Performance of Conventional PCR for Detection of *Mycobacterium tuberculosis* in Mouthwashes

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Summary

BACKGROUND

Lack of rapid, sensitive, and affordable diagnostics has greatly hampered tuberculosis control efforts in countries with high prevalence of human immunodeficiency virus (HIV) infection and anti-tuberculosis drug resistance. Although sputum smear microscopy remains the principal tool for diagnosing active Pulmonary tuberculosis, its sensitivity is quite low. The impact of sputum culture and drug susceptibility testing is limited by the long duration and complexity of the laboratory processes. Additional diagnostic challenges posed by extra-pulmonary tuberculosis, pediatric tuberculosis, and latent tuberculosis infection. *M. tuberculosis* PCR amplification in mouthwashes was compared with existing methods for diagnosis of tuberculosis.

MATERIALS AND METHODS

This study was carried out at Mbagathi Hospital, Nairobi, between January 2016 and December 2018. During the study period, all adult patients of either sex referred to the Mbagathi Hospital TB laboratory with clinical features suggestive of tuberculosis were recruited into the study. Mouthwashes were collected through rinsing with normal saline. Mouthwash results were compared with that of reference standard culture, Ziehl–Neelsen (ZN) smear microscopy the GeneXpert.

RESULTS

Of the 300 patients that fitted the study inclusion criteria, acceptable specimen samples were obtained from 210 patients whereby 165 patients whose cultures were read as either positive or negative had their results analyzed.70 (42.4%) patients were both culture and ZN smear-positive whereas 87(52.7%) were both culture and ZN smear negative.7(4.2%) patients were culture negative but ZN positive whereas 1(0.6%) was culture-positive but ZN smear negative.69(41.8%) patients were positive for both culture and PCR whereas 80(48.4%) were negative for both cultures and PCR.14 (8.4%) patients were, however, negative for culture but PCR positive.2(2.4%) of the patients were culture-positive but PCR negative.66 (40.7%) of the patients tested positive for both culture and GeneXpert whereas 87(53.7%) were both culture and GeneXpert negative. 2(1.2%) of the

patients were culture negative but positive for GeneXpert and lastly,7 (4.3%) of the patients were culture-positive but GeneXpert negative. 45(27%) of the patients had their cultures contaminated. The test performances were as follows:100%,94%,92% and 94% for culture,90.1%,99%,90% and 91% for ZN smear, 83%,97%,81% and 83% for PCR and 97.1%,92%,88% and 90.1% for the GeneXpert respectively.

CONCLUSIONS

PCR test accurately and rapidly detected *M. tuberculosis* - specific DNA sequences of small numbers of mycobacteria in mouthwashes and was easily manipulable. Further refinements of the test may improve the diagnosis of tuberculosis in resource-constrained countries.

Keywords: *Polymerase chain reaction, tuberculosis, Ziehl–Neelsen, Mouthwashes, GeneXpert, Mycobacterium tuberculosis*

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Introduction

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* (M. *tuberculosis*), is a devastating disease that is over a century old and is responsible for the highest number of deaths from the infectious disease globally. [1, 2] Over 2 million deaths are caused by the disease and in 2018 alone, there were over 10 million new infections. [1, 2] The disease generally affects the lungs but can also affect other parts of the body.[2, 3, 4, 5] Most infections present without symptoms, what is referred to as latent tuberculosis. [2, 5]

A steady decline in the global rate of TB had been observed but this was reversed in the 1980s following the emergence of the acquired immunodeficiency syndrome (AIDS) that also prompted the development of drug resistance.[1, 2] The only available vaccine against TB, the Mycobacterium Bovis bacilli Calmette-Guérin (BCG) vaccine has varied and sub-optimal efficacy in adults.[22]

Early identification of TB is critical to receive timely treatment, reduce poor health outcomes, and further transmissions.[31,42] Sputum smear microscopy remains the principal tool for diagnosing active Pulmonary tuberculosis but its sensitivity is quite low.[23] The impact of sputum culture and drug susceptibility testing(DST) is limited by the long duration and complexity of the laboratory processes.[23,29]There are additional diagnostic challenges posed by extra-pulmonary tuberculosis, pediatric tuberculosis, and latent tuberculosis infection.[23,29,31,42] Besides, the methods require highly skilled personnel and specialized laboratory infrastructure.[29,42,43,30]

The recently rolled GeneXpert MTB/RIF assay, an automated nucleic acid amplification test can detect smear-negative, culture-positive tuberculosis near the point of care but presents variable sensitivity coupled with infrastructural demands. [35, 29] Other WHO-approved tools including fluorescent microscopy, loop-mediated isothermal amplification, and line probe assays have promising applications but are associated with inherent challenges.[8, 50]

Few studies have addressed the role of PCR amplification tests in smear negative TB patients.[24] Worrying low sensitivity (72%) has been observed with NAAT(Nucleic acid amplification technique) on sputum with low bacilli load in some countries through the tests are affordable and fast. [8,15]Owing to its sticky

texture, sputum is tedious, risky, and difficult to handle, as well as not easily obtainable from some patients. [50] Pediatric, extra-pulmonary, and smear-negative pulmonary infections pause great challenge of paucibacillary disease. [50] Moreover, sampling sputum in a big population is particularly not easy when carrying out large evaluation studies.[50]There is a need to explore alternative specimen samples to replace the tedious and risky sputum samples.[50]

Mycobacteria are known to associate with diverse biological and environmental surfaces and this may lead to their accumulation on oral epithelia in sufficient quantities.[28] Previously, oral (buccal) swabs have been evaluated as alternative non-sputum samples for the detection of tuberculosis.[12] Buccal swabs have been used to detect TB in tuberculosis patients and non - human primates (NHP, Saimiri sciureus) that do not produce sputum and those presenting with cutaneous lesions. [7,28]Oral specimens are very easy to collect as they are painless, noninvasive, non - aerosol producing, and take only seconds to complete without stringent privacy or isolation. [8, 59] Mycobacterial species have previously been successfully identified using the secA gene. [61] Pneumocystis pneumonia was also successfully identified in oral washes .[19] In the current study, clinically confirmed outpatients were evaluated for mouthwash detection of TB using conventional PCR.

Phenotypic tests

Owing to its slow growth, identification and drug susceptibility testing of M. *tuberculosis* require several weeks. [12, 21] Thus, the delays in obtaining results drive the prolongation of potentially inappropriate anti-tuberculosis therapy, contributing to the emergence of drug resistance, reduction in treatment options, and increased treatment duration with associated costs, resulting in increased mortality and morbidity [21] For these reasons, novel diagnostic methods are needed for the timely identification of M. *tuberculosis* and the determination of the antibiotic susceptibility profile of the infecting strain. [21]

Molecular methods offer enhanced sensitivity and specificity, early detection, and the capacity to detect mixed infections with improved turnaround time, cost-effectiveness, and are amenable for point-of-care testing. Despite this, phenotypic susceptibility testing is still needed for the determination of drug susceptibility and quantification of the susceptibility levels of a given strain towards individual antibiotics. [21] Considering this, current phenotypic methods should therefore be used in combination with the genotypic methods for anti-tuberculosis susceptibility testing.[21]

Get expert assay

The GeneXpert assay is widely used for M.tuberculosis detection. It detects both the presence of TB and the associated rifampicin resistance. [22]This is a useful test in confirming culture positive cases and although not as sensitive in smear negative cases,the test does not discriminate between HIV associated positive and negative cases of TB in adults.[22] The test provides accurate results and can allow rapid initiation of MDR-TB treatment, pending results from conventional culture and DST.[22]

The main advantages of the GeneXpert test include; reliability when compared to sputum microscopy and the speed of getting the result when compared with the culture test. [22] Although sputum smear microscopy is both quick and cheaper, it is often unreliable, particularly in HIV-positive people [23] Although culture gives a definitive diagnosis, results take weeks rather than hours. [23]The test presents disadvantages including the requirement of

stable electricity supply, the 18-month shelf life of the cartridges, constant instrument re-calibration, and operational temperature ceiling. [23]

PCR Assay

PCR amplification techniques have been recognized as appropriate ways to diagnose infectious diseases. The applications detect M. *tuberculosis*-specific DNA sequences and thus, small numbers of mycobacteria in clinical specimens rapidly and reliably.[26]

PCR is the method of choice for the diag nosis of tuberculosis in cases where the suspicio n is high but ZN staining is negative.[26] When the sample is positive in ZN staining, PCR permits a distinction between *M. tuberculosis* complex and other mycobacterial in fections. [26]

Current systems for clinical diagnostic applications are mainly PCR-based which can however only be used in hospitals and are still relatively complex and expensive. [24] Nucleic acid testing for infectious diseases at the point of care is beginning to enter clinical practice in developed and developing countries; especially for applications requiring fast turnaround times, and in settings where a centralized laboratory approach faces limitations. [24] However, integrating sample preparation with nucleic acid amplification and detection in a cost-effective, robust, and user-friendly format remains challenging. [24]

There are various challenges associated with PCR diagnostics that and these include; false-positive reactions resulting from contamination with DNA fragments of previous PCRs (amplicons) and false-negative reactions caused by inhibitors. Quality control of the PCR mix and the performance of the amplification itself is therefore mandatory if routine PCR is to replace culture for the diagnosis of tuberculosis. [25]

Materials and Methods

Study design, setting and population

This was a cross-sectional study. It was performed at Mbagathi Hospital, Nairobi, between January 2016 and December 2018. During the study period, all adult patients (over 18 years) of either sex referred for TB evaluation that also consented to the study were recruited. A total of 300 patients were sampled, according to the sample size calculation.

Sample size determination

It was envisaged that the detection of difference for PCR in sputum and oral washes was 99% and 73%.[8]

According to Fisher's static test[63]

$n= Z^2p\ (1-p)/d^2 = (1.96)2\ 0.73(1-0.73)$

Where: n = sample size =303

z = standard normal variable =1.96

p = prevalence proportion =0.73

d = Level of precision =0.05

Patient data and specimen collection

A questionnaire was administered and the consenting patients provided demographic and clinical information. Early morning sputum specimen samples were collected and brought along with the patient whereas spot mouthwashes were made under the instruction of the laboratory technician.

Both sputum and mouthwashes were collected in ɣ - irradiated disposable plastic containers. The molecular analysis of this study involved the use of PCR to detect TB in mouthwashes. Mouthwashes were collected by gargling 10ml of normal saline following a 5

times vigorous cough. Samples were concentrated by centrifugation at 3000- g, and the resulting pellet was frozen at - 20 C within 8 h after collection.

The two sputum and mouthwash samples were placed in a cooler box and immediately transported to the Kenya Medical Research Institute (KEMRI) - Center for Respiratory Diseases Research (CRDR), Nairobi, for processing and preservation. CRDR is one of the research centers in KEMRI that is mandated with carrying out research activities in respiratory diseases. The center houses a TB laboratory with the capacity to perform both molecular and phenotypical investigations. Previously, the center has been involved in various multinational collaborative research activities including evaluation of diagnostic kits and clinical trials.

Potions of concentrated samples collected after centrifugation were frozen at -20^0 C within 8 hours of collection,with the mouthwashes having been treated with 1% dithiothreitol (Prob Diagnostics,Unit 7,Westwood Court,Clayhill Neston and Cheshire,UK).

Microbiological investigation for M. tuberculosis

Investigations for *M. tuberculosis*, including AFB smear examination and culture, were done on all sputum specimens of patients recruited to the study.

Microbiological processing of sputum specimens

A direct smear was prepared for the auramine fluorescent stain and, where positive, the ZN stain was performed for confirmation. The respiratory specimens got subjected to autolysin/sodium hydroxide (4%) for 30 minutes at room temperature with rocking. After neutralization with 20 ml of 0.067M sodium phosphate buffer (pH 5.3), the mixture was centrifuged at 2750 6g for 30 minutes. After discarding the supernatant, 400–500 ml of the sediment was obtained.

About 60 ml of the sediment was inoculated into two Lowenstein-Jensen (LJ) medium culture bottles and incubated at 37˚C for up to eight weeks. Solid medium slants were considered positive when visible colonies grew. The colonies were further confirmed as mycobacteria by the ZN stain. Cultures positive for AFB were identified by the AccuProbe hybridization assay (Gen-Probe, San Diego, California, USA), according to the manufacturer's instructions. For mycobacteria other than M. tuberculosis, conventional biochemical tests were performed for identification. The rest of the sediment was transferred to an Eppendorf tube and stored at 220˚C if not immediately processed.

Molecular investigations

After centrifugation at 15 000 g for 10 minutes, mouthwashsupernatants were decanted. DNA extraction was done by boiling at 100' C for 10 minutes. Existing primers and PCR conditions were utilized in this study.

Primer preparation details:

For secA gene PCR; commercially synthesized primers; Mtu.Forward1 (5-GAC AGY GAG TGG ATG GGY CGS GTG CAC CG - 3' and Mtu.Reverse3 (5'-ACC ACG CCC AGC TTG TAG ATC TCG TGCAGC TC-3' were commercially synthesized (Midland Certified Reagent Company, Midland, Texas).

The following M13 primers were prepared for sequencing of the secA gene for *M.tuberculosis*: M13 Forward, 5'-GTA AAA CGA CGG CCA G-3'; M13 Reverse, 5'-CAG GAA ACA GCT ATG AC-3'.

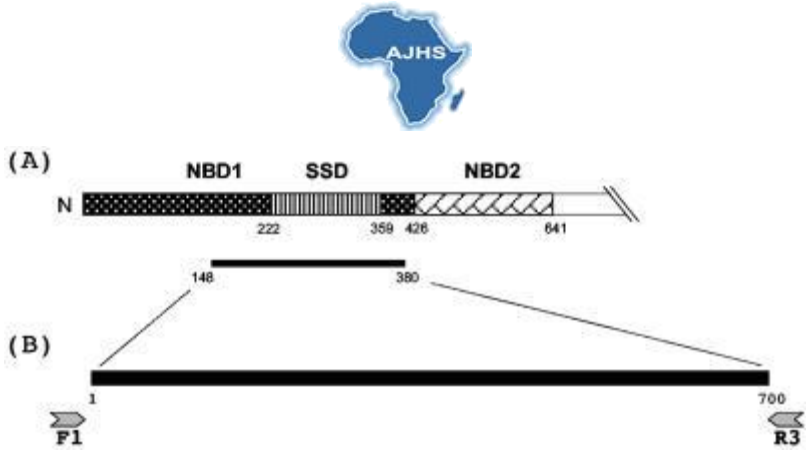

Figure 1: 649-amino-acid schematic representations; (A) The SecA1 protein from M. tuberculosis contains two nucleotide-binding domains (NBD1 and NBD2) and a substrate specificity domain (SSD). The SSD is embedded in NBD1. The thin black bar shows the region of the protein coded by the secA1 gene region targeted in the assay. Numbers indicate amino acid residues. (B) Primers Mtu.Forward1 (F1) and Mtu.Reverse3 (R3) was used to generate a 700-bp fragment from the secA1 gene. Numbers indicate the nucleotide position in the amplified fragment of secA gene sequences.

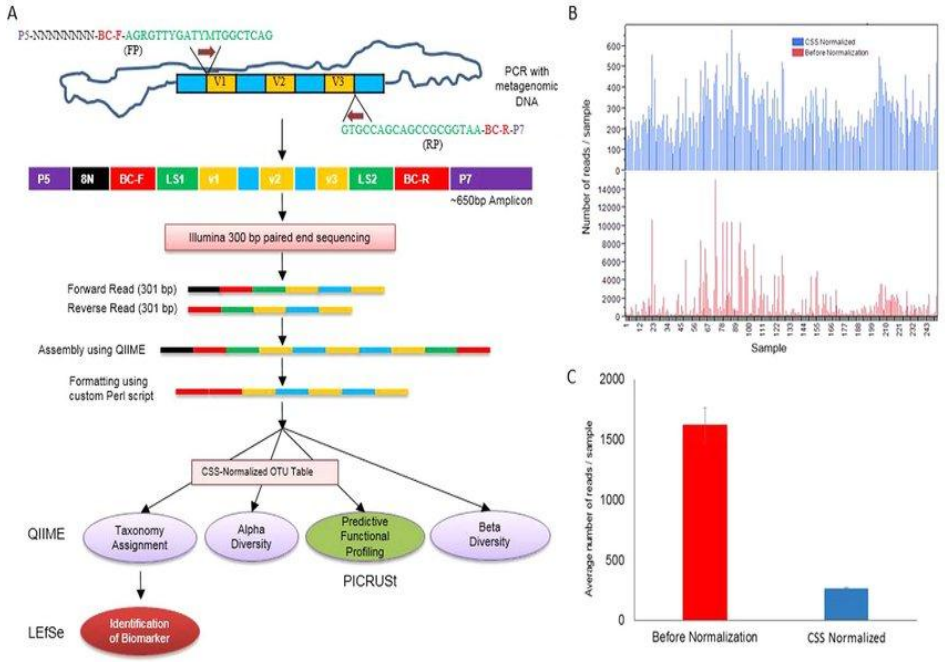

Figure 2: Schematic strategy for 16S rRNA sequencing. (a) Flow diagram representing library preparation, formatting reads acceptable to QIIME analysis which was further analyzed using LefSe and PICRUSt. *Normalized OTU table was used for PICRUST analysis. (FP = forward primer, RP = reverse primer, V = variable region of 16s RNA gene, P5 and P7 = Illumina sequencing primers, N = random nucleotide, BC-F = barcode of forward primer, BC-R = barcode of reverse primer, and LS = linker sequence). (b) Bar graph showing reads distribution of 252 samples before (red) and after normalization (blue) of OTU table. (c) Bar graph showing an average number of reads per sample with standard error before (red) and after normalization (blue). (https://www.researchgate.net/figure/Schematic-strategy-for-16S rRNA-sequencing-a-Flow-diagram-representing-library_fig1_303086665)

For sequence comparison, a set of universal bacterial primers for 16S RNA were also proposed as 5'-TGCCAGCAGCCGCGGTAATAC-3' (515-535, forward) and 5'-CGCTCGTTGCGGGACT-TAACC-3' (1107-1087, reverse) (E Coli, J01859). These primers are highly conserved among a wide range of bacteria and amplify a 593-bp fragment of the 16S rDNA.

Briefly, PCR amplifications were performed in a Perkin-Elmer 9600 Thermocycler (Perkin Elmer, Foster City, California, USA) with a reaction mix containing 2.5 mM MgCl2, 1 pmol of forwarding primer, 1 pmol of reverse primer, 1 U of uracil N-glycosylase (UNG) (Roche Diagnostics), 5 µl of extracted DNA, 1 U AmpliTaq Gold polymerase (Perkin Elmer, Foster City, California, USA) and ultrapure water to a final volume of 25 µl.

Amplification parameters consisted; 30^0 C for 10min, 10 min at 95^0 C and 49 cycles of 1 min at 95^0 C, 1 min at 65^0C, 1 min at 72^0C, and a final incubation step of 10 min at 72^0C. Negative controls included ultrapure water for every amplification reaction mixture.PCR products were visualized by UV illumination of ethidium bromide-stained 2% agarose gel following electrophoresis. The purification of the remaining PCR product was achieved with a Microcon-100 micro concentrator (Millipore, Bedford, Mass.), following the manufacturer's instructions.

Ethical consideration

The ethical approval of this study was provided by the Scientific Ethical Review Committee of KEMRI (KEMRI – SERU) accordingly with the stipulation that samples from the study and the patient data were well protected and used for research purposes under consent from the patient as indicated in the study. It was clear that procedures in this study did not infringe discomfort nor disrupt normal patient care.All data were anonymously handled and remained confidential

Statistical analysis

SPSS version 21.0; $\chi 2$ tests and kappa statistics were employed for the analysis with p-value <0.05 considered statistically significant. Mouthwash PCR, smear microscopy, and the GeneXpert demonstrated high sensitivity and specificity values.

Results

Of the 300 patients that fitted the study inclusion criteria, 210 provided adequate samples as required. Out of these, 165 patients whose cultures were read as either positive or negative were analyzed. This is because the culture was the Gold-standard (or reference standard) among the tests performed. Amongst these,70 (42.4%) patients were both culture and ZN smear-positive whereas 87(52.7%) were both culture and ZN smear negative.7(4.2%) of the patients were culture negative but ZN positive whereas 1(0.6%) were culture positive but ZN smear negative.69(41.8%) patients were positive for both culture and PCR whereas 80(48.4%) were negative for both cultures and PCR.14 (8.4%) were, however, negative for culture but PCR positive.2(2.4%) of the patients were culture-positive but PCR negative.66 (40.7%) of the patients tested positive for both culture and GeneXpert whereas 87(53.7%) were both culture and GeneXpert negative. 2(1.2%) of the patients were culture negative but positive for GeneXpert and lastly,7 (4.3%) of the patients were culture-positive but GeneXpert negative. 45(27%) of the patients had their cultures contaminated.

Table 1: Performance of ZN SMEAR, PCR, and Gene Expert Compared to Culture as the Gold Standard

	CULTURE -TB						
TB	POSITIVE	NEGATIVE	Sensitivity	Specificity	PPV	NPV	K
POSITIVE	70	0	1	0.940594	0.921053	0.940594	0.964912
NEGATIVE	6	95					
95% CI							
(n=165)			82–100%	82–100%	82–100%	82–100%	82–100%
ZN smear							
POSITIVE	70	7	0.909091	0.988636	0.897436	0.915789	0.951515
NEGATIVE	1	87					
95% CI							
(n=165)			82–100%	82–100%	82–100%	82–100%	82–100%
PCR							
POSITIVE	80	14	0.831325	0.97561	0.811765	0.833333	0.90303
NEGATIVE	2	69					
95% CI							
(n=165)			82–100%	82–100%	64–81%	82–100%	82–100%
Gene expert							
POSITIVE	66	7	0.970588	0.925532	0.88	0.90625	0.944444
NEGATIVE	2	87					
95% CI							
(n=165)			82–100%	82–100%	82–100%	82–100%	82–100%
Kappa (K) values are within the confidence index at 95%.							

The performance of each test was 100%,94%,92% and 94% for culture,90.1%,99%,90%,and 91% for ZN smear, 83%,97%,81% and 83% for PCR and 97.1%,92%,88% and 90.1% for the GeneXpert respectively.

Discussions

This study determined the performance of ZN smear, PCR and the GeneXpert in comparison with sputum cultures from TB suspect patients reporting at Mbagathi Hospital, Nairobi between January 2016 and December 2018. The sensitivity, specificity, positive and negative predictive values of each method of 100%,94%,92% and 94% for culture, 90.1%, 99%, 90% and 91% for ZN smear, 83%,97%,81% and 83% for PCR and 97.1%, 92%,88% and 90.1% for the GeneXpert respectively, varied from the earlier reported sensitivities of 57% to 76% in smear-negative specimens using commercial molecular assays with high specificity (specificity; 97% - 99%).[8] In both cases the results were obtainable within 4 h. Recently in Uganda, the sensitivity of PCR for TB detection was reported at 87%. [72] It is often difficult to diagnose TB in patients who fail to produce a productive cough.[57] Such patients are either missed out or take a long time to have their cultures grow, posing a danger of infecting other people in the community.53 types of mouthwash present an optional

specimen sample that is more easily accessible and attainable at the point of care, less difficult to handle and is easily processed.[8,47,66]

Recent studies have shown that throat washing is a promising candidate for 2019-nCoV screening and monitoring due to its non-invasiveness and reliability together with high sensitivity confirming an overlap in use of oral mucosa and mouthwashes for diagnosis of the two diseases.[7,12,28,44,64]Detection of M. *tuberculosis* DNA in oral mucosa has previously been reported in human and non-human primates.[12, 7] This demonstrated that M. *tuberculosis* DNA is a common occurrence in oral mucosa and washes. [12, 7]

The most widely used target for MTB identification is the IS6110, a plasmid-carried, transposable element that exhibits low copy numbers; 25 copies per MTB genome and is liable to miss out in some strains. The target is a valuable marker for studying the spread of M. *tuberculosis* through fingerprinting.[5] However,Mycobacterium spp. have been successfully identified using the SecA sequences.[68] Earlier, Davies *et al* successfully targeted the gene for TB inpatients.[8]Successful detection of MTB DNA in mouthwashes supports the role of environmental stress in enabling M. *tuberculosis* secret virulence adhesions for attachment to epithelial cells, thus ensuring the beneficial pathological and immunological roles of the secA pathway both in mycobacteria and another bacterium. [73]

Many patients struggle to produce adequate sputum for testing, especially in active case-finding scenarios, a situation demanding the use of easy-to-collect, non-invasive alternative samples. M. *tuberculosis* cells or DNA are deposited non-specifically on oral surfaces.[8] Although there may be fewer bacilli on average in the oral cavity, the oral sample is less viscous and less complex.

There is continued search and multi-centered evaluations of various strategies for scaling up diagnosis and the associated rise in TB drug resistance.[28,31]Considerable interest has been directed to the use of molecular probes with the most recent one being the GeneXpert rolled out by WHO in 2011.[57]

Although nucleic acid-based diagnostic tests such as PCR are quite sensitive, the tests often require isolation and concentration of nucleic acids from biological samples.[53] DNA was extracted from mouthwash pellets through boiling for 10 minutes at 100°C. Commercial purification kits are difficult to use in low-resource settings because of their cost and insufficient laboratory infrastructure .[53]Despite its known possession of a tough cell wall, mycobacterial DNA has been successfully extracted through heat treatment of sputum.[39] Precautious heat treatment has been in practice as a low-cost approach for successful DNA extraction as high-temperature exposure is known to cause damage to cell membranes and cell walls. [10] Temperature fluctuations have however been reported to result in sub-optimal DNA quality and therefore emphasizing the need for enhanced precautions.[10]

Additional work may however be necessary to adapt mouthwashes for use on automated diagnostic platforms, such as GeneXpert MTB assay. Thus, a reliable POC diagnostic device could reduce transportation needs and risk of spreading infections.[55]As a limitation, the study did not evaluate PCR on sputum due to a lack of adequate reagents. It was also not possible to have intra-strain comparisons to associate positive results with either lung or oral infection.

Conclusion

The use of mouthwashes for PCR detection of TB DNA is a non -invasive technique that would be safe and less involving in accurately and reliably detecting tuberculosis.

Recommendations

The use of molecular testing with mouthwashes is promising if modified into a rapid, inexpensive format, preferably integrating low-cost approaches. Protein secretion can be targeted for developing drugs against tuberculosis. Strain typing of mouthwashes and paired sputum samples could rule out whether the TB infection is an oral infection or a lung infection. A reliable POC diagnostic device could reduce transportation needs and risk of spreading infections. The sample is applicable for widespread testing at a very early stage of disease in clinics, emergency departments (EDs), airports and aged care facilities where ultra-fast screening with high accuracy is necessary. Besides improving PCR speed, developing a multiplexed PCR-based assays for other respiratory diseases can reduce the psychological burden of COVID-19 for both patients and healthcare workers, and the government.

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