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FUNGAL CONTAMINANTS ARE RESPONSIBLE FOR DELAY IN DIAGNOSIS OF TUBERCULOSIS

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ABSTRACT: Back ground: The purpose of this study was to investigate possible fungal growth containments during Mycobacterium tuberculosis cultures in solid media leads to delay in diagnosis of tuberculosis. A total of 800 clinical sputum samples were collected from the out patients attending to district tuberculosis center Warangal and Karunalayam care and treatment center for HIV Karunapuram Warangal. Method: The cultures were grown on Lowenstein-Jensen [LJ] medium after decontamination by using NACl-NAOH method for sputum. Growth was reported every day in the first week, from 2nd to 8th week reporting was done continues week. Fungus was found in the early of first week from 3 day of inoculation and identified contaminates were carried in to fungal media and the growth was observed between 3-5days for filamentous fungi. Result: 45contaminates were found from 2013- 2018 all were identified as fungal culture and no bacterial contamination was able to found. Conclusion: Based on this study we recommend that there is a need for improving aseptic techniques to be maintained during transporting the sputum sample, opening the sample, decontamination, inoculation and solid media preparation in order to reduce the level of fungal contaminants in TB cultures so as to improve the detection rate**.**

KEYWORDS: Fungal contamination, Fungal identification, Mycobacterium tuberculosis, L-J media, Liquid media, Decontamination.

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1.INTRODUCTION

Tuberculosis [TB] continues to be one of the most fatal infections of the present time despite of well-planned therapeutic regimen recommended by the World Health Organization [WHO][1]. The most accomplished TB control Programmes in developing countries are reaching only 56 % case detection rate by direct sputum microscopy [2]. A simplest approach for improving the level of case detection is to upgrade the performance of existing inexpensive diagnostic tools, without making them too complicated and expensive. Though, gold standard for pulmonary TB diagnosis remains culture, but in most of the developing countries diagnosis relies on microscopy for acid-fast bacilli [AFB] in sputum smears because it is simple, inexpensive and provides rapid results. Unfortunately, this technique has a low sensitivity [22– 43 % for a single smear [3] and up to 60 % under optimal conditions [4]] when compared with that of cultures. The threshold for detection of AFB in sputum samples under optimal conditions is between 104 and 105 bacilli ml21; TB programme conditions and technical constraints further decrease this yield [5].A major problem when dealing with tuberculosis has been a difficulty in diagnosis due to slow growth of mycobacterial cultures, which subsequently explains the slow process of evaluating the susceptibility of this microorganism to antibiotics. Using current tools, a primary culture is obtained in two to four weeks on average, and antibiotic susceptibility is determined after an additional two to four weeks[6,7]culture remains the gold standard for detecting TB and drug sensitivity testing [8].However, the effectiveness of culture systems is greatly undermined by contamination with bacteria and fungi [9]Contamination reduces the proportion of interpretable results, thereby limiting the diagnostic value of culture systems. Contaminated cultures must be repeated, at additional cost to public health systems, which delays or ultimately prevents TB diagnosis. Decontamination techniques have been implemented at all stages of specimen collection and processing. Antimicrobial and antifungal oral rinse solutions can be used prior to sputum expectoration [9], and decontamination using cetrimide, oxalic acid, and sulfuric acid can be used during sputum processing [10, 11]. A variety of decontamination methods have been developed for various M. tuberculosis culture media but the effective sample processing method for successful cultivation of M. tuberculosis a mixture of NaOH, sodium citrate and N-acetyl L-cysteine is added to digest the sample for the unbeaten recovery of causative organism [12]. Samples were processed for decontamination was followed by centrifugation to concentrate the sample, before being inoculated on to specific media. Lowenstein Jansen [LJ] medium is widely used for cultivation of mycobacteria [13] however microorganism other than mycobacteria may grow. These may out-compete mycobacteria for nutrients and other growth requirements and will grow to over shadow mycobacteria the organism of interest, as a result, false negatives may be reported in TB cultures. This study was aimed to isolating and characterizing the fungal organisms responsible for overgrowth and contamination. The study also focused on determining the susceptibility of these organisms to various antibiotics on solid culture media.

2. MATERIALS AND METHODS

The study was conducted at the Department of Microbiology, Sri Shivani College of Pharmacy which is located in rural Warangal district of Telangana state, India. With the support of District tuberculosis center which is attached to MGM hospital Warangal and Karunalayam care and treatment for HIV center we were able to progress the work. Project was funded by Department of Science and technology SERB division to study the prevalence of tuberculosis among the tuberculosis among the HIV-TB co-infection and drug resistance for period of 3 years from 2013-2016.

Strains: M. tuberculosis $H_{37}Rv$ were used as standards to compare the growth of the new cultures from various specimens after proper decontamination.

Clinical materials: A total of 800 consecutive clinical samples were collected from suspected cases of tuberculosis attending the DMCs Warangal and KCTC,during August 2013 to September 2018.

Inclusion Criteria

1. Patients with cough more than 2 weeks and/or blood stained sputum.
2. New sputum positive pulmonary tuberculosis patients before initiation of treatment.

Exclusion Criteria

1. Patient already under anti-tubercle drug treatment.
2. Inadequate sample volume [< 2ml].
3. Sample not representative of lower respiratory tract.
4. Patient denying participating in the study. Decontamination

Sputum collection

Tb Patients reported at clinic would be clinically examined and information recorded in the clinical formats. HIV reactive persons counselled and tested at ICTC centre which is attached to MGM Hospital Warangal] were screened for tuberculosis and their samples were collected.

Sputum transport

Required amount of the samples placed in a vial then it is sealed with parafilm. Then it is placed in box with sufficient gel packs and completely sealed complete address of the destination is mentioned on the box and posted. Use a sterile container or falcon tubes clean and free from contamination before and after collecting the samples .Do not open them prior to collecting the sample. Examine the sample bottle for cracks, missing seal or other signs that its sterility may be compromised, if any of these indications are found, discard the bottle and use a suitable one. Samples shall be kept in a refrigerator or cooler to maintain a temperature of -20^0c.

Bacterial Straining

ZN sputum Smear preparation AFB sputum smear preparation done in Bio safety cabinet level II at SSCP lab. One drop of each suspended pellet was used to prepare slides for AFB microscopy using the Ziehl– Neelsen stain. Each slide was coded, read blindly by a qualified technician and reported

according to the National Tuberculosis Program and WHO/IUATLD standards [WHO, 1998]. Smears were reported as follows: Grade 0 where no Acid Fast Bacilli [AFB] is observed in a total of 200 oil immersion fields, Scanty [Sc] where 1–9 AFB in 100 microscopic fields [few bacilli] is observed; 1+ with 10–99 AFB in 100 fields; 2+ with 1–10 AFB per field in at least 50 fields; 3+ with more than 10 AFB per field in at least 20 fields. Each slide will be coded, examined and graded according to the RNTCP guidelines

Decontamination Method

Two mL of a mixture composed by 1.0 mL 1.0% N-acetyl-L-cysteine [Merck, Germany] in 2.9% citric acid and 1.0 mL 4.0% NaOH were added to 2 mL volumes of each respiratory specimen and vortexed in a tube for 15–20 seconds and incubated at 37 °C for 20 minutes. Phosphate buffer pH 6.8 [Stanton] was then added and the tubes centrifuged at 3000 g for 15 minutes. The supernatant was then carefully discarded, and the sediment resuspended in 1–2 mL of phosphate buffer pH 6.8. This last suspension was used to inoculate culture media and to prepare smears for microscopic examination.

Preparation of Solid Media [LJ]

Mineral salt solution: [Potassium dihydrogen Phosphate [0.15%]. Magnesium Sulfate [0.015%], Magnesium Citrate [0.0375%], L-Asparagine [0.45%], Glycerol [0.75%], dissolve the ingredients by heating and autoclave at 121ºc for 25 minutes to sterilize this solution keep indefinitely and may be stored in suitable amounts. Malachite Green Solution: [Distilled water [100ml]. Malachite green [4gms]. Prepared a 2%solution of malachite green in sterile water with sterile precautions by dissolving the dye in the incubator for 1-2 her this solution can be stored indefinitely and should be shaken be for us. After preparation of malachite green is added to the mineral salt solution.Egg suspension: Fresh egg solution [15-20] was added to the media and mixed them. According to the procedure. And poured the breeze bottles, put in to the Hot Air Oven and observed the media bottles with [70-80] temperature. Then Preserved at- 4ºC. The media was allowed to incubate for eight weeks after inoculation, as the mycobacterium grows slow when compared with other bacteria. A loop of Mycobacterial culture was inoculated on to media separately in respective bottles under aseptic conditions. After inoculation, the media were allowed to incubate at 37ºC over the growth period.

Middle Brook Media [Liquid Media]

Middle brook 7H9 Broth purchased from Himedia, Mumbai [Ammonium sulphate 0.05%, Disodium phosphate 0.25%, Monopotassium phosphate 0.1%, Sodium citrate 0.01%, Magnesium sulphate 0.005%, Calcium chloride 0.00005%, Zinc sulphate 0.0001%, Copper sulphate 0.0001%, Ferric ammonium citrate 0.004%, L-Glutamic acid 0.05%,Pyridoxine 0.0001%, Biotin 0.00005%]is a liquid growth medium especially used for cultivation of Mycobacterium tuberculosis.Glycerol: Glycerol [2ml] added to the media, dissolves the ingredients by heating and autoclave at 121ºc for

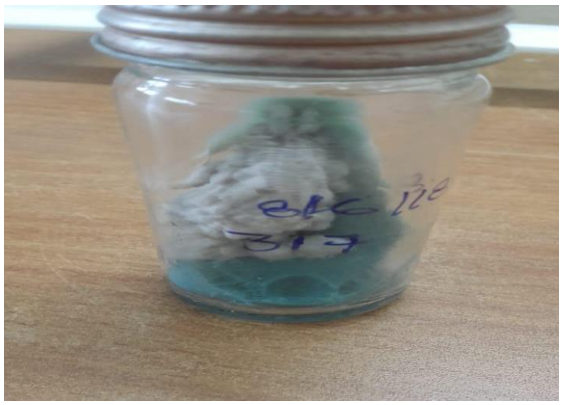
15 minutes to sterilize this solution keep indefinitely cool it.

OADC: bovine Albumin Fraction v [2.50gm], Dextrose [1.00], Catalyze[0.002gm], Oleic acid [0.025gm], sodium chloride [0.425gm], distilled water [50ml].The OADC were added to the cool medium And poured the test tube sunder aseptic condition. LJ media was inoculated in Middle brook 7H9 media liquid growth medium especially used for cultivation of Mycobacterium tuberculosis. Cultures were reported from the day 3 after inoculation and for every alternate day [5th, 7th, 9th, and 15th] up to 21 days.Individually to the freshly prepared sterile media and mixed well. A loop of diluted Mycobacterial subcultures were inoculated into sterile medium separately in respective tubes under aseptic conditions and mixed properly using Vortex. After inoculation, the culture tubes were allowed to incubate at 37ºC over the growth period. (15, 16, 17,18,19,20,21,22,23,24)

Laboratory Methods

Bacterial Contaminants

Tubes were incubated at 37°C for a maximum of 8 weeks. Cultures showing no growth after 8 weeks of incubation were reported as negative. Liquefied or discolored[dark green] LJ media or LJ slants with colonies of non-acid-fast bacteria were considered contaminated. Internal quality control for microscopy and LJ was performed as previously described. For a subset of 45 contaminated LJ tubes, culture liquid was inoculated on blood agar and incubated at 37°C for 48 h. After growth was detected, an isolated colony was picked for further characterization using Gram staining. Aspergillus Niger was found to be the most contaminating fungi in LJ medium. Bacterial contamination is more predominant than fungal contamination in LJ media.



Culture and Isolation of Fungi

A colony from contaminated slants was inoculated on SDA with chloramphenicol to inhibit growth of other contaminating bacteria and observed the colony morphology. These isolates were grown on PDA for 3 days at 37°C; A conidial suspension was prepared with 1ml of phosphate buffer [PH 7.4] and 0.05% Tween 20 [PBST]. This suspension was used for growth on PDA medium. The petri dishes were incubated at 37oC and observed for colonial growth. Only one colony was selected

from each plate and grown in PDA agar slants at 37°C. The conidia of the monospores were observed in sterile water at 4°C. Subsequently, the cover slip was carefully separated from the agar, placed on the slide with a drop of lacto phenol cotton blue stain and observed under microscope. The diameter of 30 conidia for each Aspergillus section isolate grown on PDA and incubated at 37°C for 3 days was measured with a calibrated ocular micrometer [Olympus America Inc.]. SDA media was further used to culture Candida species: Organisms from contaminated TB slants were directly inoculated on SDA medium and grown for three days and observed for colony morphology. The germ tube test was done by using: Test tubes of 12 x 75 mm and Pasteur pipettes, 3 drops of fresh pooled human serum were dispensed into tubes. To the tubes, serum obtained from the serology laboratory Mulago and with a sterile wooden applicator stick, a yeast colony was lightly touched and the stick placed into serum. The yeast was suspended in serum and the stick discarded in a discard container. The test was incubated at 35°C for 3 hours. After 3 hours, a drop of the suspension was placed on a clean microscope slide. A clean cover glass was placed over the suspension and then examined under microscope using lower objective

Reasons for contamination

Contamination could be probably due to factors such as Contaminant within the sample, Inoculating and pipetting methods, contaminants in the laboratory cabinet or individual media tubes and the sensitivity of the different inhibitors present in the media, deviation from sample processing SOP.

3. RESULTS AND DISCUSSION

A total of 800 samples were analyzed for this study no bacterial contaminates only fungal contaminants were identified. Based on this study the cultures were grown on Lowenstein-Jensen [LJ] medium after decontamination by using NACl-NAOH method for sputum. Growth was reported every week till 8th week and identified growth by conventional morphology and biochemical methods. Contamination of fungus was found in the early of first week from 3 day of inoculation and identified contaminates were carried in to fungal media and the growth was observed between 3-5days for fungal contaminants. Cultures on SDA were also monitored for three weeks for possible growth of filamentous fungi. Fungal contaminants included were normal flora that inhabit the respiratory system such as the A. fumigatus, C. Albicans, Mucoor Aspergillus. In this study we are observed 45contaminates were found from 2013- 2018 all were identified as fungus and no bacterial contamination was able to found. Cultures contamination is a problem during the growth of MTB and allows the overgrowth of fungus present in the sputum specimen, which can potentially mask the presence of M. tuberculosis [25]. As per reports Lowenstein-Jensen has been previously shown to have a high contamination rate despite its cheap cost and applicability in low income setting [26]. A total of 200 samples were analyzed for this study. Bacterial and fungal contaminants were identified as below. Generally, the level of contamination was high for all the samples analyzed. These organisms included normal flora that inhabit the respiratory system such

as Staphylococci and Streptococci. The fungi Isolated included A. fumigatus and C. albicans.[27] Mitali reported that treatment of specimens with 4 per cent NaOH yielded minimum recovery of pure cultures, while use of 2 per cent NaOH produced higher number of contaminants compared to other methods of decontamination. Addition of N-acetyl L-cystein [NALC] coupled with 2 per cent NaOH to the samples for decontamination provided fairly reasonable recovery, but the highest number of M. tuberculosis cultures could be obtained when the specimens were treated with tri-sodium phosphate and benzalkonium [TSPB]. Among the sputum positive cases recovery of growth of M. tuberculosis was higher with greater number of bacilli present in the specimens. Regarding the influence of culture media, BLM produced not only rapid growth, but reasonably higher rate of isolation of M. tuberculosis. [28] **Out of 143 microbiological sputum samples, 104 were found to be positive for acid fast bacilli by both Modified petroff's method and Hypertonic Saline Sodium Hydroxide method [HS-SH] of concentration and decontamination and 39 samples were negative by both these methods. Sensitivity, Specificity, Positive predictive value & Negative predictive value of the HS-SH method was found to be 100%.(29,30,31)

4. CONCLUSION

Major threat to the sensitivity of mycobacterial culture due to fungal contaminates. Contamination of mycobacterial culture media may occur during collection, transportation, processing or overgrowth by fungi and bacteria. Culture contamination will reduce the proportion of interpretable results and diminishes the diagnostic value of culture technique. It is therefore important to understand which fungi are more common contaminants of Mycobacterial cultures. The isolated bacteria and fungi may not just be contaminants but possible causative agents of infection/co-infections in TB/ TB like diseases especially among patients whose immune system is compromised and which delays in diagnosis of infections and leads to death of the patients. RECOMMENDATION: Laboratory personnel need to be routinely sensitized about the occurring contaminants during growth on L-J, so that they observe the most required precautionary measures in accordance with the RNTCP guidelines. More studies need to be done to put into consideration other mycobacterial contaminants It is also worth following up certain organisms such as Aspergillus and Mucor among others since they may be the actual cause of TB like disease or co infection with TB that is likely to complicate management of such diseases more so among immune compromised individuals.

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CONFLICT OF INTEREST

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Conflict of interest : There are no conflicts of interest.

Ethical approval : Ethical approval obtained from DTC centre Warangal.

REFERENCES

1. Phanouvong SVH, Blanc L, Wondemagegnelm E, Rägo L, Moore T, Mboussa J, Norval P, Matji R, et al. Operational guide for national tuberculosis control programmes on the introduction and use of fixeddose combination drugs. World Health Organization, Geneva, Switzerland. 2002
2. WHO Global Tuberculosis Control: Surveillance, Planning, Financing. WHO Report. WHO/HTM/TB/2005.349. Geneva: World Health Organization.
3. Apers L, Mutsvangwa, J., Magwenzi J, Chigara N, Butterworth A , Mason P. & Van der Stuyft, P., et al. A comparison of direct microscopy, the concentration method and the Mycobacteria Growth Indicator Tube for the examination of sputum for acid-fast bacilli. Int J Tuberc Lung Dis 2003; 7, 376–381.
4. Toman, K. Tuberculosis Case-Finding and Chemotherapy. Case Detection, Treatment and Monitoring – Questions and Answers, 2nd edn. Geneva: World Health Organization. 2004.
5. IUATLD, 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 2005,9, 355–361.
6. Bello AK, Njoku CH. Tuberculosis: current trends in diagnosis and treatment. Niger. J. Clin. Pract. 2005,8:118 –124.
7. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH.. Manual of clinical microbiology, 1999, 9th ed. ASM Press, Washington, DC.
8. Kalema N, Boon SD, Cattamanchi A, Davis JL, Andama A, Katagira W, Everett C, Walter N, Byanyima P, Kaswabuli S, Worodria W, Huang L, et al. Oral antimicrobial rinse to reduce mycobacterial culture contamination among tuberculosis suspects in Uganda: a prospective study. PLoS; 2012,
9. Gautam G, Singh TSK, Sharma TD, Ghimire M, Regmi SM. Decontamination of sputum sample for the isolation of mycobacterium tuberculosis. J. Gandaki Med. College 2010,.3:32–35.
10. Peres RL, Palaci M, Loureiro RB, Dietze R, Johnson JL, Maciel EL, et al. Reduction of contamination of mycobacterial growth indicator tubes using increased PANTA concentration. Int. J. Tuberc. Lung Dis. 2011,15:281–283.

11. Buijtels PC, Petit PL. Comparison of NaOH-N-acetyl cysteine and sulfuric acid decontamination methods for recovery of mycobacteria from clinical specimens. J Microbiol Methods 2005; 62 : 83-8.
12. McClean M, Stanley T, Stanley S, Maeda Y, Goldsmith CE, Shepherd R, et al. Identification and characterization of breakthrough contaminants associated with the conventional isolation of Mycobacterium tuberculosis. J Med Microbiol. 2011;60[Pt 9]:1292-8.
13. Asmar S, Drancourt M. Rapid culturebased diagnosis of pulmonary tuberculosis in developed and developing countries. Front Microbiol. 2015;6:1184.
14. Kampala Tushabe John Vianney1 , Katuura Esther2 and Iramiot J. Stanley3 ;Bacterial and Fungal Contaminants in Mycobacterium tuberculosis Cultures at National Tuberculosis Referral Laboratory- Microbiology Research Journal International 2017,19[5]: 1-8,.
15. Murray P. R., Baron E. J., Jorgensen J. H., Pfaller M. A., Yolken R. H., (Eds.), 8th Ed., 2003, Manual of Clinical Microbiology, ASM, Washington, D.C.
16. Lowenstein E., Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig., 1931, 120:127.
17. Jensen K. A., Zentralb. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig., 1932; 125:222.
18. Gruft, Health Lab. Sci., 1971;,8:79.
19. Gruft, Am. Rev. Respir. Dis., 88:412. 6. Boisvert H., 1960, Ann. Inst. Pasteur, 1963; 99:600.
20. MacFaddin J. F., Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria, 1985, Vol. 1, Williams and Wilkins, Baltimore.
21. Boisvert H., Ann. Inst. Pasteur, 1960,99:600.
22. Kent P. T and Kubica G. P., , Public Health Mycobacteriology: A Guide to the level III Laboratory, USDHHS, Centers for Disease Control, Atlanta, Ga. 1985
23. Forbes B. A., Sahm A. S. and Weissfeld D. F., Bailey & Scotts Diagnostic Microbiology, 10th Ed., Mosby, Inc., St. Louis, Mo,1998,.
24. Cernoch P., Enns R., Saubolle M. and Wallace R., 1994, Cumitech, 16A, Laboratory Diagnosis of the Mycobacterioses coord , Ed., Weissfeld , ASM, Washington, D. C.
25. Isenberg, (Ed.), Clinical Microbiology Procedures Handbook, Vol. I, ASM, Washington, D. C. R;1992.
26. Mitali Chatterjee, Susmita Bhattacharya, Kalpana Karak, and Sujata G. Dastidar; Effects of different methods of decontamination for successful cultivation of Mycobacterium tuberculosis:Indian J Med Res.2013 Oct; 138[4]: 541–548
27. Bhattacharya S, Ray R, Roy Chowdhury N, Dasgupta A, Dastidar SG. Comparison of a novel bilayered medium with the conventional media for cultivation of Mycobacterium tuberculosis. Indian J Med Res 2009; 130 : 561-6.

28. Das .D, Dwibedi. B, Kar S.K ; Spot or early morning sample for mycobacterial culture: which?Int J Tuberc Lung Dis, 18 [3] [2014], pp. 310-311
29. Cohen T, van,Helden PD, Wilson D, Colijn C, McLaughlin MM, Abubakar I, WarrenRM. M ixed-strain Mycobacterium tuberculosis infections and the implications for tuberculosis treatment and control. Clin Microbiol, 2012; Rev 25:708–719.
30. Zetola NM, Modongo C, Moonan PK, Ncube R, Matlhagela K, Sepako E, CollmanRG, Biss on GP;Clinical outcomes among persons with pulmonary tuberculosis caused by Mycobacterium tuberculosis isolates with phenotypic heterogeneity in results of drug-susceptibility tests. J Infect Dis 2014;209:1754–1763.
31. Gan M, Liu Q, Yang C, Gao Q, Luo T;. Deep whole-genome sequencing to detect mixed infection of Mycobacterium tuberculosis. PLoS; 2016.