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Breakthrough Contamination Of Lowenstein Jensen Media In A Tuberculosis Culture Laboratory Of Tripura, North East India.

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Abstract:
Background: "Breakthrough contamination" is the overgrowth of resistant non-mycobacterial contaminants in primary culture mainly owing to long incubation period of the later causing physical masking and competition effects. In 2022 Mycobacterial Culture laboratory was initiated in Tripura Medical College for comprehensive study of M.tuberculosis (MTB) through gold standard LJ culture. However since inception the laboratory has noted contamination of few inoculated media with breakthrough contaminants. This study was conducted with an aim to isolate and identify these contaminants growing on LJ media during M. tuberculosis solid culture in both pulmonary and extra pulmonary samples.
Method: 115 LJ media bottles were inoculated of which 81 (70.5%) were pulmonary samples and 34 (29.5%) were extra-pulmonary samples. Characterization of contaminating bacteria and fungi was carried out after ruling out mycobacterial growth. Collected data was entered in Microsoft excel 2007 and analyzed using SPSS version 15. The results were expressed as frequency and percentage as and where applicable.
Results: Overall contamination rate was 6%. Frequency was found to be higher in pulmonary samples as compared to extra pulmonary samples. Majority were bacterial in origin. All contaminants were breakthrough as none grew as mixture along with acid-fast organisms. However on molecular detection, three samples were confirmed for M. tuberculae and rifampicin sensitivity, four were confirmed for M. tuberculae but rifampicin indeterminate, two showed invalid results and one was negative for M. tuberculae.
Conclusion: This is indicative of the masking effect that can lead to false negative results and decrease the overall sensitivity of LJ culture.

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I. Introduction:

As per the World Tuberculosis Report, 2022 by WHO, India's TB incidence is 210 per lakh population as compared to 256 per lakh in the base line year 2015. An 18% decline was shown which was better than the global average of 11% [1].This significant stroke was achieved by the timely detection and management of tuberculosis patients at a war footing pace. On this path, diagnostic laboratories stood as a colossal pillar working under the umbrella guidelines set by Revised National Tuberculosis Control Program, novel methods of early detection and in-depth research. To this similar mission, the Tripura Medical College Tuberculosis laboratory has been working since, providing support to the state of Tripura. Molecular testing laboratory was set up in 2020 and then in 2022 Mycobacterial Culture laboratory was initiated for comprehensive study and confirmation of Mycobacterium tuberculosis as well as atypical mycobacteria through gold standard LJ culture and susceptibility testing. However since the inception, the TMC MTB culture laboratory has noted contamination of few inoculated media with breakthrough contaminants. "Breakthrough contamination" is the overgrowth of resistant non-mycobacterial contaminants in primary culture mainly owing to the long incubation period of the later causing physical masking and competition effects, thereby reducing the proportion of interpretable results. In most cases, such cultures are required to be repeated at additional cost which increases the cost effectiveness as well as the time to diagnose tuberculosis [2].The presence of Acid Fast Bacilli is confirmed by ZN staining in which *M. tuberculae* appear in serpentine cords of varying length. If the culture smear shows no AFB, then the growth is generally discarded by considering it as a contaminant.[2] Generally in patients with complicated tuberculosis, there is co-infection with pathogenic bacteria or fungi, but interestingly their presence on LJ media in presence of malachite green must always be taken as contaminants. As per

McClean et al. the predominant contaminants on LJ media include *Pseudomonas aeruginosa, Serratiamarcescens, Escherichia coli, Enterococcus fecalis, Staphylococcus species*[3]. But overall, there are very few studies on this topic and data as well as knowledge regarding contaminants growing on LJ media is limited.

II. Objective:

To isolate and identify bacterial and fungal contaminants growing on LJ media during M. tuberculosis solid culture in both pulmonary and extra pulmonary samples.

III. Methodology:

All routine sputum samples were received in duplicate for this study by the Tuberculosis culture laboratory, department of Microbiology from the DMC center of Tripura Medical College and BRAM Teaching Hospital, Hapania from May'2022 to December'2022. Before processing, all sputum samples were graded by Bartlett's scoring method for quality analysis [4].Here we microscopically looked for the presence of neutrophils and squamous epithelial cells. Sputum specimens with a score of ≤0 were rejected as per criteria, rest of the samples accepted for further processing. No consent was taken from any patient because this study was conducted on contaminated LJ bottles that were thereafter discarded.The pulmonary samples that are sent to TB laboratory are contaminated to varying degrees and have high chances of overgrowing before the mycobacteria due to its long incubation period as compared to commensals. Therefore a homogenization and decontamination process is imperative to liquefy the sputum and rule out commensals. Hence Ziehl-Neelsonstaining was done and all sputum specimens were processed routinelyby the NALC/NaOH decontamination protocol using N-acetyl-L-cysteine (NALC) and 4%(w/v) NaOH decontamination step followed by conventional detection of M. tuberculosis through extended culture on paired Lowenstein–Jensen (LJ) agar [8]. Samples from same candidates were also processed by TrueNat for Reverse Transcription Polymerase Chain Reaction (RT PCR).Readymade LJ culture bottles from HIMedia were used for isolation. Inoculation of the samples on LJ media was done by following standard protocol. After inoculating the media, incubation was done at 397°Cup to 8 weeks. The slopes were observed weekly till the colonies grew and any growth was checked for acid fast bacilli by performing ZiehlNeelson staining. The non AFB samples were considered as contaminants and any growth was further processed as per gross inspection, staining and biochemical reactions.Contaminating non-acid fast growth detected in any culture bottle was first Gram stained and then inoculated in blood agar, nutrient agar and macConkey agar and incubated at 37°C for 48hours. On observation of growth in culture plate, gram staining was done following by biochemical tests (Oxidase, indole, urease, citrate, TSI, germ tube test etc.) depending on the findings. Collected data was entered in Microsoft excel 2007 and was analyzed using SPSS version 15. The results expressed as frequency and percentage as and where applicable.

IV. Results:

A total of 115 samples were collected from patients and inoculated in Lowenstein Jensen media in the duration between May'2022 and February'2023. Out of these 81 (70.5%) were pulmonary samples and 34 (29.5%) were extra-pulmonary samples. Out of total inoculated LJ media, 7 (6%) showed growth of contaminant within the first week, composing mainly of gram negative bacilli. Of these six were pulmonary (sputum) samples and one was extra pulmonary (gastric lavage). (Figure 1) The Reverse Transcription Polymerase Chain Reaction (RT-PCR) results specific for *M. tuberculae* obtained for the same samples were also correlated. Three samples were confirmed for *M. tuberculae* and rifampicin sensitive on molecular testing, four samples were confirmed for *M. tuberculae* but rifampicin indeterminate, two samples showed invalid results and one sample was negative for *M. tuberculae*. However, none grew as mixture along with acid-fast organisms in solid LJ culture even after extended incubation of 8 weeks.

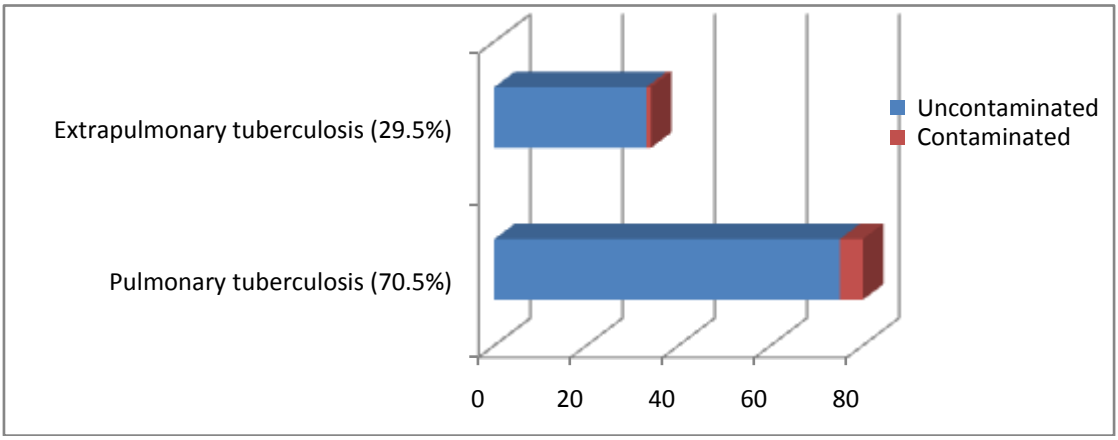

Figure 1: Distribution of pulmonary & extra pulmonary samples with presence & absence of breakthrough contamination.

Sample contamination was higher in first three months (5/7), declined in next three months (2/7) and no contaminants were isolated in last two months. Only one specimen grew two different isolates, namely *Aerobic Sporulating Bacillus* (ASB) and *Candida albicans.* Rest all bottles showed single type of bacterial organism. So with regard to the seven paired LJ slopes, three contained *Pseudomonas aeruginosa*, two contained *Aerobic Sporulating Bacilli* (ASB), two contained *Candida albicans* and one contained *Serratiamarscescens*. All contaminating organisms were confirmed by isolation and suitable biochemical testing methods and tabulated as given. (Table 2)

Seriel number	Sample	Bacterial contaminant
1	Gastric lavage	*Pseudomonas aeruginosa*
2	Sputum	*Aerobic Sporulating Bacilli (ASB)*
3	Sputum	*ASB + Candida albicans*
4	Sputum	*Candida albicans*
5	Sputum	*Pseudomonas aeruginosa*
6	Sputum	*Pseudomonas aeruginosa*
7	Sputum	*Serratiamarscescens*

Table 1: Contaminants isolated from Lowenstein Jensen slants.
***Significance could not be calculated due to small sample size.**

V. Discussion:

Even with the advent of fast detection molecular techniques for tuberculosis, culture still remains the gold standard for confirmation. Especially in resource limited settings and developing countries which bear the brunt of tuberculosis burden, the indispensability of culture laboratories cannot be ruled out. However, due to the heavy load of commensals and environmental contaminants, due to inhalational process, pulmonary samples have very high predisposition to contamination in comparison to extra pulmonary samples [5].These contaminants may out-compete mycobacteria for the purpose of important nutrients and other growth requirements and these will grow to over shadow the organism of interest, by virtue of which, false negatives may be reported in TB cultures. Our study reflects this finding with majority of contaminated growth pertaining to sputum samples (~86%) and only one extra-pulmonary sample i.e. gastric lavage.As per studies conducted in Kenya (Okumu et al, 2017) [6]and India (Sharma et al, 2017) [7],there is a higher incidence of contamination in egg based solid media. This was in convergence to our finding of almost 6% contamination in LJ media which is, however, within the permissible limit[8].As per McClean et al, there has been doubling of breakthrough contamination rate from 5% to 10% between the years 2000-2005 [3]. However in one of the studies conducted by Lumb et al, the contamination rate was found to be 4%. Overall worldwide where studies have been conducted primarily on pulmonary samples, the contamination rates varies from 4% to 30.1%.[3,9,10]All samples were tested by molecular method (RT PCR) specific for *Mycobacterium tuberculae*. Three samples showed positive for *M. tuberculae* and rifampicin sensitivity, four samples were confirmed for *M. tuberculae* but rifampicin indeterminate, two samples showed invalid results and one sample was negative for *M. tuberculae*. On solid culture, none showed mixed growth with *Mycobacterium tuberculae* even on extended culture of 8 weeks after isolation of contaminant. This might be the indication of a masking effect hampering culture methods and calls for further studies.

As per studies, the important contaminants isolated in India and worldwide[2,3,11] were also evident in our study with*Pseudomonas aeruginosa*showing the highest preponderance of 42.8%, followed by *Aerobic spore bearers* i.e. *Bacillus spp.* and *Candida albicans* which we obtained 28.5% each and also Serratiamarcescens with 14.28%.The reasons behind the higher incidence of contamination of LJ cultures in laboratories maybe due to various reasons such as inadequate cleaning of body site, space limitation in the laboratory, improper sample handling, inadequate sterilization, breakdown of instruments, electricity failure and inherent deficiency of NALC/NaOH method to remove contaminants. Most of these are unique and commonly encountered in developing countries.[2] The study by McClean et al has shown that NaOH at a concentration of 2 or 4 % was unable to eliminate all contaminants tested, with the exception of *Pseudomonasaeruginosa*. Athough NALC/NaOH method acts as a better decontaminant, the low shelf life of NaOH and reduced viability of mycobacteria due to the process can lead to false negative results [3].Use of LJ media with chlorhexidine or other antibiotic agents are the latest methods by which contamination can be controlled further. Even though this was outside the scope of our current study, future study in this regards will be carried out in order to reach an effective and sustained procedure of combating contaminants in the TB culture laboratory.

VI. Conclusion:

Breakthrough contamination of mycobacterial culture media can be caused by diverse genera of bacteria and fungi. Standardization of decontamination methods nationwide, proper laboratory disinfection, repeated trainings of technical staff, antibiotic fortified culture media and timely upgradation of standard operating procedures is required to control the same. Our mycobacterial culture laboratory is the second in the state and newly set up with freshly trained technicians. This is evident in the observation that there was higher contamination rate in the first three months after laboratory initiation which gradually declined and no contaminations have been detected in the last two months. This was achieved by strengthening the sample collection process and decontamination protocols with proper and routine disinfection of the laboratory. Standard operating procedures as per RNTCP are stringently followed. However this is a pilot study and further studies on larger sample size and longer duration have to be carried out.Furthermore, the fragility of initial laboratory infrastructure in developing countries can only be addressed in a stepwise manner and this study is a baseline data on the contaminating organisms that were isolated and encountered in similar laboratories intended to aid similar resource limited set-ups.

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