Effects of Sputum Quality on Xpert® MTB/RIF Results in The Detection of Mycobacterium Tuberculosis from Persons Presumed To Have TB in EAPHLN Project Operational Research Study Sites in Kenya

*Orina F.[1], Mwangi M.[2], Githui W.A[1], Ogaro T[5], Kiptoo M.[3], Sang W.K.[4], Kariuki J.N.[2], Wanzala P.[2]

1. *Center for Respiratory Disease Research (CRDR), Kenya Medical Research Institute*
2. *Center for Public Health Research (CPHR), Kenya Medical Research Institute*
3. *KEMRI Graduate School of Health Sciences (KGSHS), Kenya Medical Research Institute*
4. *Center for Microbiology Research (CMR), Kenya Medical Research Institute*
5. *Division of TB, Leprosy & Lung Disease, Ministry of Health Kenya*

Corresponding author Email: orinafred@gmail.com

The East Africa Public Health Laboratory Networking Project (EAPHLNP) is a regional project involving five East African countries, Namely: Burundi, Kenya, Rwanda, Uganda and Tanzania and it is supported by the World Bank.

Summary

BACKGROUND

The common problem in tuberculosis (TB) management is mis-diagnosis or under-diagnosis of cases leading to high morbidity and mortality. In order to reverse this, new diagnostic tools for detection of Mycobacterium tuberculosis (MTB) the causative agent of TB disease have been developed. However, in the evaluation process of these tools many studies have not considered attributes of sputum quality in their testing algorithm.

OBJECTIVE

This study aimed at evaluating the effect of sputum quality in detection of MTB when using Xpert® MTB/RIF (GeneXpert) among patients presumed to have pulmonary TB.

METHODOLOGY

Between February 2013 and August 2014 a total of 3585 Spot and morning sputum specimens were collected from 1918 persons presumed to have pulmonary TB enrolled in nine East Africa Public Health Laboratory Networking (EAPHLN) Project study sites in Kenya. The mean age was 40 (+17SD) years ranging between 18 and 95 years. Some of these specimens (512) were rejected and 3073 were analyzed. The specimens were appropriately packaged and transported to KEMRI Mycobacteriology research laboratory where they were macroscopically characterized into muco-purulent; mucoid, salivary or blood stained. The sputum specimens having reddish color was labeled as blood-stained sputum. Each specimen was processed for GeneXpert testing and culture.

RESULTS

Upon macroscopic characterization, out of the 3073 specimens received, 46.1% were mucoid, 44% salivary, 7.5% muco-purulent, while 2.4% were blood stained. Bivariate analysis revealed that there was a significant association between sputum quality and gender ($p<0.001$), age ($p=0.022$), specimen type ($p<0.001$), and HIV status ($p=0.003$). Performance of GeneXpert on the different specimen categories, muco-purulent (85.7%; CI 95%, 67.4-100%) and mucoid (85.3%, 95%CI: 77.393.3%) specimens had higher sensitivity when compared to salivary specimens

(76.7%, 95%CI: 64.1-89.3). However when stratified by HIV status, GeneXpert detected more MTB on salivary specimens produced by HIV positive (85.7%, 95%CI: 67.4-100%) patients than those from HIV negative patients (71.4%, 95%CI: 52.1- 90.7%).

CONCLUSION

By macroscopic characterization, any sputum specimen type from HIV Positive or Negative persons presumed to have tuberculosis can be used in diagnosis of tuberculosis regardless of sputum quality classification. However, the sensitivity of GeneXpert was higher in morning sputum specimens that were muco-purulent and mucoid with high MTB yield than in spot sputum specimens which were salivary with low MTB yield. Also, GeneXpert sensitivity was higher, though not significant, in salivary specimens from HIV positive individuals than those of HIV negative individuals. Sputum specimen quality assessment should be considered as an integral part of routine laboratory diagnosis of TB especially in HIV negative individuals.

Keywords: Specimen quality, Sputum, Tuberculosis, Xpert® MTB/RIF, GeneXpert

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Introduction

Tuberculosis (TB) mainly occurs in low-income and middle-income countries, where sputum microscopy is the primary method for diagnosing pulmonary tuberculosis [1]. A major drawback of conventional microscopy, especially Zeihl Neelsen (ZN), is the relatively low sensitivity compared to sputum culture which is used for definitive diagnosis of TB [2, 3, 4]. The limited culture facilities and the long turn-around times involved with sputum culture hinder use of culture for routine diagnosis.

New diagnostic methods are being developed for detection that includes rapid-culture systems, antigen detection, immune-based assays, and nucleic-acid amplification tests [5]. The Xpert® MTB/RIF assay (GeneXpert) is a rapid molecular diagnostic and automated test that can detect both TB and rifampicin resistance, within two hours after starting the test, with minimal hands-on technical time [6, 7]. The GeneXpert assay is a hemi-nested realtime PCR method that detects Mycobacterium tuberculosis (MTB) and rifampicin resistance.

Due to its ability to detect MTB better than microscopy [8] the GeneXpert is rapidly being adopted by many countries including Kenya. Implementation on a large number particularly in developing countries is an attempt to provide better diagnosis of TB in the high risk population to achieve the millennium development goal global 6 target [9]. Sputum is the preferred specimen for diagnosis of pulmonary tuberculosis since it is non-invasive. Discolored sputum is commonly interpreted by both patients and physicians as a clinical sign for the presence of infection [10, 11].

However, for optimal laboratory diagnosis of pulmonary TB, patients need to appropriately expectorate sputum from deep within the lungs in order to optimize the chances of having a high- acid fast bacilli (AFB) density in submitted sputum [12]. Ideally, a good sputum specimen consists of recently-discharged material, with minimum amounts of oral or nasal material. The macroscopic appearance of sputum samples may vary considerably, and many laboratories use a time-honored descriptive classification of mucoid, muco-purulent, and bloody [8, 13, 14]. Poor quality specimens are mainly thin and watery or composed largely of saliva [12, 15].

Despite the fact that, the presence of blood in sputum has been described as an indicator for pulmonary TB especially in developing countries [16, 17], different studies have shown that, hemoptysis can also result from other conditions including but not limited to bronchitis and pneumonia most common, but also *lung neoplasma, aspergilloma, bronchiectasis, coccidioidomycosis, pulmonary embolism, pneumonic plague* and *cystic fibrosis* [17, 8, 19].

To ensure desirable treatment outcomes, limit TB transmission and obtain successful TB control, early diagnosis of TB is critical for proper and early

identification of cases [20]. This can be achieved by provision of good quality sputum specimen.

The objective of this study was to assess if the quality of sputum specimens affected the detection of MTB when using GeneXpert for diagnosis of TB. The results from this study will provide guidance on collection of the most appropriate quality sputum specimen for diagnosis of pulmonary TB.

Methodology

We analyzed sputum specimens from HIV positive and negative persons presumed to have TB enrolled in nine East Africa Public Health Laboratory Networking Project study sites (Malindi, Lamu, Busia, Kitale, Nyahururu, Wajir, Machakos, Narok and Kisii) in Kenya.

The study was approved by the Kenya Medical Research Institute (KEMRI) Ethical Review Committee. All participants provided written consent before being included into the study. After medical examination, enrolled participants were instructed on sputum collection procedure by hospital staff, oriented on the procedure and then requested to provide spot and morning sputum. The spot specimen was collected when the patient attended the clinic for the first time of recruitment whereas the morning was collected early morning sample at home the next day. The specimens were appropriately packaged and transported to KEMRI Mycobacteriology research laboratory.

On arrival at KEMRI, sputum specimens were logged in the laboratory data management system using a custom made electronic data management system. An acceptance and rejection criteria was used and specimens with respective accompanying documentation, correctly labeled with collection date and study participant particulars were accepted. The specimens were then classified macroscopically, by skilled retrained laboratory staff as previously described by Yoon and collogues [21] i.e. clear and watery appearance without any viscosity was categorized as saliva while the differentiation between mucoid and purulent sputum was based on a five-point sputum color chart (BronkoTest; Heredi lab Inc., Salt Lake City, UT, USA). Colors 1 and 2 were regarded as mucoid and colors 3 to 5 as muco-purulent sputum. The sputum specimens having reddish color was labeled as blood-stained sputum.

When the sputum specimens were heterogeneous, the predominant portion was considered to be the quality of sputum specimen. Presence of food particles was also recorded.

Sputum specimen processing for GeneXpert was done according to manufacture recommendations. Briefly, the reagent buffer containing NaOH and isopropanol was added in a 21 ratio to at least 2 ml of the specimen. The mixture was incubated for 15 min with intermittent hand mixing. Two milliliters of the resulting liquefied inactivated sample was added to the Xpert® MTB/RIF cartridge (Cepheid, Sunnyvale CA, USA). The cartridge was placed in the instrument module, and the automated processes initiated. Results were automatically generated within 2 hours and reported as MTB-negative or -positive (with semi-quantification) and RIF sensitive or resistant, error or invalid.

Specimens were also processed for culture by the standard NALC-NaOH digestiondecontamination method; briefly, an equal volume of 2% NaOH and 2.6% Sodium citrate containing 0.5% NALC was added to each tube, mixed by vortexing and then incubated at room temperature for 15 minutes. Within the incubation period the tubes were inverted several times then vortexed at regular intervals. Phosphate-buffered saline (pH 6.8) was added up to 45ml and then centrifuged at 3000 X g for 15 minutes. The supernatant was carefully decanted off and the sediment re-suspended in 1.5ml of phosphate-buffered saline, this was vortexed and used in inoculation into Lowenstein-Jensen (LJ) culture media and Mycobacterium Growth Indicator Tube (MGIT). The MGIT contained Middlebrook 7H9 broth, supplemented with MGIT Growth

Supplement and PANTA (BD) and incubated at 37ºC up to 42 days in the automated BACTEC 960 machine (Becton Dickinson). Positive cultures were subjected to Zeihl-Neelsen (ZN) staining to confirm the presence of AFB and further immune-chromatographic analysis for identification of Mycobacterium tuberculosis complex [22].

The data analysis was performed using IBM SPSS, version 21.0 for Windows. Descriptive statistics such as mean, standard deviation and range were used to analyze continuous variables while frequencies and proportions were used to analyze categorical variables. Pearson‖s Chi-square was used to test for the association

between quality of specimen and different independent factors. Sensitivity and specificity of the GeneXpert in relation to specimen quality was calculated for each and expressed as percentages with 95% confidence intervals.

Results

In this study, 3585 specimens from 1918 people presumed to have TB were received. The mean age was 40 (+17SD) years ranging between 18 and 95 years. Some of these specimens, 512 were rejected and 3073 were analyzed. The gender distribution among participants, whose specimens were analyzed, was comparable with the males having a proportion of 52.9%.while females 47.3%. 2506 specimens obtained from individuals whose HIV status was available of which 664 (26.5%) of the specimens were from HIV positive individuals. Same individuals gave the spot and morning specimens hence the overlap in the HIV status occurred when the specimens were categorized by HIV status.

Distribution Specimen Quality by Gender, Age, Specimen Type and HIV Status

Table :1 presents the distribution of specimen by quality. Mucoid (46.1%) and salivary (44%) specimens contributed to over 90% of all specimens analyzed with a small proportion of specimens being muco-purulent (7.5%) and bloodstained (2.4%) *(Table:1)*

Table 1: *Distribution Specimen Quality*

Specimen quality	Total
	% (95% CI)
Muco-purulent	7.5 (6.6-8.4)
Mucoid	46.1 (44.3-47.9)
Bloodstained	2.4 (1.9-2.9)
Salivary	44.0 (42.2-45.8)
Total	3073

Table 2: *Distribution Specimen Quality by Gender and Age*

Specimen quality	Gender		Age in years		
	Male % (95% CI)	Female % (95% CI)	<30 yrs % (95% CI)	30-49 yrs% (95% CI)	>50 yrs% (95% CI)
Muco-purulent	8.3 (6.9-9.7)	6.8 (5.5-8.1)	6.4 (4.8-8.0)	7.6 (6.1-9.1)	8.6 (6.6-10.6)
Mucoid	48.4(45.9-50.9)	44.1(41.5-46.7)	45.2(41.9-48.5)	45.7 (42.8-48.6)	50.3 (46.7-53.9)
Bloodstained	3.5 (2.6-4.4)	0.9 (0.4-1.4)	3.1 (2.0-4.2)	1.7 (1.0-2.5)	1.9 (0.9-2.9)
Salivary	**39.8(37.342.3)**	**48.3(45.6-51.0)**	45.3 (42.0-48.6)	45.0 (42.1-47.9)	39.1 (35.6-42.6)
Total	**1524**	**1357**	**890**	**1147**	**729**

Table 2 presents the distribution of specimen quality by gender and age. Overall distribution of specimen quality by gender was significantly different (p<0.001). The males had a significantly higher likelihood of producing bloodstained sputum (3.5%, 95% CI : 2.6 - 4.4) as compared to females (0.9%, 95% CI : 0.41 .4), whereas females had a significantly higher likelihood of producing salivary specimens (48.3%, 95% CI : 45.6 - 51.0) than males (39.8%, 95% CI : 37.3 - 42.3).

Distribution of specimen quality by age was significantly different (p=0.022). A higher proportion of the people presumed to have TB aged 50 years and

above produced mucoid specimens (50.3%, 95%CI: 46.7-53.9) compared to those aged 30 years (45.2%, 95%CI: 41.9-48.5) and those aged between 30-49years (45.7%, 95%CI: 42.8-48.6). A lower proportion of the people presumed to have TB aged 50 years or more produced salivary specimens (39.1%, 95%CI: 35.6-42.6) than those aged less than 30 years (45.3%, 95%CI: 42.0-48.6) and those aged between 30-49years (45.0%, 95%CI: 42.1-47.9).

Table 3 presents the distribution of specimen quality by specimen type and HIV status. Distribution of specimen quality by specimen type was significantly different (p<0.001). The likelihood of producing muco-purulent specimens was significantly higher in morning (8.8%, 95%CI: 7.4-10.2) than spot (6.2%, 95%CI: 5.0-7.4). Similarly, the likelihood of producing mucoid specimens was significantly higher in morning (49.0%, 95%CI: 46.5-51.5) than spot (43.1%, 95%CI: 40.6-45.6).

The likelihood of producing salivary specimens was significantly higher in spot (48.6%, 95%CI: 46.1-51.1) than morning (39.6%, 95%CI: 37.2- 42.0). Distribution of specimen quality by HIV status was significantly different (p=0.003). The likelihood of producing bloodstained specimens was significantly higher in HIV negative patients (2.9%, 95%CI: 2.1-3.7) than HIV positive (0.5%, 95%CI: 0.0-1.0).

Table 3: *Distribution Specimen Quality By Specimen Type and HIV Status*

Specimen quality	Specimen type		HIV status	
	Spot %(95% CI)	Morning %(95% CI)	Positive %(95% CI)	Negative %(95% CI)
Muco-purulent	6.2 (5.0-7.4)	8.8(7.4-10.2)	8.4(6.3-10.5)	7.5(6.3-8.7)
Mucoid	43.1(40.6-45.6)	49.0(46.5-51.5)	46.4(42.6-50.3)	45.1(42.8-47.4)
Bloodstained	2.1(1.4 to 2.8)	2.6(1.8-3.4)	0.5(0.0-1.0)	2.9(2.1-3.7)
Salivary	48.6	(46.1-		
51.1)	39.6(37.2- 42.0)	44.7(40.9-48.5)	44.4(42.1-46.7)	
Total	**1507**	**1566**	**664**	**1842**

Diagnostic Performance of Genexpert in Different Specimen Quality

A total of 839 sputum specimens with both GeneXpert and culture results were used to determine the diagnostic performance of GeneXpert in different specimen quality categories *(Table 4)*. The sensitivity of GeneXpert in salivary specimens (76.7%; 95% CI, 64.1-89.3) was lower than in mucopurulent (85.7%; 95% CI, 67.4-100) and mucoid (85.3%; 95% CI, 77.3-93.3) specimens. However, there was no significant difference in sensitivity of GeneXpert by specimen quality, but a higher likelihood of detecting MTB in muco-purulent and mucoid specimens than salivary and blood stained specimens.

Table 4: *Diagnostic performance of GeneXpert in different specimen quality*

Specimen quality	Sensitivity, %(95% CI)	Specificity, %(95% CI)	ppv	npv	n		
Muco-purulent	12/14	85.7(67.4-100)	31/38	81.6(69.3-93.9)	63.2	93.9	52
Salivary	33/43	76.7(64.1-89.3)	264/288	91.7(88.5- 94.9)	57.9	96.4	331
Mucoid	64/75	85.3(77.3-93.3)	324/370	87.6(84.2-91.0)	58.2	96.7	445
Blood stained	3/4	75.0(75.0-100)	7/7	100	100	87.5	11
Total	112/136	82.4(76.0- 88.8)	626/703	89.0(86.7 -91.3)	59.3	96.3	839

Key: **ppv**=positive predictive value, **npv**=negative predictive value, n=number, **CI**=confidence Interval

Diagnostic performance of microscopy and negative (83.3; 95% CI, 74.7-91.9%). By GeneXpert in different specimen quality by specimen quality, salivary specimens produced HIV status by HIV negative patients had a GeneXpert

Table 5 presents diagnostic performance of sensitivity of **(71.4;** 95% CI, 52.1- 90.7%), GeneXpert in different specimen quality by HIV while in HIV positive patients GeneXpert status. Only 447 sputum specimens with sensitivity was (85.7; 95% CI, 67.4-100%). In corresponding HIV status, GeneXpert and contrast, the sensitivity in muco-purulent culture results were classified by quality. produced by HIV negative (85.7; 95% CI, Sensitivity of GeneXpert was higher in HIV 59.8-100 %) was higher than in HIV positive positive (84.4; 95% CI, 71.8-97.0%) than HIV (75.0; 95% CI, 32.6-100 %).

Table 5: *Diagnostic Performance of GeneXpert in Different Specimen Quality by HIV Status*

HIV status	Specimen quality	Sensitivity(95% CI)		Specificity(95% CI)		ppv	npv	n
Positive	Muco-purulent	3/4	75.0(32.6-100)	12/13	92.3(77.8-100)	75	92.3	17
	Salivary	12/14	**85.7**(67.4-100)	54/63	85.7(77.1 94.3)	57.1	96.4	77
	Mucoid	12/14	85.7(67.4-100)	68/83	81.9(73.6-90.2)	44.4	97.1	97
	Total	27/32	84.4(71.8-97.0)	134/159	84.3(78.7-90.0)	51.9	96.4	191
Negative	Muco-purulent	6/7	85.7(59.8-100)	17/18	94.4(83.8-100)	85.7	94.4	25
	Salivary	15/21	**71.4**(52.1-90.7)	145/160	90.6(86.1-95.1)	50	96	181
	Mucoid	37/42	88.1(78.3- 97.9)	168/191	88.0(83.4-92.6)	61.7	97.1	233
	Bloodstained	2/2	100	6/6	100	100	100	8
	Total	60/72	83.3(74.7- 91.9)	336/375	89.6(86.5-92.7)	60.6	96.6	447

Key: ***ppv*** = positive predictive value, ***npv*** = negative predictive value, ***n*** = number, ***CI*** = confidence Interval

Discussion

To our knowledge, this is the first time, to describe the effect of different sputum specimen qualities on performance of GeneXpert MTB/RIF assay in detection MTB in diagnosis of pulmonary TB. From our study results, most of the specimens submitted for analysis were mucoid and salivary with small proportions of muco-purulent and bloodstained specimens. The most appropriate specimen with reference to quality for diagnosis of TB using

GeneXpert was muco - purulent, followed by mucoid, bloodstained and salivary, respectively. Muco-purulent specimens are known to be the ideal specimens for MTB detection [14]. Our results showed that, generally there was no significant difference in the performance of GeneXpert on specimen quality and HIV status. This indicates that regardless of the quality of sputum specimen, GeneXpert can be used to adequately diagnose TB in both HIV positive and negative persons presumed to have TB. When stratified by specimen quality the likelihood of detection is greater in muco-purulent and mucoid sputum specimens than salivary sputum specimens.

Tuberculosis incidence rates are higher for males‖ adults than in females [23]. Our results also show that the male gender produces good quality specimen (purulent and mucoid) as compared to female gender who mainly produce salivary specimens. Long et al showed that biological reasons in females with TB influenced their ability of producing a productive cough than in males. However, when using the GeneXpert, the probability of detecting MTB was higher in male gender than in female gender irrespective of specimen quality. Bloodstained specimens were produced mainly by males with no MTB detected. This indicates that the hemoptysis could have resulted from other medical conditions that we did not investigate.

Despite the fact that the number of specimens was small from the older age group (>50 yrs) they produced better quality specimen in contrast to younger participants. The importance of strictly following sputum specimen collection instructions may have been better understood by the older age group than the younger group. Further investigation is required to elucidate this.

The morning specimen had a higher likelihood of being muco-purulent and mucoid, while the spot specimen being salivary. This reemphasizes the need of using the morning specimen for TB diagnosis even with GeneXpert also as seen with smear microscopy and culture [24, 25]. As WHO recommends switching to same-day diagnosis with spot-spot specimens for countries that have successfully implemented the policy of twospecimen (morning-spot) case-finding strategy [26], countries should pilot the strategy to ascertain its usefulness before making decisions on a wider scale-up especially when the GeneXpert will be included.

HIV-positive patients with smear-negative tuberculosis are more likely to die during or before diagnosis than HIV-negative patients because of their immuno-suppression [27]. Lower bacillary load seen in the sputum of HIV-infected patients [28] prevents TB diagnosis especially by AFB microscopy. The detection of MTB in salivary specimens known to be inadequate in TB diagnosis [12] by the GeneXpert, especially in HIV positive patients will help in tuberculosis control. Caution must be exercised not to reject salivary specimens submitted by HIV positive individuals. Presence of blood in sputa is a symptom in diagnosis of TB. In this study MTB was not detected in bloodstained specimens both by GeneXpert and culture indicating its presence could be as a result of other medical conditions [16, 17].

Generally, for better management of patients, health workers attending to people presumed to have TB must emphasize on production of good quality specimens (muco-purulent) for definitive and evidence based diagnosis of TB. Appropriate and sufficient instructions as well as the importance of sputum quality should be given to all presumed to have TB especially those younger than 50 years of age and also HIV negative individuals not to compromise proper management of the disease. Training is necessary for staff involved in sputum collection on how to instruct patients and persons presumed to have TB to provide proper specimens for TB diagnosis and management.

In conclusion, the sensitivity of GeneXpert was higher in morning sputum specimens that are more likely to be muco-purulent and mucoid with high MTB yield than in spot sputum specimens which are more likely to be salivary with low MTB yield. However, GeneXpert sensitivity was higher, though not significant, in salivary specimens of HIV positive individuals than those of HIV negative individuals. Sputum specimen

quality assessment should be considered as an integral part of routine laboratory diagnosis of TB especially in HIV positive individuals.

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