Sputum Smear Concentration May Misidentify Acid-Fast Bacilli As *Mycobacterium Tuberculosis* in HIV-Infected Patients

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Background: Tuberculosis (TB) diagnosis in most resource-limited settings still depends on smear microscopy for identification of acid-fast bacilli (AFB). However, recently developed molecular diagnostics that test for the presence of *Mycobacterium tuberculosis* (Mtb) DNA have been shown to be superior for confirmation of TB diagnosis.

Methods: At regular clinical visits over a 12-month period, we collected sputa from HIV-infected patients presenting with signs or symptoms of TB at 2 Nigerian clinics. Sputa were stained for AFB and tested using the Genotype MTBDRplus to confirm the presence of Mtb. Other species were identified using 16S rRNA sequence.

Results: In 56% (233/415) of AFB-positive patients, Mtb was confirmed. The patients on antiretroviral therapy were less likely than those not on antiretroviral therapy to be infected with Mtb [odds ratio (OR) = 0.25, $P = 0.003$]. In a multivariate logistic regression model using clinical features and diagnostic results, abnormal respiratory findings on auscultation (OR = 3.28, $P = 0.03$) and a direct sputum smear grade >3/100 (OR = 6.4, 4.6, $P < 0.02$) were significant predictors of Mtb infection. Concentrated sputum smear was predictive of Mtb infection only at the highest grades (2+, 3+). Interestingly, among 65 samples that could not be confirmed for Mtb, 32 (49%) were found to contain other, possibly novel, actinomycetes, including atypical Mycobacteria, *Rhodococcus* spp, *Nocardia* spp, and *Corynebacterium* spp.

Conclusions: We conclude that concentrated sputum smears may misidentify other bacteria as Mtb in HIV-infected patients. The use of molecular diagnostics could reduce unnecessary or inappropriate treatment and improve identification of pathogens in resource-limited settings with high HIV burden.

Key Words: HIV/TB coinfection, concentrated AFB smears, acid-fast bacilli, genotype MTBDRplus, actinomycetes in HIV

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INTRODUCTION

Before the HIV pandemic, tuberculosis (TB) was deemed an infectious disease on the path to extinction but is now the leading cause of mortality among HIV-infected individuals.[1] Nigeria, grappling with Africa's second largest burden of HIV and TB, has identified TB/HIV coinfection management as a health priority.[2,3] Current diagnostics notoriously lack sensitivity and specificity for accurately detecting TB in HIV-infected patients due to HIV-associated immunosuppression.[4] Presently, the diagnosis of pulmonary TB in resource-limited settings (RLSs) is based on the microscopic examination of sputum smears stained for acid-fast bacilli (AFB). The phenol-based stain resists decolorization with acid/alcohol, resulting in the characteristic red/pink color of bacteria with high cell wall mycolic acid content.[5] A more sensitive technique, and the gold standard of TB diagnosis, is the growth of *Mycobacterium tuberculosis* (Mtb) on culture medium.[6] However, culture methods are costly, time consuming, and require specialized equipment and training, thus limiting both utility for patient care and likelihood of timely treatment in RLS.[7]

AFB sputum smear microscopy generally has a high specificity but low sensitivity.[8] The sensitivity is limited because it is often difficult for patients to produce high-quality sputum specimens or due to the low quantity of bacteria present

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within the sample.[9,10] Typically, sputa from HIV-uninfected patients with TB infection contain high numbers of Mtb bacilli, resulting in positive AFB smears because their immune responses tend to concentrate the bacteria by forming granulomas.[11,12] However, HIV-infected patients are more likely to develop paucibacillary disease due to poor granuloma formation as a consequence of CD4 T-cell and macrophage dysfunction.[13] Evidence suggests that concentrating bacilli in sputum samples through chemical digestion, with bleach or sodium hydroxide, and centrifugation (termed concentrated sputum smears) increases AFB smear sensitivity.[14–16]

At the time of our study, the World Health Organization (WHO) utilized the following diagnostic criteria for TB: 2 of 3 positive AFB smears from a nonimmunocompromised patient or a single AFB-positive smear from an HIV-infected patient.[17] In 2010, the WHO changed its recommendations such that a single positive AFB smear is sufficient to diagnose pulmonary TB regardless of HIV status, further highlighting the challenges related to the poor sensitivity of sputum smear microscopy.[18]

Although Mtb is a highly prevalent acid-fast organism among HIV-infected patients, other acid-fast bacteria may also be present, such as nontuberculous or atypical mycobacteria. In particular, *Mycobacterium avium–intracellulare* complex is well known to cause disseminated disease among severely immunocompromised patients.[19,20] Additionally, other members of the actinomycetes order, such as *Rhodococcus* or Nocardia, with mycolic acid–containing cell walls can yield an AFB-positive smear.[5,21] Although infrequently pathogenic in healthy individuals, these microbes can cause disease in immunocompromised hosts.[19,22,23] Furthermore, immunocompromised patients may have opportunistic colonizers with mycolic acid cell walls in their upper respiratory tract.[24] Given the high prevalence of TB in RLS, AFB-positive smears are assumed to be Mtb, and speciation is rarely performed.[18] However, this automatic classification may lead to unnecessary or incorrect treatment in a subset of cases.

Recently, several tests that simultaneously identify Mtb and the corresponding genetic markers of drug resistance have been described. Reports from studies performed in RLS indicate the tests have both high sensitivity and specificity.[25] One such test is the line probe assay Genotype MTBDRplus (Hain Lifescience GmbH, Nehren, Germany). MTBDRplus identifies a segment of 23S rDNA intended for detection of Mtb and mutations in resistance conferring genes—*rpoB* for rifampicin, *katG* gene, and *inhA* promoter region for isoniazid.[26] The test has been successfully used in several settings globally and was reported to have a high specificity (95%–100%).[27–29]

In the study described here, we used the Genotype MTBDRplus test to identify TB infection among a cohort of HIV-infected patients presenting with signs or symptoms of TB infection at 2 clinics in Nigeria over a 12-month period. Additionally, for AFB smear positive cases that did not contain Mtb DNA, we identified the bacterial species responsible for the AFB-positive (AFB+) smear result. This study provides a valuable insight into the identity of potential bacterial species that may result in a misdiagnosis of TB among HIV-infected patients in RLS.

METHODS

Patient Recruitment

Two geographically distinct Harvard/APIN PEPFAR care and treatment centers in Nigeria were chosen as sites for this study: the Nigerian Institute for Medical Research (NIMR) in Lagos (southwest zone) and Jos University Teaching Hospital (JUTH) in Jos (north–central zone). During routine clinic visits, HIV-infected patients suspected of pulmonary TB were asked to participate in the study; after the completion of informed consent, the patients were then asked to provide 3 sputum samples. Recruitment occurred between June 2009 and June 2010 at NIMR and from August 2009 to November 2010 at JUTH. The ethical approval for the study was obtained from the Institutional Review Boards at NIMR, JUTH, and Harvard School of Public Health (Protocol #16430-103).

Sputa were stained both directly and after concentration with the modified Petroff method for AFB using the Ziehl–Neelsen procedure.[30,31] The sputum smears were graded for the amount of bacteria present on the slide according to the International Union Against TB and Lung Disease. If <10 bacilli were counted within a 100 high power fields (HPF), the actual number of bacilli were reported (fraction); 10–99 bacilli/100 HPF was designated as 1+; 1–10 AFB per 50 HPF

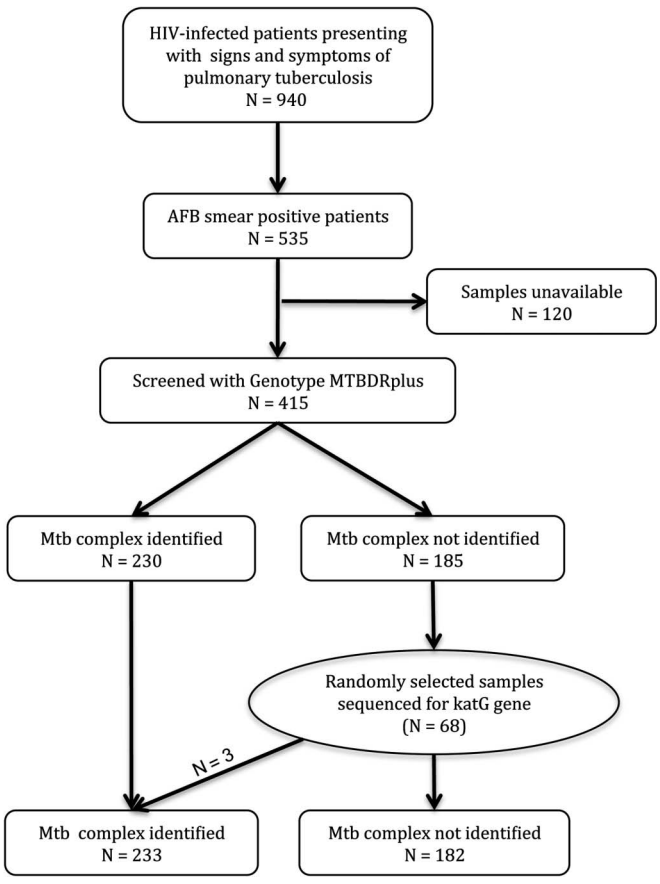

FIGURE 1. Patients presenting with signs and symptoms suggestive of pulmonary TB.

TABLE 1. Baseline Characteristics of Mtb Confirmed Vs Mtb-Free (Reference) Patients

Characteristic	All Patients N (%) or Median [IQR]	Unadjusted		Adjusted (N = 349)*	
		OR	*P*	OR	*P*
Median age	36.8 [30.6, 44.2]	0.99	0.53	1.02	0.39
Site					
Lagos	209 (50.4)	1.00		1.00	
Jos	206 (49.6)	2.15	**<0.01**	0.20	0.12
Gender					
Male	168 (40.5)	1.00		1.00	
Female	247 (59.5)	0.86	0.46	3.12	0.31
Median CD4 count (cells per milliliter, N = 382)*	180 [70, 349]	1.00	0.17		
CD4 <200	211 (55.5)	1.00		1.00	
CD4 ≥200	169 (45.5)	0.75	0.16	3.05	0.35
Viral load (median $\log_{10}$ copies per milliliter, N = 383)*	4.3 [2.3, 5.3]	1.12	0.12		
≤400 Copies per milliliter (undetectable)	99 (25.8)	0.80	0.34	1.74	0.40
On ART	163 (39.3)	0.76	0.19	0.25	**0.003**
Median time on ART (mos)	20.3 [6.7, 39.1]	1.02	**0.03**		
On NNRTIs	136 (83.4)	0.59	0.22		
Virally suppressed (N = 148)*	84 (56.8)	0.79	0.48		
Cotrimoxazole					
Receiving cotrimoxazole	272 (65.5)	0.74	0.16	0.49	0.09
Eligible† and receiving (N = 211)*	139 (65.9)	1.00	0.99		
Clinical TB diagnosis (N = 389)*	286 (73.5)	2.98	<0.01	3.29	0.00

Bold values indicate $P < 0.05$.

*Four hundred and fifteen patients were recruited; however, not all CD4 counts, viral loads were available for the time around sputum collection (t_0). The actual number of patients included in the unadjusted analysis is noted next to the characteristic. Adjusted analyses also included the following interaction terms: viral load and ART (OR: 1.09, $P = 0.91$), site, and age (OR: 1.04, $P = 0.15$), age and CD4 count (OR: 0.97, $P = 0.2$), age and gender (OR: 0.96, $P = 0.15$), gender and CD4 count (OR: 2.04, $P = 0.17$), cotrimoxazole treatment and CD4 count (OR: 2.01, $P = 0.19$), and site and ART (OR: 6.8, $P = 0.00$).

†Cotrimoxazole eligibility was defined per 2009–2010 guidelines: CD4 count <200 cells per milliliter (multivariate model $P < 0.001$).

was designated as 2+; >10 AFB per 20 HPF was designated as 3+. Patients with at least 1 AFB-positive sample were enrolled in the study, and their samples were tested with the Genotype MTBDRplus (HAIN, Nehren, Germany) as previously described.[32]

Phylogenetic Analyses

DNA amplification and sequencing methods are described in detail in the **Supplemental Methods** (see **Supplemental Digital Content,** http://links.lww.com/QAI/A396). Sequences were analyzed using Lasergene 8 (DNASTAR, Madison, WI) and compared with those of the National Center for Biotechnology Information (NCBI) database, using the NCBI's basic local alignment search tool (BLAST). *katG* amplicons were compared to ascertain that the Mtb gene was amplified, confirming the presence of Mtb. The 16S rRNA sequences were analyzed for chimeric polymerase chain reaction amplicons using the UCHIME algorithm with default parameters.[33] Nonchimeric sequences were compared with the 16S rRNA Greengenes database[34] using BLAST. The top database match with >99% homology is reported unless otherwise noted (Table 3).

Greengenes Nearest Alignment Space Termination algorithm was used to align the 16S rRNA sequences to a template sequence with default parameters, except that minimum match length was adjusted to 400 bp.[36] Sequences shorter than 400 bp were not used. Important pathogenic species in the genera and Nearest Alignment Space Termination obtained 3 near-neighbor sequences were included in the alignment. Minor manual corrections were made for the best alignment. Neighbor-joining trees were constructed under the Kimura 2-parameter model, with sequence gaps stripped before analysis and 1000 bootstrap iterations using the Mega 5.05 software package.[37] Redundant branches were removed from the final phylogenetic trees for the sake of simplicity.

Data from Clinical Records

The date of sputum collection was designated as t_0 and clinical characteristics closest to t_0 were evaluated. Viral loads and CD4 counts closest to t_0 were obtained from patient records, and were considered for inclusion in the analyses if they occurred in the time period ranging from 5 months before 2 months after t_0. The lower limit of detection for plasma viral load is 400 copies per milliliter (Roche, Amplicor v.1.5). Chest radiograph results were considered within a 90-day window before and after t_0. Cotrimoxazole drug pickup was evaluated within a 60-day window before t_0. Antiretroviral therapy (ART) eligibility was in accordance with the Nigerian National ART guidelines in 2009–2010: CD4+ cell count <200 cells per cubic millimeter or active TB (the degree of immune suppression determined timing). Patient records were reviewed for signs or symptoms of

TABLE 2. Association Between Clinical Findings, Chest Radiograph, and Sputum Smear Grade in Mtb Confirmed Vs Mtb-Free (reference) Status

	Direct Sputum Smear (n = 191)						Concentrated Sputum Smear (n = 225)					
	Mtb Confirmed	Mtb Free	Unadjusted		Adjusted		Mtb Confirmed	Mtb Free	Unadjusted		Adjusted	
Characteristic	N (%)	N (%)	OR	*P*	OR	*P*	N (%)	N (%)	OR	*P*	OR	*P*
Site (reference Lagos)	54 (53.5)	37 (41.1)	1.64	0.09	0.34	0.13	68 (53.1)	36 (37.1)	1.92	0.02	2.66	0.07
Positive finding on the CXR	68 (67.3)	43 (47.8)	2.25	0.01	1.96	0.20	88 (68.7)	45 (46.4)	2.54	<0.01	1.27	0.64
Abnormal findings on auscultation	20 (19.8)	6 (6.7)	3.46	0.01	3.28	**0.03**	25 (19.5)	6 (6.2)	3.68	<0.01	3.81	**0.02**
Blood in sputum	5 (4.9)	5 (5.6)	0.88	0.85			6 (4.7)	5 (5.1)	0.90	0.87		
Cough	92 (91.1)	71 (78.9)	2.73	0.02	2.12	0.12	117 (91.4)	75 (77.3)	3.12	<0.01	1.88	0.17
Fatigue	22 (21.8)	11 (12.2)	2.00	0.08	2.10	0.11	27 (21.1)	11 (11.3)	2.09	0.06	2.85	**0.02**
Shortness of breath	9 (8.9)	6 (6.7)	1.37	0.57			14 (10.9)	6 (6.2)	1.86	0.22		
Chest pain	19 (18.8)	13 (14.4)	1.37	0.42			26 (20.3)	14 (14.4)	1.51	0.25		
Night sweats	25 (24.7)	16 (17.8)	1.52	0.24	1.03	0.94	36 (28.1)	18 (18.6)	1.72	0.10	0.83	0.65
Weight loss	43 (42.6)	24 (26.7)	2.04	0.02	1.71	0.14	54 (42.2)	27 (27.8)	1.89	0.03	1.65	0.16
Malaise	19 (18.8)	15 (16.7)	1.16	0.70			23 (18.0)	16 (16.5)	1.11	0.77		
Aches	5 (5.0)	6 (6.7)	0.73	0.61			5 (3.9)	6 (6.2)	0.62	0.44		
Fever	53 (52.5)	36 (40.0)	1.66	0.08	1.32	0.41	71 (55.5)	41 (42.3)	1.70	0.05	1.20	0.58
Loss of appetite	12 (11.9)	8 (8.9)	1.38	0.50			13 (10.2)	9 (9.3)	1.10	0.83		
AFB sputum smear grade*												
0	33 (26.6)	52 (57.8)	1.00	—	1.00	—						
1/100–3/100	10 (8.1)	9 (10.0)	1.75	0.27	3.10	0.15	7 (5.5)	13 (13.4)	1.00		1.00	
4/100–6/100	26 (20.1)	13 (14.4)	3.15	<0.01	6.40	**0.01**	18 (14.1)	22 (22.7)	1.52	0.46	0.71	0.61
7/100–1+	32 (25.8)	16 (17.8)	3.15	<0.00	4.64	**0.02**	62 (48.4)	61 (62.9)	1.89	0.21	1.32	0.62
2+, 3+	23 (18.5)	0 (0.0)	Predicts Mtb perfectly (23 observations excluded from the model)				41 (32.0)	1 (1.0)	76.1	<0.01	52.6	**<0.01**

Bold values indicate $P < 0.05$.

*Smear grade: fractions = numerator is the actual number of bacilli counted in a 100 fields of view, 1+ = 10–99 bacilli in a 100 fields, 2+ = 1–10 AFB per fields in at least 50 fields, 3+ ≥10 AFB per field in at least 20 fields (multivariate models $P < 0.001$).

pulmonary TB, including chest pain, cough lasting >2 weeks, fever, night sweats, and weight loss. Diagnosis of pulmonary TB and its signs and symptoms were evaluated within a 30-day window before and after t_0.

Statistical Analyses

Univariate statistics and multivariate logistic regression models were generated using Stata version 10.1 (College Station, TX) to examine the association between baseline characteristics (site, age, gender, CD4 count, viral load, ART status, cotrimoxazole treatment) and Mtb confirmation. The significance level for predictor variables in the univariate analyses was set at $P \leq 0.05$. The multivariate logistic regression model was built manually in stepwise fashion following the procedure described by Hosmer and Lemeshow[38] and included the relevant baseline characteristics with $P \leq 0.2$ in the univariate analysis. The final model also included biologically meaningful interaction terms (site and age, site and ART status, age and CD4 count, age and gender, gender and CD4 count, and cotrimoxazole treatment and CD4 count) that were found, by means of the likelihood ratio test, to have a significant effect on the model.

Clinical features (eg, blood in sputum, findings on auscultation, cough, fatigue, shortness of breath, chest pain, night sweats, weight loss, malaise, aches, fever, loss of appetite), chest radiograph (CXR) results and direct and concentrated smear grades were evaluated as predictor variables for confirmation of Mtb with molecular techniques utilizing univariate methods. Variables with P values ≤ 0.20 in the univariate analyses were selected as candidates for the multivariate logistic regression model. For all the multivariate models, the threshold of significance was set at $P \leq 0.05$.

RESULTS

Among 940 patients with signs or symptoms of TB, 535 produced at least 1 AFB+ sputum sample (Fig. 1). Genotype MTBDRplus was performed for 415 patients, whereas specimens were unavailable for 120. The Genotype MTBDRplus results confirmed the presence of Mtb in 230 patients (55.4%), where 224 had an MTBDRplus result for at least 1 drug and 6 hybridized only the Mtb control band. Of the 68 samples that did not amplify with the Genotype MTBDRplus test, only 3 (4.5%) were identified as Mtb positive through independent *katG* sequencing. In total, of the 415 AFB smear positive

TABLE 3. Bacterial Species Identified Among AFB+, Mtb DNA-Negative Sputum Samples*

Genera	Species	Number of Samples in Which Species Was Found	Other Species Found Within Sample
Mycobacteria (N = 6)	*Mycobacterium ratisbonense/M. aubagnense* (98%)†	2	
	M. hodleri (97%)†	1	
	M. duvalii	1	*D. cinnamea*
	M. avium	1	
	Mycobacterium vanbaalenii/Mycobacterium vaccae	1	*Corynebacterium xerosis*
Corynebacteria (N = 14)	*C. xerosis/Corynebacterium amycolatum*	4	
	C. jeikeium (97.7%)†	1	
	C. argentoratense	3	*C. xerosis*
	C. mucifaciens	1	
	Corynebacterium variabile	1	
Rhodococci‡ (N = 12)	*Dietzia maris*	3	*C. variabile, R. ruber, C. matruchotti* (97%)†
	Dietzia timorensis	1	
	R. erythropolis (N = 1; 95%)†	5	
	R. ruber	2	*C. xerosis*
Other (N = 5)	*Nocardia higoensis*	1	
	Rothia mucilaginosa	3	
	Gordonia terrae	1	
	No 16-s rDNA amplified	33	*n/a*
	No. samples examined	65	

*Identification based on approximately 400-bp variable region of 16S rRNA.
†Percent sequence similarity with the closest named isolate was 99% unless otherwise noted.
‡*Dietzia* is closely related to *Rhodococcus*, many clinical isolates were previously misclassified as *Rhodococcus* through microbiological tests.[35]

patients, 233 (56.1%) were confirmed as Mtb positive and 182 patients as Mtb-negative (Fig. 1); this patient stratification was used in subsequent analyses.

The study population comprised approximately 60% women with a median age of 36 years (IQR: 30.6–44.2; Table 1). Over half (55.5%) of the study population had a baseline CD4+ cell count <200 cells per cubic millimeter and the median baseline viral load was 4.3 $\log_{10}$ copies per milliliter (IQR: 2.3–5.3; Table 1). At the time of sputum collection, the majority of the patients had not yet started ART but were receiving cotrimoxazole prophylaxis.

In univariate analyses, Mtb infection was significantly associated (at the $P < 0.05$ level) with clinical site [odds ratio (OR) for Jos vs Lagos = 2.15; $P < 0.01$], median time on ART (OR: 1.02; $P < 0.03$), and having a clinical diagnosis of TB (OR: 2.98; $P < 0.01$; Table 1). In the multivariate model, adjusting for baseline characteristics, clinic site no longer remained a significant predictor of Mtb confirmation (Table 1). Lack of ART (OR: 0.25; $P = 0.003$) remained a significant positive predictor of Mtb; on average, non-ART patients were 4.0 times more likely to be Mtb positive compared with those on ART. This association between ART and TB varied by site, whereby the ART patients at Jos were more likely (OR = 1.73) to be confirmed for Mtb than patients not on ART. Patient visit records were examined for clinical evidence of pulmonary TB diagnosis or initiation of TB treatment. The patients confirmed for Mtb with molecular methods were significantly more likely to be diagnosed with pulmonary TB than those in whom Mtb was not found, when controlling for baseline characteristics (OR = 3.29; $P < 0.01$; Table 1).

The finding that clinical diagnosis of TB was significantly associated with molecular diagnosis of TB prompted us to examine whether Genotype MTBDR-plus confirmed Mtb infected patients had different clinical characteristics than the Mtb-negative, but AFB+ population. Two separate models were constructed, differing in the mode of AFB smear—direct vs concentrated. When examining data in patients for whom direct sputum smear were available, unadjusted logistic regression analyses indicated that the following clinical features and available diagnostic methods at presentation were associated with confirmed Mtb: positive CXR, abnormal findings on auscultation, cough and weight loss, direct sputum smear grade >3/100 (Table 2). However, in a multivariate analysis, controlling for direct sputum AFB smear grade, the only clinical feature that remained a significant predictor of Mtb was an abnormal finding on pulmonary auscultation. Moreover, as the concentration of AFB increased in the sputum, measured by direct sputum AFB smear grade, the more likely it was that Mtb infection was confirmed with molecular techniques. The association between direct sputum smear and confirmed Mtb strengthened progressively above grade 3/100; a weaker trend was observed with concentrated sputum smear grades, which was only predictive of TB at the highest concentrations (2+ and 3+). In a multivariate analysis controlling for

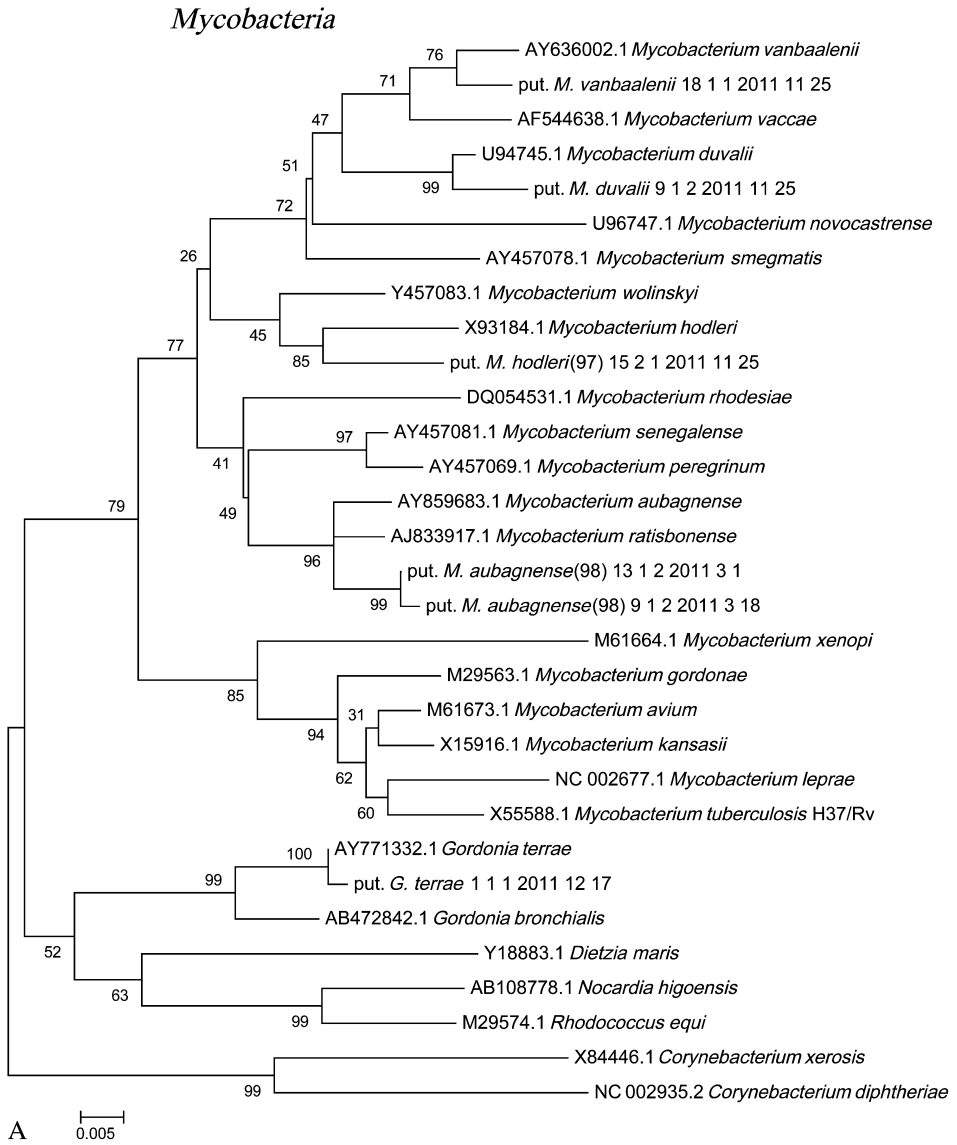

FIGURE 2. Phylogenetic analysis of 16S rRNA sequences identified in this study. Selected reference sequences from the Greengenes database were used as references.[34] Bootstrap values indicated at the nodes. Trees are grouped according to Genera listed in Table 3.4: A, Mycobacteria; B, Corynebacteria; C, Rhocodocci. Nomenclature: Sequences identified in this study are preceded with 'put,' for putative, followed by the species name of the closest matched isolate in the Greengenes database, and a laboratory sample code. Percent identity with the nearest database match is >99% unless specified after the matched name (in parentheses). Reference sample designation begins with their NCBI accession number, followed by the species name.

concentrated sputum smears, reports of fatigue and abnormal respiratory auscultation were found to be significant predictors of Mtb.

To examine what bacilli other than Mtb could be responsible for AFB+ smears, we examined the 16S rDNA sequences in the samples. DNA amplification and sequencing were successful in 49% (N = 32) of the cases (Table 3). The majority of identified bacteria were of the suborder Corynebacterineae, pointing to the selectiveness of our polymerase chain reaction to relevant actinomycetes, and not any bacterium. Six atypical mycobacterial species, other than Mtb, and other bacteria previously reported to cause pulmonary infection, including *Corynebacterium mucifaciens*, *Rhodococcus rubber*, and *Nocardia shimofusiensis* were identified. Because 2 clones of each sample were examined, we were able to identify >1 species in 7 of the patient samples (Table 3).

Most 16S rRNA sequences in this study matched a known database isolate at ≥99% homology. However, 3 *Mycobacterium* spp, 2 *Corynebacterium* spp, and 1 *Rhodococcus* spp were ≤98% homologous to matched isolates prompting additional phylogenetic analyses. Phylogenetic comparisons divided by genera are presented in Figure 2. The majority (97%) of our sequences clustered with the sequences of BLAST-matched species in the evolutionary trees. Some species, however, have different evolutionary distances from the common ancestor of their closest matched known isolate: putative (put) *Mycobacterium aubagnense*, put; *Mycobacterium hodleri*, put; *Mycobacterium duvalii*, put; *Corynebacterium argentoratense*, and *Corynebacterium jeikeium* (Figs. 2A, B). Furthermore, a put *Rhodococcus erythropolis* (95) shares the closest common ancestor with *R. rubber* not *R. erythropolis*, with low bootstrap values and a greater evolutionary distance (Fig. 2C). The

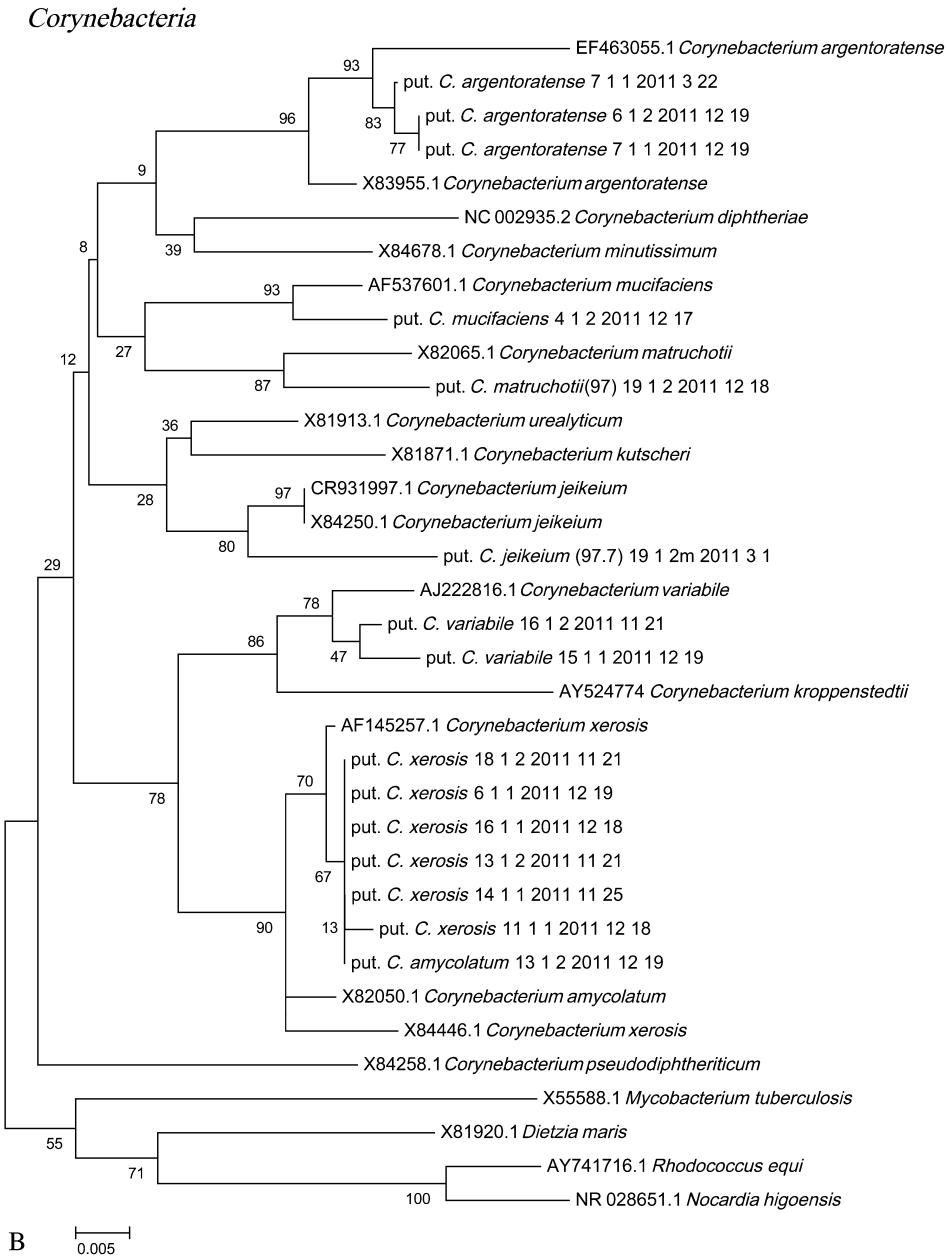

FIGURE 2. (Continued)

phylogenetic analyses of 16S rRNA infer that our sequences could be from unique species.

DISCUSSION

HIV-infected patients who have TB often have low or negative grade AFB sputum smears. However, the diagnosis of TB in many RLS, where the HIV prevalence is consequently the highest, still largely depends on AFB sputum smear microscopy. The results of our study highlight the benefits of using a molecular diagnostic in confirming the presence of Mtb infection, where the AFB direct smear was of a low grade or negative. Such use of molecular tests can greatly improve care for patients in Nigeria, where there is a high burden of HIV–TB coinfections, and in potentially other RLS.

Additionally, our results show that one should be cautious when using sputum concentration techniques to increase the sensitivity of sputum smear microscopy. We have demonstrated the presence of other AFB+ organisms in a subset of our HIV-infected patients. The majority of the microbes identified through sequence analyses in this study contained mycolic acid cell walls and are capable of retaining dye after strong acid-alcohol wash (acid-fast), thereby explaining the AFB+ smear results. These organisms are globally distributed and have been isolated from clinical specimen in prior studies from Nigeria and elsewhere.[39–43] It is possible that these non-Mtb species are opportunistic infections, leading to pulmonary infection with

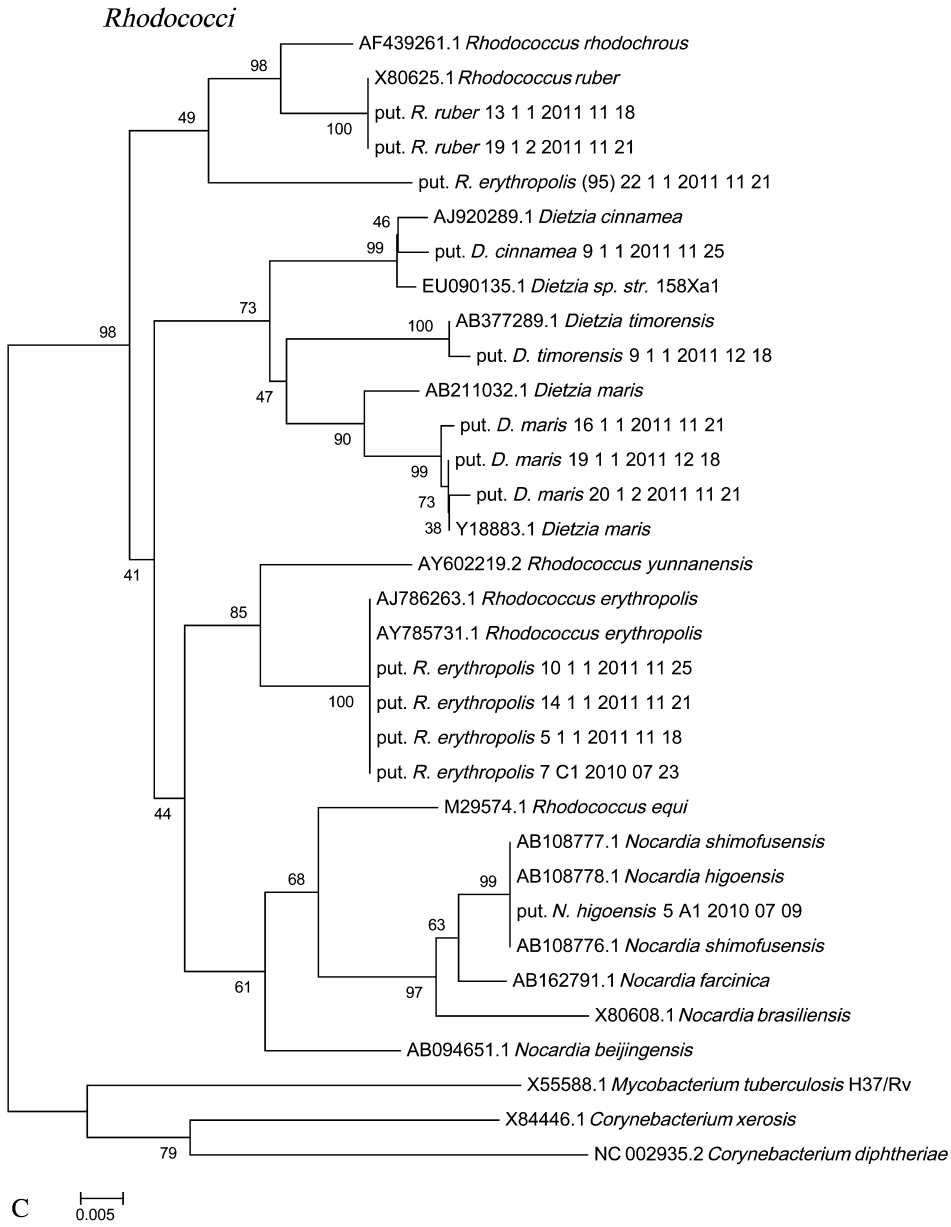

FIGURE 2. (Continued)

signs or symptoms similar to Mtb or they may simply represent colonizers that exist in parallel with a pulmonary infection. The presence of most of these organisms has been demonstrated in HIV-infected individuals before[19,20,23]; however, the resources for species or genotypic distinction are often lacking in RLS, making the likelihood of misidentification as Mtb through AFB more likely.

Based on analyses of demographic and clinical characteristics at t_0, the Mtb-positive patients only differed significantly from the Mtb-negative patients only in terms of ART status. Differences in the protective effect of ART between the Jos and Lagos study sites could stem from the variations in ART dispensing at those sites. For example, patients at Jos might have been initiated on ART immediately upon presentation with symptoms of respiratory infection, whereas NIMR physicians might have commenced ART after TB diagnostics and treatment were completed. The majority of patients seemed to be immunocompromised at the time of presentation, as indicated by CD4 counts at t_0. This finding is not surprising given that most of the patients recruited for this study were just entering the APIN/Harvard PEPFAR program at the time of sputum collection, and the majority of these patients presented for medical care for their TB-like syndrome. Although only a little over 50% were confirmed for Mtb, half of the Mtb-free individuals harbored different, potentially pathogenic, acid-fast bacteria. Both multivariate models using clinical features and available diagnostics as predictor variables included data from a subset of patients.

Although the direct sputum AFB smears with >3 bacilli in 100 high-powered (×1000 magnification) fields generally

confirmed molecular results, our study indicates that low-grade AFB smears are misleading. This finding is particularly relevant among HIV-positive populations where low smear grades tend to prevail.[4] Accordingly, in our population, the highest proportion (37.3%) of direct sputum AFB smears was graded 0 (ie, no bacilli found); for those patients with a direct AFB smear of grade 0, Mtb could only be detected in the concentrated smear. In most previous studies, concentration of sputum smears has been shown to improve AFB smear sensitivity.[14–16] However, a study performed in Uganda concluded that concentration does not significantly increase the sensitivity for Mtb detection in an HIV-infected population.[44] We observed that concentration might actually compromise the specificity by misidentifying other acid-fast organisms as Mtb (Table 3).

Typically, AFB found in the sputum of individuals in TB endemic areas are considered Mtb infection; however, due to their immunocompromised status, HIV-infected patients may become infected by other typically less pathogenic organisms. The potential for opportunistic pathogens to cause disease in HIV-infected individuals makes the diagnosis of TB more complex. Few studies have reported on the etiologies of non-Mtb pulmonary infections from AFB+ HIV-infected patients, particularly from RLS where funding for such analyses and laboratory infrastructure are limited. Given that RLS are also the areas with the highest burden of HIV infection, the need for additional data is even more imperative. Similarly, identification of potentially novel species is hard to achieve with limited infrastructure. Our sequences, even though limited to a segment of 16S rRNA, are chimera-free and seem to be distinct, pointing to previously uncharacterized species. Furthermore, when analyzed phylogenetically, the sequences cluster unusually (*R. erythropolis*) or have different evolutionary distances from the common ancestor of their closest sequences match (*C. jeikeium*, *Mycobacterium augbanense*, and *M. hodleri*). Our findings suggest that AFB+ sputum smears in immunocompromised patients can potentially result in misdiagnosis and lead to inappropriate treatment in a subset of patients.

Finding an effective diagnostic tool for TB, particularly in settings where culture equipment and expertise are not widely available, has been a WHO priority for many years. Our analyses highlight that monitoring symptoms is not a sufficiently reliable way to identify Mtb infection in all HIV-infected patients and that effective diagnostic techniques are critically needed. Molecular diagnostic techniques that can rapidly confirm Mtb infection while also identifying drug resistance, such as the Genotype MTBDRplus test, may remedy this problem and lead to improved patient care.

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