center. Thanks to friends Mr. Saroj Shrestha and Mr. Bikash Shakya.

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