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Bacterial and Fungal Contaminants in *Mycobacterium tuberculosis* Cultures at National Tuberculosis Referral Laboratory-Kampala

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Authors' contributions

This work was carried out in collaboration between all authors. Author TJV designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author KE and IJS managed the analyses of the study and critical reviews. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aim: This study was aimed at identification of fungal and bacterial contaminants in Tuberculosis cultures at the Uganda National Reference Laboratory (NTRL).
Methods: The cultures were previously grown on Lowenstein-Jensen (LJ) medium in bottle slants. The method used involved conventional culture methods and biochemical characterization of the contaminants. Bacterial cultures were carried out in both aerobic and carbon dioxide incubators where required and to the respective temperatures of the organisms. Growth was observed between 24-48 hours for bacterial and up to 72 hours for yeast contaminants. Cultures on SDA were also monitored for three weeks for possible growth of filamentous fungi.
Results: The identified bacteria mostly included; *S. pyogenes, S. aureus, E. coli, P. aeruginosa, K. pneumoniae,* and least isolated were*; S. marcescens, H. influenza, S. pneumoniae* and *B. subtilis*

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Fungal contaminants were *A. fumigatus* and *C. albicans.*
Conclusion: Based on this study a relatively increased level of contamination was noted. We recommend that high level of aseptic techniques be maintained in addition to the current standards in order to reduce the level of bacterial and fungal contaminants in TB cultures so as to improve the detection rate of *Mycobacteria tuberculosis* by culture method.

Keywords: Bacteria; contaminants; Mycobacterium tuberculosis.

1. INTRODUCTION

Tuberculosis (TB) is one of the main killer diseases in Uganda [1]; The treatment is complicated due to poor health care and multidrug resistant strains [2]. There is no documentation at National TB Reference Laboratory Uganda about the specific contaminants in TB cultures. There is need to understand the common contaminants for improved isolation of Mycobacteria.

Lowenstein Jansen (LJ) medium is widely used for cultivation of mycobacteria [3] 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.

Tuberculosis remains a major public health problem in Uganda with an annual incidence of 330 cases of all forms and 136 new smear positive cases per 100000 per year [4]. The expected case load per year is 102,000 [5]. The 2010 Global WHO Report ranked Uganda the 16th among the twenty-two countries with the highest TB burden. Uganda like most of Sub-Saharan African countries is battling with the dual Tuberculosis and HIV/AIDS epidemic and this has complicated treatment of the later.

The HIV prevalence in the general population is 7.3%, majority of these are in the age range of 15-46 years [6]. It is estimated that about 60% of the cases are co-infected with TB (National HIV Behavioral Sero-Survey 2005). This dual epidemic has resulted in a fourfold augmentation in the notification numbers of TB cases in the region [7].

According to a report by a humanitarian organization Medecins Sans Frontieres [8], about 300 new infections and 5000 deaths each year occur. It affects the most productive age of between 15-49 years causing a loss in economy. The problem has been worsened by HIV whereby immune-compromised patients are more susceptible to the infection and the complication of the dual infection leads to many deaths [9]. The resistance of the bacterium to the existing drugs, and the mixed infections that suppress detection of the bacterium worsens the situation hence the need to identify, characterize and document the contaminants in TB cultures and improve methods of diagnosis in Uganda [3].

Molecular diagnostic methods are the main stay in diagnosis of TB in developed countries. This is because they are effective and efficient however; they are costly particularly to the developing countries and require skilled personnel in their use [10,11].

In most laboratories in developing countries including Uganda, diagnosis of TB largely relies on the direct microscopic examination of sputum specimens [11]. Despite the specificity of the technique, it has low and variable sensitivity [11] and cannot be relied on to identify other non-targeted mixed microbes which are also sometimes drug resistant strains. Mycobacterial culture is a better method since it is more sensitive in identifying mixed microbial growth; however, growth of TB bacilli on traditional solid medium requires 4-8 weeks and consequently delays appropriate treatment in absence of a confirmed diagnosis [12].

This study was aimed at isolating and characterizing the bacterial and 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

2.1 Study Site

The study was majorly carried out at National Tuberculosis Reference Laboratory (NTRL) Kampala. Phenotypic characterization was done from the National TB laboratory and fungal isolation at Makerere University Mycology Laboratory located in Mulago hospital complex. NTRL is located in Kampala, it processes and

tests patients' specimen from across the country to provide quality testing and quality control to ensure local laboratories are following standard operating procedures. Other Laboratories in Uganda rely on NTRL under the Ministry of Health to provide quality assurance for diagnosis of TB and MDR-TB, and to provide Training on new Innovations in Diagnosis. It has a Supranational Status and official accreditation of the WHO Global Laboratory Initiative.

2.2 Sample Size and Sampling Procedures

The sample size was calculated based on previous data showing that the existing prevalence is around 31% in lowland areas of Uganda [3].The sample size was calculated using the formula by Cochrane;

$n = 1.96^2 P_{exp} (1-P_{exp})/d^2$ [13].

Where

n = required sample size,
P_{exp} = expected prevalence and
d = desired absolute precision formula.

$$n = \frac{4x0.31\ (1-0.31)}{0.05^2}$$

n=342

Samples were randomly selected among the LJ slants that showed growth as an indicator of contamination from the NTRL. However, of the 342 samples, only 200 samples were used for this study based on available resources.

2.3 Design

A cross sectional study was carried out.

The solid culture medium, Lowenstein-Jensen Medium supplemented with malachite green dye to inhibit the growth of some contaminating bacteria and provide a contrast against which colonies of mycobacterium are easily seen was used. Inoculations were made on Blood agar, MacConkey agar, Chocolate, Sabouraud dextrose agar (SDA) and Potato Dextrose Agar to observe microbial growth and subsequent characterization was carried for each contaminant.

2.4 Sample Collection

The samples were obtained from previous cultures on LJ media that showed contamination at NTRL. The contaminated slants were collected and stored in an incubator at 37.5℃. A total of 200 samples (contaminated slants) were used for this study.

2.5 Laboratory Methods

2.5.1 Bacterial contaminants

Routine clinical sputum specimens that had been sent to the NTRL for TB culture by the conventional cultural isolation of *M. tuberculosis* were obtained from patients (n=200). All sputum specimens were processed routinely for the conventional detection of *M. tuberculosis* using a 4% (w/v) NaOH decontamination step, which involved extended culture on Löwenstein–Jensen (LJ) agar. Consecutively contaminated LJ slopes were identified and set aside for further processing as part of this study. Bacterial contaminants were isolated by subculture of LJ slopes onto Columbia blood agar (Oxoid), supplemented with 5% (v/v) defibrinated horse blood, MacConkey agar, and Chocolate agar for 24–48 h at 37℃ under aerobic conditions.

Identification was done by observation of colony morphology, Gram staining, and biochemical characterization.

2.6 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℃; A conidial suspension was prepared with 1ml of phosphate buffer ($_P$H 7.4) and 0.05% Tween 20 (PBST). This suspension was used for growth on PDA medium. The petri dishes were incubated at 37^{o}C and observed for colonial growth. Only one colony was selected from each plate and grown in PDA agar slants at 37℃. The conidia of the monospores were observed in sterile water at 4℃. 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℃ 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℃ 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.

2.7 Biochemical Characterization

Biochemical identification of bacterial isolates was done using standard methods [14,15]. Briefly, the tests employed were catalase, oxidase, motility test using motility indole urea medium, reactions on triple sugar iron agar (TSI), urease, nitrate reduction, indole, methyl red (MR), Voges Proskauer (VP), citrate utilization, lysine decarboxylase, and sugar fermentation tests.

Drug susceptibility testing (DST) using identification/differentiation discs was performed with the disc diffusion method on Mueller Hinton Agar (MHA) (Oxoid, Hampshire, United Kingdom) plates as recommended by the clinical laboratory standards institute [16]. Three colonies were emulsified into sterile saline and the turbidity of the suspension adjusted to the 0.5 McFarland standard. The antimicrobial discs (Oxoid, Hampshire, United Kingdom) used included Optochin (5 µg), bacitracin (10 µg) and X & V factors.

Study profile showing the schematic workflow

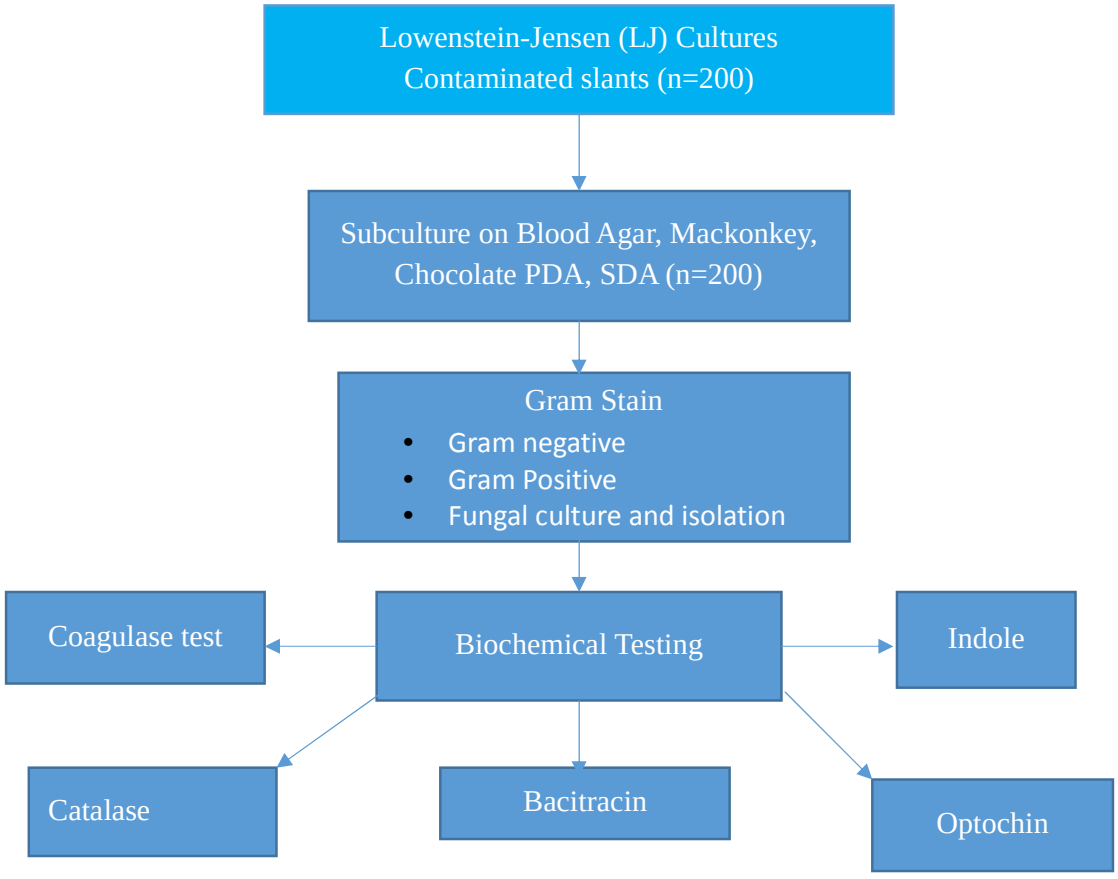

Fig. 1. Schematic representation of workflow for isolation of bacterial and fungal contaminants in TB cultures from 200 samples obtained from the National Tuberculosis Reference Laboratory (NTRL) in Kampala Uganda

3. RESULTS

A total of 200 samples were analyzed for this study. Bacterial and fungal contaminants were identified as below (Fig. 2).

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*.

4. DISCUSSION

Contamination of TB cultures is a problem in that it allows the overgrowth of another bacterium or fungus present in the sputum specimen, which can potentially mask the presence of *M. tuberculosis* [17]. Compared to other culture media, Lowenstein-Jensen has been previously shown to have a high contamination rate despite its cheap cost and applicability in low income setting [11].

This study found out that there was an overall high level of contamination of all the culture obtained from NTRL as all the samples showed considerable growth of either normal flora or pathogenic microorganisms. All the bacterial contaminants were identified in all the 200 samples analyzed.

Biochemical identification was done with emphasis put on two most occurring fungal-like microorganisms that were characterized and identified as *Aspergillus fumigatus* and *Candida albicans.* As a matter of fact, *Candida albicans* has been shown to be among the colony forming fungal contaminants in *Mycobacterium tuberculosis* cultures [18] and this strongly agrees with findings from this study.

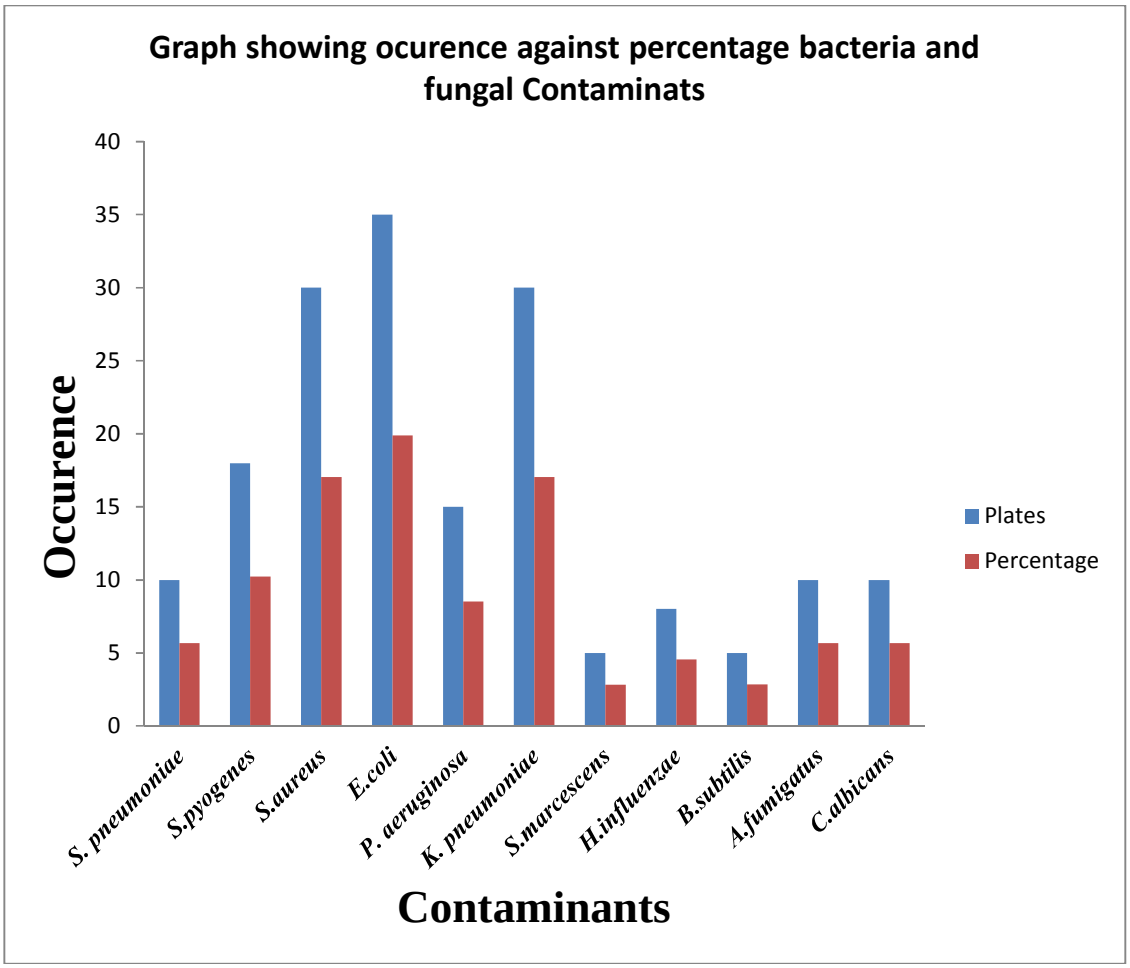

Fig. 2. Frequency of occurrence of bacterial and fungal contaminants isolated from LJ slopes

Occurrence of fungal contaminants unlike the bacterial ones was observed, an indicator of possible contamination from the environment or infection due to such fungi that mimic mycobacterial disease. *Escherichia coli* were the most occurring microorganism with the least occurring being *Bacillus Subtilis*. Among the fungal contaminants, *Aspergillus fumigatus* was the most occurring.

Escherichia coli being the most dominant bacterial contaminant in this study is comparable to a study done in the UK at the Northern Ireland Mycobacterium Reference Laboratory (NIMRL) that identified *Pseudomonas aeruginosa* as most occurring isolated contaminant [17]. In another study, the antibiotic penicillin has been shown to be effective at reducing contamination by *Pseudomonas aeruginosa* [3].

In addition, Gram-negative organisms such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Serratia marcescens* and Gram-positive *Staphylococcus aureus* identified in this study from LJ slants agree with findings from studies by McClean et al. [17], with the exception *Staphylococcus epidermidis*. Furthermore, *Serratia* and *Staphylococcus* species were similary identified in antibiotic free LJ culture tubes collected from individuals from Mbarara regional referral hospital [3] and in a hospital in France [18]. Among the least occurring contaminants in this study was the Gram-positive hemolytic colony forming *Bacillus subtilis* bacteria. The Bacillus species was similary isolated as one of the contaminants in an Indian study by Kumar et al. [19]. This points to the fact that the organisms observed in this study are among the most common contaminants in *M. tuberculosis* cultures.

The existing method of LJ TB culture needs to be used while aware of the possible breakthrough contaminants for effective and quality results to ensure that there are no any contaminants as some of the occurring organisms were normal flora indicating that the treatment procedures for methods mentioned were not 100% effective at inhibiting these organisms. It has been noted that certain normal flora are able to survive the action of 4% sodium hydroxide (NaOH) [19] which points to possibly the reason why these contaminants were identified in culture.

The contamination could as well be probably due to factors such as, contaminant within the sample, inoculating and pipetting procedures, contaminants in the laboratory cabinet or individual media tubes and the sensitivity of the different inhibitors present in the media. This study did not answer these questions as it was aimed at identification of these contaminants but not tracking the source of the contamination.

The microbial contaminants isolated in this study differed with those obtained using pyro-sequencing in another study [20] with the only exception being *Pseudomonas, Haemophilus and Klebsiella* species. The differences could be due to the high sensitivity and specificity as well as wider dynamic range of molecular based identification methods in that study.

As has been noted previously [18], no single method of mycobacterial culture is one hundred percent contaminant free but aseptic techniques and proper decontamination procedures can help minimize the level of contaminants and improve detection of *Mycobacterium tuberculosis* in culture such as chlorhexidine-based decontamination method.

5. CONCLUSION

Culture contamination presents a major threat to the sensitivity of mycobacterial culture. Contamination of mycobacterial culture media may occur during collection, transportation, processing or overgrowth by oro-pharyngeal bacteria and/or fungi. Culture contamination reduces the proportion of interpretable results and diminishes the diagnostic value of culture technique. It is therefore important to understand which bacteria and fungi are common contaminants of Mycobacterial cultures so as to device means of making the media more selective against such bacteria and fungi.

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.

6. RECOMMENDATION

Laboratory personnel need to be routinely sensitized about the occurring contaminants so that they observe the most required precautionary measures in accordance with the NTRL guidelines.

More studies need to be done to put into consideration other mycobacterial contaminants

i.e. mycobacterium other than tuberculosis (MOTTs) for example *Mycobacterium canetti*. More research should be done to trace the sources of contamination especially for those organisms that don't constitute the normal flora in order to devise means to completely or greatly reduce on the level of contamination at NTRL.

Molecular biology techniques involving PCR need to be employed in further studies as these have been proved more sensitive and effective as compared to the conventional methods [21] since the conventional methods such as microscopy have been shown to have low sensitivity [22] and could probably lead to misdiagnosis of *Mycobacterium tuberculosis* among patients.

It is also worth following up certain organisms such as Aspergillus and Klebsiella 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.

ETHICAL APPROVAL

Ethical clearance was received from the Faculty Ethical Review Committee of Kyambogo University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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