Sputum bacterial load predicts multidrug-resistant tuberculosis in retreatment patients: A case-control study

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ABSTRACT

Background: Rapid and effective diagnosis of multidrug-resistant tuberculosis (MDR-TB) is an essential component of global tuberculosis control, but most MDR-TB cases are still not diagnosed.

Objective: To assess if patient sputum bacterial load can be used to identify patients that are at higher risk of having multidrug resistant TB.

Methods: We used a case-control study and multivariable logistic regression models to investigate associations between MDR-TB and sputum bacterial load, as measured by semi-quantitative microscopy and automated time to detection of liquid culture. We assessed data from retreatment TB patients with MDR-TB (cases) and without MDR-TB (controls) at a reference laboratory in Cameroon.

Results: MDR-TB was associated with a smear microscopy grade of 3+ (odds ratio, 21.9; 95% confidence interval [CI] 6.2-76.8) or 2+ (odds ratio, 10.8; 95% CI 2.9-40.7), as compared to a result of 1+, scanty or smear-negative, among 80 MDR-TB cases and 521 controls. MDR-TB was associated with automated time to detection of ≤160 hours (odds ratio, 2.2; 95% CI, 1.1-4.7) as compared to >160 hours among a sub-population of 47 cases and 350 controls.

Conclusions: A higher sputum bacterial load is associated with MDR-TB in retreatment patients in Cameroon.

INTRODUCTION

The increasing prevalence of multidrug-resistant tuberculosis (MDR-TB) has exacerbated the ongoing public health challenge of tuberculosis control.[1] In spite of significant increases in the detection of MDR-TB in recent years, global control efforts are still limited by insufficient laboratory testing capacity; in 2013, only 28% of the 480,000 estimated incident MDR-TB cases were diagnosed worldwide. [2] Improvements in screening methods to identify the patients most at-risk of MDR-TB may help to increase testing efficiency and reduce time to diagnosis of MDR-TB cases. Better and faster diagnosis, combined with initiation on appropriate treatment, will also help to reduce disease transmission.

Previous TB treatment is the most significant known risk factor for MDR-TB, and the prevalence of MDR-TB also varies significantly by geographical region.[3] The development of drug resistance by drug selection pressure may be related to several factors, including drug supply and quality of care.[4] In addition, a significant amount of evidence now indicates that both patient and bacteria-specific variables are important contributors to the development of drug resistance. [5–8]

Genetically-based drug resistance in *Mycobacterium tuberculosis* occurs exclusively as a result of chromosomal mutations, and recent studies indicate that the rate at which these bacteria acquire drug-resistant mutations may be much higher than previously estimated. [9–12] Importantly, models developed to predict the evolution of MDR-TB *in vivo* reveal that patients with higher bacterial burdens, who have correspondingly more potential for drug-resistant mutations to be present, are at greater risk to develop MDR-TB than patients who have lower bacterial burdens. [9,10]

Clinically, more severe pulmonary tuberculosis disease is characterized by an increase in the amount of lung area involved, density of lesions, and cavitation apparent on chest x-ray, which is associated with higher rates of smear-positivity for acid-fast bacilli [13] and reduced time to detection in culture. [14]

By integrating the prediction that patients with higher bacterial burdens are more likely to develop MDR-TB, with evidence that pulmonary TB patients with more severe disease produce larger numbers of bacteria in their sputum, we aimed to determine whether patients with higher sputum bacterial loads are more likely to be MDR-TB cases. We assessed this using a case-control study of archival data from a TB reference laboratory in Cameroon.

STUDY POPULATION AND METHODS

Study population and setting

The study population consisted of patients from whom sputum specimens were received at the Tuberculosis Reference Laboratory Bamenda in Cameroon for drug susceptibility testing (DST). The lab serves four regions of the country, representing a total population of ~7.8 million people. The National TB Program (NTP) guidelines recommend DST for all previously treated cases of TB, including those classified as relapses, treatment after loss to follow-up, and treatment after failure, following WHO case definitions.[2] DST coverage for the four regions during 2012 and 2013 was estimated from NTP data as 67% of smear-positive retreatment patients; estimating the coverage of smear-negative retreatment patients is not possible from this data but is likely much lower since only ~7% of specimens received for DST during this period were smear negative. In line with WHO recommendations,[15] the NTP uses a standard 6-month rifampin-throughout regimen for new cases, and a standard 8-month regimen for retreatment cases.

Study Design

This was an unmatched case control study of archived routine laboratory data. Two case definitions were used. In the main analysis, case patients were defined as those with TB bacilli resistant to both isoniazid and rifampin, and control patients were those with TB bacilli susceptible to both or monoresistant to one of the drugs. In the secondary case definition, cases were defined as those with TB bacilli resistant to rifampin and controls were those with bacilli susceptible to rifampin. Both case definitions have clinical and diagnostic value; current World

Health Organization (WHO) guidelines recommend that patients with rifampin-resistant TB (RR-TB) are eligible for MDR-TB treatment.[16] Specimens received from June 15, 2012 to September 30, 2013 were included in the analysis. The study was approved by the National Ethics Committee of Cameroon.

Laboratory procedures

Sputum specimens were processed using the N-acetyl-L-cysteine–NaOH (NALC-NaOH) method.[17] From the re-suspended pellet, a concentrated smear was prepared and examined for acid-fast bacilli by fluorescence microscopy, 0.5mL was cultured on mycobacterial growth indicator tubes (MGIT) using the BACTEC MGIT 960 system, and ~0.1mL was cultured on Löwenstein-Jensen media. Rapid molecular DST for isoniazid and rifampin resistance was performed using the Genotype MTBDR*plus* line probe assay according to the manufacturer's instructions. Smear-positive specimens were processed and tested directly by the MTBDR*plus* assay [18]. Smear-negative, culture-positive specimens were tested for *M. tuberculosis* complex by MPT64 antigen detection (Standard Diagnostics, Korea), and if positive, then tested by MTBDR*plus* assay. Following WHO guidelines, any specimen with any resistance on the rapid assay was also tested using the proportion method on Löwenstein-Jensen media for isoniazid and/or rifampin resistance, with critical concentrations of 0.2mg/L and 40mg/L respectively. [19,20] Resistance for isoniazid or rifampin by either the MTBDR*plus* assay or the phenotypic proportion method was considered as resistant; [21] no specimens that were resistant by rapid testing were sensitive by phenotypic testing (Supplementary Figure 1). The laboratory participates in annual external quality assurance for DST under the WHO global project for TB drug resistance surveillance with excellent results.

Description of variables

Patients: As shown in Figure 1, during the study period the laboratory received sputum specimens from 739 retreatment patients that were smear and/or culture positive for acid-fast bacilli; of these, specimens from 665 patients were confirmed to be positive for *Mycobacterium tuberculosis* complex and had interpretable DST results. Among these, 601 patients (90.2%) had known TB treatment history, sex and age; 397 of these (66.1%) also had results for automated liquid culture time to detection.

Laboratory results: Patients who had at least one specimen positive for *Mycobacterium tuberculosis* complex by Genotype MTBDR*plus* assay and/or proportion method DST were considered to have TB. Microscopy results were obtained on the same day as specimen processing, which was before the DST results were available. Smears were graded semi-quantitatively according to the IUATLD/WHO scale for fluorescence microscopy and categorized as 3+, 2+ or 1+/scanty/negative. The automated liquid culture (BD MGIT 960) instrument records the time to detection (TTD) of culture positivity in hours; this variable corresponds to the number of colony-forming units (CFU) per milliliter and therefore provides a measure of the density of viable bacilli in the sputum. [22,23]

Data extraction and statistical analysis

Patients for inclusion in the analysis were identified from the electronic laboratory database, and patient information and specimen results were verified against the paper laboratory registers. Univariable and multivariable logistic-regression models were used to estimate odds ratios and the associated 95% confidence intervals. Age, sex and any variables that were found to be significant in the univariable analysis were included in the multivariable analysis, as indicated for each analysis. Epidata Analysis (v. 2.2), and Stata (v. 9.2) software were used for all analyses. The STROBE recommendations were followed for reporting these data.[24]

RESULTS

In the multivariable analysis of 80 cases and 521 controls (Table 2), MDR-TB was associated with a smear microscopy grade of 3+ (odds ratio, 21.9; 95% CI 6.2-76.8) or 2+ (odds ratio, 10.8; 95% CI, 2.9-40.7), treatment failure (odds ratio, 10.2; 95% CI, 5.4-19.2), and originating from the Southwest region (odds ratio, 6.4; 95% CI, 2.2-18.9). The distribution of MDR-TB cases as compared to non-MDR-TB controls is shown as a function of smear microscopy grade and type of retreatment case in Figure 2.

The multivariable analysis revealed an association between higher smear microscopy grade and MDR-TB that was independent of whether the patient was categorized as failure of treatment. To further explore the association between sputum bacterial load and MDR-TB among patients not on treatment at the start of the

retreatment regimen, we performed a separate analysis including only relapse and return after default patients and excluding treatment failures. In the multivariable analysis of these 48 cases and 469 controls, MDR-TB was associated with a smear microscopy grade of 3+ (odds ratio, 21.4; 95% CI, 2.9-160) or 2+ (odds ratio, 9.7; 95% CI, 1.2-79.8) and originating from the Southwest region (Supplementary Table 2).

When any rifampin-resistance rather than resistance to both isoniazid and rifampin was used as a case definition, there were 92 cases and 509 controls. In the multivariable analysis, rifampin-resistant TB was associated with a smear microscopy grade of 3+ (odds ratio, 11.9; 95% CI 4.7-30.4) or 2+ (odds ratio, 5.1; 95% CI, 1.8-14.2), treatment failure, originating from the Southwest, and being female (odds ratio, 1.8; 95% CI, 1.1-3.0), (Supplementary Table 3).

For the sub-population of patients with specimens that also had data on time to detection of liquid culture, including 47 cases and 350 controls, the median time to detection for MDR-TB cases was 139 hours (interquartile range [IQR], 106- 190), while for non-MDR-TB controls the median time to detection was 166 hours (IQR, 116-236). In the multivariable analysis, MDR-TB was associated with a time to detection result of ≤160 hours (odds ratio, 2.2; 95% CI, 1.1-4.7) and treatment failure, as shown in Table 3.

DISCUSSION

In this study of tuberculosis retreatment patients in Cameroon, we found an association between multidrug-resistant TB and higher sputum bacterial load, as assessed by both semi-quantitative sputum microscopy of acid-fast bacilli and automated time to detection of culture. An association between higher smear microscopy grade and rifampin-resistant TB was also observed; this finding is important since rifampin-resistance is frequently used as a surrogate for rapid diagnosis of multidrug-resistant TB.[25] These results are consistent with previous reports of an association between MDR-TB and smear-positivity as compared to smear-negativity at baseline diagnosis[26,27] and a report of higher numbers of MDR-TB patients among patients with higher microscopy smear grades;[28] these results are also consistent with previous reports of an association between cavitary disease and MDR-TB since cavitary pulmonary TB is associated with higher sputum bacterial load.[29,30]

This work was motivated by predictions that higher patient bacterial load is associated with multidrug resistance[9,10] and evidence that higher patient bacterial load corresponds to higher sputum bacterial load.[13,14] However, the relationship between patient bacterial load and sputum bacterial load in pulmonary TB is not simple; it depends on several factors, including patient comorbidities and physiology. For example, patients that are severely immunocompromised are more likely to have disseminated TB with fewer TB bacilli in their sputum; [31,32] in addition, in many patients highly smear-positive TB does not emerge predictably over time but instead develops quickly. [33] Similarly, the relationship between sputum bacterial load and drug resistance may depend on a range of factors, including setting-specific MDR-TB prevalence, and local policies and resources that influence how early in the course of the disease TB and MDR-TB are detected by available diagnostics. In this study, the association between higher sputum bacterial load and MDR-TB was observed for TB retreatment patients with different treatment histories, including both patients that were failures on treatment as well as patients that were categorized as relapse or return after default patients and not on treatment at the time of diagnosis. If the association between higher sputum bacterial load and MDR-TB is also observed in other settings and patient populations, this would provide additional evidence that higher patient bacterial load leads to a higher probability for the development of MDR-TB.

There were several potential limitations to this study. These data are from a single diagnostic laboratory, representing a limited geographical area. The number of case patients was relatively small, which resulted in relatively large confidence intervals for the various analyses; nevertheless, the 80 cases and 521 controls in the primary analysis provided 80% power to detect an odds ratio of 2 with 95% confidence, assuming 25-48% exposure in the control population. There are known weaknesses in the specimen collection and transport system for specimens sent to this lab in a resource-limited setting; however, our analyses indicated that these factors are unlikely to lead to systematic bias in the results. Bacterial viability remained high (94% of smear-positive diagnostic specimens were culture positive) and the culture contamination rate was low (2.1% of diagnostic specimens), as shown in Supplementary Figure 2. Also, in Cameroon, an estimated 38% of TB patients are HIV co-infected,[2] but information about patient HIV-status is not routinely collected in the lab and was not included in this analysis. A recent meta-analysis found a marginal positive association between HIV-infection and MDR-

TB globally (odds ratio 1.24; 95% CI 1.04-1.43) ;[34] due to the relatively weak nature of this association, a similar trend in our setting is unlikely to significantly influence the association between MDR-TB and higher smear microscopy grade observed here, particularly since HIV-infected patients are more likely to produce paucibacillary sputum than HIV-negative patients. Also, in this analysis of laboratory data, patient clinical data such as radiography results, history of confirmed or potential exposure to MDR-TB, duration and type of symptoms at presentation, and co-morbidities such as diabetes and substance abuse were not evaluated.

Geographical region was found to play a role in the current study. Systematic drug susceptibility testing of retreatment patients started in the Southwest region at the end of 2012, and the coverage during the study period was estimated as 21% of smear-positive retreatment patients; in contrast, for the other three regions the testing coverage was estimated as ≥75% of smear-positive patients in each region. It is possible that the relatively few specimens sent for testing from the Southwest were more likely to be from chronic patients that had survived for longer periods of time with drug-resistant disease prior to the start of systematic drug resistance testing. In spite of this effect of region on the prevalence of MDR-TB, there was a strong independent association of higher sputum bacterial load and MDR-TB across these four diverse regions of the country.

In this analysis, we used graded smear microscopy results obtained after specimen concentration prior to inoculation for culture. The advantage of using the concentrated smear result for this analysis is that these results were generated by a single laboratory with good quality control systems in place, ensuring consistency among the semi-quantified results. It was not possible to perform an analysis using direct microscopy results from the peripheral laboratories because many sites either did not indicate the microscopy result on the request form or instead reported the direct microscopy result from a different specimen obtained from the patient than the one sent to the reference laboratory for testing. It will be important to analyze the association between MDR-TB and direct smear microscopy results in future work, as this information is of more practical significance for many TB programs.

It will be of interest to assess whether the association between RR/MDR-TB and higher bacterial load that has

been observed here is also found to apply in other populations and/or settings, such as in patients who have never taken TB treatment or in places where laboratory testing for RR/MDR-TB is not yet widely available. This association may also be of use to ensure those with higher bacterial loads are prioritized for drug susceptibility testing.

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Table 1. Characteristics of TB retreatment patients according to whether multidrug-resistant tuberculosis (MDR-TB) was diagnosed

Characteristic	All patients (n=601)	MDR-TB (n=80)	Not MDR-TB (n=521)
Age, years	36 (29-46)	36 (30-45)	36 (29-46)
Female	206 (34)	34 (43)	172 (33)
Region			
Littoral	406 (68)	55 (69)	351 (67)
Northwest	115 (19)	11 (14)	104 (20)
Southwest	14 (2)	7 (9)	7 (1)
West	66 (11)	7 (9)	59 (11)
Type of retreatment patient			
Relapse	412 (69)	41 (51)	371 (71)
Return after default	105 (17)	7 (9)	98 (19)
Treatment failure	84 (14)	32 (40)	52 (10)
Smear positivity grade			
Negative	40 (7)	1 (1)	39 (8)
Scanty	35 (6)	0 (0)	35 (7)
1+	77 (13)	2 (3)	75 (14)
2+	140 (23)	17 (21)	123 (24)
3+	309 (51)	60 (75)	249 (48)

Data are median (IQR) or number (%).

Table 2. Associations of multidrug-resistant TB (MDR-TB) with age, sex, region, type of retreatment patient, and acid-fast bacilli smear-positivity

Variable	Unadjusted odds ratio (95% CI)	P Value	Adjusted odds ratio (95% CI)*	P Value
Age				
≤ 35 yrs	1.0		1.0	
>35 yrs	1.0 (0.6-1.6)	0.9	1.0 (0.6-1.7)	1.0
Sex				
Male	1.0	1.0	1.0	
Female	1.5 (0.9-2.4)	0.1	1.5 (0.8-2.5)	0.2
Region				
Littoral	1.0		1.0	
Northwest	0.7 (0.3-1.3)	0.3	0.9 (0.4-1.9)	0.8
Southwest	6.4 (2.2-18.9)	<0.001	5.8 (1.6- 20.9)	0.008
West	0.8 (0.3-1.7)	0.5	0.6 (0.2-1.5)	0.3
Type of retreatment patient				
Not failure	1.0		1.0	
Failure	6.0 (3.5-10.2)	<0.001	10.2 (5.4-19.2)	<0.001
Smear-positivity grade				
Negative/Scanty/1+	1.0		1.0	
2+	**6.9 (2.0-23.9)**	**0.003**	**10.9 (2.9-40.7)**	**<0.001**
3+	**12.0 (3.7-38.8)**	**<0.001**	**21.9 (6.2-76.8)**	**<0.001**

*Adjusted for age, sex, region and type of patient. CI=confidence interval.

Table 3. Associations of multidrug-resistant TB (MDR-TB) with age, sex, region, type of retreatment patient, and time to detection of culture

Variable	Unadjusted odds ratio (95% CI)	P Value	Adjusted odds ratio (95% CI)*	P Value
Age				
≤ 35 yrs	1.0		1.0	
>35 yrs	1.2 (0.7-2.2)	0.6	1.1 (0.5-2.2)	0.9
Sex				
Male	1.0		1.0	
Female	1.0 (0.5-1.9)	1.0	1.0 (0.5-2.1)	1.0
Region				
Littoral	1.0		1.0	
Northwest	0.5 (0.2-1.2)	0.1	0.7 (0.3-1.9)	0.5
Southwest	3.9 (0.9-17.0)	0.07	3.1 (0.5-18.8)	0.2
West	0.5 (0.1-1.6)	0.2	0.3 (0.1-1.2)	0.1
Type of retreatment patient				
Not failure	1.0		1.0	
Failure	22.8 (10.3-50.6)	<0.001	22.9 (9.8-53.5)	<0.001
Time to detection, automated liquid culture				
> 160 hours	1.0		1.0	
≤ 160 hours	**2.3 (1.2-4.4)**	**0.01**	**2.2 (1.1-4.7)**	**0.04**

*Adjusted for age, sex, region and type of patient. CI=confidence interval.

Figure 1. Study profile.

AFB = Acid-fast bacilli. DST = Drug susceptibility testing. TTD = Time to detection of automated culture.

Figure 2. Distribution of patients by MDR-TB status, sputum smear microscopy grade, and type of TB treatment history

(A) Distribution of patients by MDR-TB status as a function of smear microscopy grade. The prevalence of MDR-TB among patients with smear grades of ≤1+, 2+ and 3+ was 1.0%, 7.0% and 15.7%, respectively. (B) Among all retreatment patients tested, 26% had a smear microscopy grade of ≤1+, 23% were graded as 2+, and 51% as 3+. (C) Among patients identified as MDR-TB cases, 4% had a smear microscopy grade of ≤1+, 21% as 2+, and 75% as 3+. An association between higher smear microscopy grade and MDR-TB was observed for retreatment patients with different treatment histories, including those classified as failure of treatment, as well as those classified as relapse or return after default and who were not on treatment at baseline diagnosis.