Additional Drug Resistance in *Mycobacterium tuberculosis* Isolates From Resected Cavities Among Patients With Multidrug-Resistant or Extensively Drug-Resistant Pulmonary Tuberculosis

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The pathogenesis of increasing drug resistance among patients with multidrug-resistant or extensively drug-resistant tuberculosis undergoing treatment is poorly understood. Increasing drug resistance found among *Mycobacterium tuberculosis* recovered from cavitary isolates compared with paired sputum isolates suggests pulmonary cavities may play a role in the development of worsening tuberculosis drug resistance.

The global emergence of multidrug-resistant (MDR) tuberculosis (resistance to isoniazid and rifampicin) and extensively drug-resistant (XDR) tuberculosis (MDR plus resistance to a fluoroquinolone and an injectable drug) is an alarming issue in tuberculosis control, presenting enormous challenges that have not yet been sufficiently addressed [1, 2]. Compared with drug-susceptible disease, M/XDR tuberculosis requires prolonged medical treatment with drugs of limited proven clinical efficacy and is associated with worse outcomes [1]. An improved understanding of mechanisms through which drug-resistant cases develop, including increasing resistance among patients undergoing treatment for MDR tuberculosis [3], may improve M/XDR tuberculosis prevention and management.

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Drug resistance in *Mycobacterium tuberculosis* develops as a result of spontaneous chromosomal mutations, not as a result of horizontal gene transfer [4]. The frequencies of these mutations occur at predictable rates (between 10^{-6} and 10^{-8} mycobacterial replications) and resistance mutations for different drugs are unlinked, making additional drug resistance unlikely when $\geq$3 effective drugs are used in combination [4]. Acquired drug resistance occurs through an amplification of the above-mentioned genetic mutations by human-related error resulting in inadequate drug treatment. These include poor regimen selection, inadequate drug supply, therapy nonadherence, and nontherapeutic drug levels. In the setting of inadequate drug treatment, populations of resistant bacilli can be selected for and become the dominant strain [4]. Primary drug resistance occurs when a person with drug-resistant tuberculosis transmits disease to a susceptible host.

We hypothesized that a tuberculous lung cavity provides an environment that facilitates the development of drug resistance due to high bacterial loads, active mycobacterial replication, reduced exposure to host defenses, and potentially low drug levels [5, 6]. A prior study found additional drug resistance in *M. tuberculosis* isolates from tuberculous cavities when compared with sputum [6]. To provide further evidence of whether tuberculous cavitary lesions act as sites of acquired drug resistance, we compared drug-susceptibility testing (DST) results from paired sputum and tissue isolates.

METHODS

The study population consisted of patients with pulmonary M/XDR tuberculosis who had surgical resection performed as adjunctive treatment at the National Center for Tuberculosis and Lung Diseases (NCTBLD) in Tbilisi, Georgia, between March 2009 and December 2010. All patients with a cavitary tissue culture performed using resected lung tissue were included. General criteria for surgical intervention included M/XDR tuberculosis, failure of medical therapy, a high likelihood of disease relapse, localized lesion, and sufficient pulmonary function to tolerate surgery. Medical treatment regimens were individualized based on DST results and guided by World Health Organization criteria [7]. The NCTBLD and Emory University institutional review boards approved the study.

Sputum samples were decontaminated with N-acetyl-L-cysteine-sodium hydroxide and centrifuged; the sediment was suspended in 1.5 mL of phosphate buffer. The treated specimen was inoculated onto Löwenstein-Jensen (LJ) egg-based solid medium and the BACTEC MGIT 960 broth culture system.

Resected cavitary lung tissue was placed in a sterile container and immediately transferred to the National Reference Laboratory, which has undergone external quality assessment by the Antwerp Supranational TB Reference Laboratory annually since 2005. A 0.5–0.8-g sample of the tissue that included sections of the cavity wall and necrotic center was homogenized, decontaminated with N-acetyl-L-cysteine-sodium hydroxide, and inoculated onto LJ medium. Positive sputum and tissue cultures were confirmed to be *M. tuberculosis* complex with the Genotype MTBDR*plus* assay [8]. DST for first-line drugs (FLDs) was performed using the absolute concentration method on LJ medium with the following drug concentrations: streptomycin, 4 μg/mL; isoniazid, 0.2 μg/mL; rifampicin, 40 μg/mL; and ethambutol, 2 μg/mL [9]. Pyrazinamide testing was performed using the MGIT960 liquid broth system (100 lg/mL). DST for second-line drugs (SLDs) was performed using the proportion concentration method with the following drug concentrations: ethionamide, 40.0 μg/mL; ofloxacin, 2.0 μg/mL; para-aminosalicylic acid, 0.5 μg/mL; capreomycin, 40.0 μg/mL; and kanamycin, 30.0 μg/mL [9]. Pyrazinamide testing was performed using the MGIT960 liquid broth system (100 μg/mL).

All data were entered into a REDCap database [10] and analyzed using SAS 9.3 software. DST results for FLDs and SLDs from positive sputum and tissue cultures were compared. If the preoperative sputum culture was negative, the most recent sputum DST result was used for comparison.

RESULTS

Among 80 patients with M/XDR tuberculosis who underwent surgical resection, 50 had both preoperative sputum and resected cavitary tissue cultures performed with further analysis. The median age was 27 years (range, 17–51 years), and 70% of patients were male. A majority of patients (61%) had been treated for tuberculosis before their current episode with either FLDs (47%) or SLDs (14%), and 34% had XDR tuberculosis diagnosed at treatment initiation. The average duration of M/XDR tuberculosis therapy before surgery was 380 days. The indications for surgery were medical treatment failure (60%), high drug resistance with low likelihood of cure (28%), damaged fibrotic lung (4%), tuberculoma (4%), empyema (2%), and massive hemoptysis (2%).

Overall, 23 of 50 patients(46%) had a culture positive for *M. tuberculosis* from a resected tuberculous cavity, including 5 of 30 patients with negative preoperative sputum cultures and 18 of 20 patients with positive preoperative sputum cultures. DST for FLDs and SLDs was performed on paired *M. tuberculosis* isolates recovered from 18 of 23 (78%) patients with sputum and tissue cultures positive for *M. tuberculosis*. Additional drug resistance was found in 7 of 18 *M. tuberculosis* isolates (37%) from tissue when compared with paired sputum isolates (Table 1). Of 45 patients with complete culture and DST data, the rate of additional resistance in cavitary isolates compared with sputum *M. tuberculosis* isolates was 25% (4 of 16) and 10% (3 of 29) in patients with a positive or negative preoperative sputum culture, respectively. Further drug resistance in cavitary isolates to 2 additional SLDs was found in 3 patients and to 1 SLD in the remaining 4 patients. In 6 of 7 patients, additional drug resistance to a fluoroquinolone and/or an injectable agent was found. In all cases, patients had been receiving an antituberculosis medication from the corresponding drug class (with reported compliance), to which additional drug resistance was found. Treatment outcomes are listed in Table 1.

DISCUSSION

In a cohort of patients with chronic pulmonary M/XDR tuberculosis undergoing adjunctive surgical therapy, there was a substantial proportion of patients who had additional drug resistance in *M. tuberculosis* recovered from the cavitary lesion compared with sputum culture isolates. Our data suggest that the tuberculous cavity promotes the emergence of increasingly drug-resistant bacilli populations during treatment. In patients who already have high drug resistance and limited treatment options, the development of further drug resistant could have unfavorable clinical consequences. In our cohort, additional drug resistance to either a fluoroquinolone or an injectable agent, the 2 most important SLDs, emerged in 6 of 7 cases.

The understanding of the mechanism of acquired *M. tuberculosis* drug resistance in a cavitary lesion is limited and probably multifactorial. In a study of 6 patients with pulmonary tuberculosis who underwent surgical resection, Kaplan et al found that a single founder strain of *M. tuberculosis* may undergo genetic mutations to form heterogeneous populations of bacilli in different sections of lung [6]. In 3 patients the observed genetic mutations led to *M. tuberculosis* isolates with additional drug resistance, which were preferentially observed at the cavity surface. The authors postulated that high mycobacterial replication rates and an absence of $CD4^+$ and $CD8^+$ T cells at the cavity surface may have created an environment conducive to the development of additional drug resistance. Another potential factor and poorly studied area is the issue of SLD penetration into cavitary lesions. SLDs are in general considered to be less potent than FLDs, and drug penetration into cavitary lesions has not been clinically studied [11]. Given that tuberculosis cavitary lesions are characterized by an outer fibrotic wall and variable vascularization, decreased SLD drug penetration may occur and result in drug-selection pressure that favors the emergence of drug-resistant bacilli populations [11]. Our group is initiating a clinical pharmacological study to measure cavitary SLD levels to help answer this question.

Although the clinical ramifications of additional drug resistance developing in cavitary bacilli populations are unknown,

Table 1. Tuberculosis Treatment Received Before Surgery, Treatment Outcomes, and Comparison of Sputum and Tissue Culture Drug-Susceptibility Testing Results

Preoperative Sputum Culture Results	Treatment Since Diagnosis of M/XDR Tuberculosis Until Surgery		Time From Collection of Sputum Specimens (Used as Comparison) Until Surgery, Days	Sputum Drug-Susceptibility Testing Results[a]								Additional Resistance in Resected Lung Tissue[b]	Treatment Outcome[c]
				First-Line Drugs				Second-Line Drugs					
	Drugs Received[a]	Duration, Months		Ri	I	P	E	Et	O	Ps	Cm	K	
Negative	Mx, Cm, Ps, Cy, A, Cl, Cf	12.5	81	R	R	R	R	R	S	S	R	R	Ofloxacin	Cure
Negative	P, L, K, Pr, Ps, Cy	4	55	R	R	-	R	S	S	S	S	S	Ethionamide	Cure
Positive	L, Cm, Pr, Ps, Cy, A, Cl, Cf	15.5	12	R	R	R	R	R	S	S	S	S	Ofloxacin	Death
Positive	L, Cm, Pr, Ps, Cy, A, Cl, Cf	21	3	R	R	S	R	S	S	S	S	S	Ethionamide, Ofloxacin	Cure
Positive	P, Mx, CM, Pr, Ps, Cy, Cl, Cf	12	1	R	R	-	R	R	R	R	S	R	Capreomycin	Cure
Negative	Mx, Cm, Pr, Ps, Cy, A, Cl, Cf	11	74	R	R	R	R	R	R	S	S	S	Capreomycin Kanamycin	Treatment Failure
Positive	Mx, Cm, Ps, Cy, A, Cl, Cf	17	5	R	R	R	R	R	S	S	R	S	Ofloxacin, Kanamycin	Probable cure

Abbreviations: M/XDR, multidrug-resistant or extensively drug-resistant; R, resistant; S, susceptible; -, no drug-susceptibility performed.

[a] A, amoxicillin/clavulanate; Cf, clofazimine; Cl, clarithromycin; Cm, capreomycin; Cy, cycloserine; E, ethambutol; Et, ethionamide; I, isoniazid; K, kanamycin; Lx, moxifloxacin; O, ofloxacin; P, pyrazinamide; Pr, prothionamide; Ps, para-aminosalicylic acid; Ri, rifampicin.

[b] Additional resistance was defined as resistance in a cavitary isolate to any first- or second-line drug to which the sputum isolate was susceptible.

[c] Treatment outcomes defined using World Health Organization criteria.

it highlights the importance of repeated DST in certain patients and the concern that sputum DST results may not always accurately present bacterial susceptibility. It is plausible that in some patients, acquired drug resistance on treatment may lead to treatment failure. In a study of serial sputum isolates in 13 patients with chronic cavitary MDR tuberculosis, 4 patients developed genetic mutations conferring additional drug resistance during the course of treatment [3]. It was difficult to measure the clinical consequences of additional drug resistance in our cohort given that all patients underwent surgical resection. Although there is selection bias, an increasing number of researchers are finding favorable treatment outcomes when surgical resection is used in patients with M/XDR tuberculosis [12]. This may be due to the removal of the tuberculous cavity, which hinders effective treatment.

Study limitations included performance with only one tissue culture and no genetic sequencing of *M. tuberculosis* isolates. This precluded the identification of heterogeneous bacilli populations in cavitary lesions and the ability to rule out reinfection as a cause for increased drug resistance. However, by performing only one tissue culture, we may have underestimated the rate of additional drug resistance.

The results of the present study suggest that patients with cavitary M/XDR tuberculosis disease are prone to develop bacilli populations that have additional drug resistance. Further investigations into the reasons for selection of bacilli with additional genetic resistant mutations, mechanisms of resistance, and impact of SLD pharmacology are needed to better understand factors facilitating further development of drug resistance and may help improve treatment outcomes in those with M/XDR tuberculosis.

Notes

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