Susceptibility Testing of Extensively Drug-Resistant and Pre-Extensively Drug-Resistant *Mycobacterium tuberculosis* against Levofloxacin, Linezolid, and Amoxicillin-Clavulanate

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Pakistan is a high-burden country for tuberculosis (TB). The emergence and increasing incidence of extensively drug-resistant (XDR) TB has been reported in Pakistan. Similarly, the prevalence of multidrug-resistant TB infections with fluoroquinolone resistance (pre-XDR) is also increasing. To treat these infections, local drug susceptibility patterns of alternate antituberculosis agents, including levofloxacin (LVX), linezolid (LZD), and amoxicillin-clavulanate (AMC), is urgently needed. The aim of this study was to determine the susceptibility frequencies of drug-resistant (DR) *Mycobacterium tuberculosis* against LVX, LZD, and AMC. All susceptibilities were determined on Middlebrook 7H10 agar. A critical concentration was used for LVX (1 µg/ml), whereas MICs were determined for LZD and AMC. *M. tuberculosis* H37Rv was used as a control strain. A total of 102 *M. tuberculosis* isolates (XDR, $n = 59$; pre-XDR, $n = 43$) were tested. Resistance to LVX was observed in 91.2% (93/102). Using an MIC value of 0.5 µg/ml as a cutoff, resistance to LZD (MIC ≥ 1 µg/ml) was noted in 5.9% (6/102). Although the sensitivity breakpoints are not established for AMC, the MIC values were high (> 16 µg/ml) in 97.1% (99/102). Our results demonstrate that LZD may be effective for the treatment of XDR and pre-XDR cases from Pakistan. High resistance rates against LVX in our study suggest the use of this drug with caution for DR-TB cases from this area. Drug susceptibility testing against LVX and AMC may be helpful in complicated and difficult-to-manage cases.

Drug-resistant tuberculosis (DR-TB), which is difficult to diagnose and manage, has emerged as a serious threat to global TB control. The 16th Annual Report of World Health Organization (WHO) on Global Tuberculosis Control, published in 2011, estimates that there were 8.8 million new cases of TB worldwide in 2010, with 59% occurring in Asia (1). These include an estimated 0.65 million cases of multidrug-resistant TB (MDR-TB; *Mycobacterium tuberculosis* resistant to at least isoniazid and rifampin). By the end of 2008, 55 countries and territories had reported at least one case of extensively drug-resistant TB (XDR-TB; *M. tuberculosis* resistant to isoniazid and rifampin, with additional resistance to any of the fluoroquinolones and any one of the second-line injectable agents, i.e., kanamycin, capreomycin, or amikacin) (2).

Pakistan is a high-burden country for TB (275 cases/100,000 in 2010) and MDR-TB (3.4% of all new TB cases were MDR-TB in 2010). XDR-TB has been reported from Pakistan and is being increasingly encountered (1.5% in 2006 to 4.5% in 2009) (3).

Treatment options for patients with DR-TB and particularly XDR-TB strains are very limited. The picture is further complicated with an ever-increasing immunocompromised population, including patients living with HIV-AIDS and DR-TB in whom the mortality is high (4). The presence of pre-XDR TB (MDR-TB with additional resistance to any fluoroquinolone or to any one of the three second-line injectables) has also been reported as a prognostic factor for poor outcome (5).

In the search for newer and better treatment options, drugs that have been used for the treatment of DR-TB, including later-generation fluoroquinolones (levofloxacin [LVX], moxifloxacin), linezolid (LZD), amoxicillin-clavulanate (AMC), clarithromycin, thioridazine, and clofazimine, have been examined.

Fluoroquinolones are a determining factor in XDR definition. A comparative study of LVX and ofloxacin in MDR-TB patients demonstrated better outcomes in patients treated with LVX for

both ofloxacin-susceptible and -resistant strains (6). A meta-analysis described significantly improved treatment outcomes in XDR-TB patients who received a later-generation fluoroquinolone (7). LZD, the first oxazolidinone shown to have antimycobacterial activity *in vitro* has been used to treat DR-TB patients, including patients with XDR-TB, with favorable outcomes (8).

The WHO has classified AMC as a category V antituberculosis drug. Its susceptibility testing is not established for *M. tuberculosis*; however, AMC has been used to treat DR-TB cases where options did not allow choosing more traditional drugs (9).

Given the increasing role of LVX, LZD, and AMC in the management of DR-TB, and the paucity of information on the susceptibility of DR-TB isolates to these agents, the susceptibility of clinical DR-TB isolates from Pakistan was assessed against LVX, LZD, and AMC.

MATERIALS AND METHODS

Bacterial strains. Drug-resistant *M. tuberculosis* isolates were collected from the clinical laboratory at Aga Khan University Hospital, Karachi, Pakistan, from February 2010 to May 2011. The hospital and its clinical laboratory are accredited by the Joint Commission International Accreditation. All of the strains used here were collected prospectively. Clinical information was obtained as a part of routine laboratory practice and not specifically for this study. For the purpose of the present study, the drug-resistant *M. tuberculosis* strains used included (i) XDR strains and (ii)

Received 30 September 2012 Returned for modification 21 November 2012

Accepted 11 March 2013

Published ahead of print 18 March 2013

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doi:10.1128/AAC.02020-12

TABLE 1 Distribution of LZD and AMC MICs among *M. tuberculosis* isolates

Strain type	No. (%) of strains ^a							
	LZD MIC (μg/ml)				AMC MIC (μg/ml)			
	0.25	0.5	1	2	0.5	8	16	>16
XDR (<i>n</i> = 59)	6 (10.2)	50 (84.7)	2 (3.4)	1 (1.7)	1 (1.7)	0	1 (1.7)	57 (96.6)
Pre-XDR (<i>n</i> = 43)	5 (11.6)	35 (81.4)	3 (6.9)	0	0	1 (2.3)	0	42 (97.7)
Total (<i>n</i> = 102)	11 (10.7)	85 (83.3)	5 (4.9)	1 (1)	1 (1)	1 (1)	1 (1)	99 (97)

^a The numbers of strains for various antibiotic MICs are indicated. The LZD and AMC MICs for *M. tuberculosis* H37Rv were 0.25 and 0.5 μg/ml, respectively.

MDR strains that are also resistant to ofloxacin (MDR-OFX or pre-XDR; MDR-TB which is also resistant to any fluoroquinolone). The study included all of the XDR strains isolated in our laboratory during the study period (*n* = 59), 32 of which had undergone previous TB treatment, 1 of which was untreated, and for 26 a treatment history was not available. Pre-XDR strains (*n* = 43) were also included; 35 of these had received treatment for TB, 7 were untreated, and for one patient the treatment history was not available. Duplicate specimens from the same patients were excluded.

TB culture was performed using Lowenstein-Jensen (LJ), Mycobacterium Growth Indicator Tube (MGIT), and Middlebrook 7H10 agar for all of the specimens. *M. tuberculosis* was isolated from clinical specimens using standard microbiological procedures, and *M. tuberculosis* was identified using nitrate reduction, niacin accumulation, and PNB (*para*-nitrobenzoic acid) sensitivity analyses (10, 11). Furthermore, colonial morphology, pigmentation, and rate of growth were also observed to ensure mixed cultures of nontuberculous mycobacteria were not included.

Antibiotics. Antibiotics were procured from their manufacturers in pure form (LVX, amoxicillin, and clavulanate were from Sigma; LZD was kindly provided by Continental Pharmaceuticals, Karachi, Pakistan).

Agar medium. Middlebrook 7H10 agar (Difco, Detroit, MI) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) was used for susceptibility testing of *M. tuberculosis*.

Ethical review. The study was provided an exemption of ethical approval by the Institutional Ethical Review Committee.

Drug susceptibility testing. An agar proportion method was used for determining the susceptibility of the study strains to all tested antibiotics. Middlebrook 7H10 agar supplemented with 10% OADC was used for all susceptibility testing. *M. tuberculosis* H37Rv was used as a sensitive control.

Resistance to LVX was assessed using a critical concentration of 1.0 μg/ml, i.e., susceptible at ≤1.0 μg/ml and resistant at ≥2.0 μg/ml (12). Critical concentrations for LZD and AMC have not been defined. A multicenter laboratory validation study suggested 1.0 μg/ml as a cutoff for determining resistance to LZD in liquid culture medium system (13). More recently, wild-type MIC distributions were described, and 0.5 μg/ml was defined as an epidemiological cutoff value for LZD against *M. tuberculosis* in Middlebrook 7H10 agar (14). In the present study, therefore, MIC values were determined for both LZD and AMC that were tested at 0.06 to 16 μg/ml and 0.25 to 16 μg/ml, respectively. Resistance to LZD was determined using a MIC value of 0.5 μg/ml as cutoff (susceptible, ≤0.5 μg/ml; resistant, ≥1.0 μg/ml). In the absence of any recommended cutoff for AMC, MIC values were used as such to show the MIC distribution among these strains.

Data management and analysis plan. The data obtained were entered into the statistical software SPSS version 19.0 (SPSS, Inc., Chicago, IL). Frequencies of susceptibility results were calculated for all three drugs tested.

RESULTS

A total of 102 *M. tuberculosis* isolates were tested using LVX, LZD, and AMC. Of these, 59 (58%) were XDR isolates and 43 (42%)

were pre-XDR isolates. Among these 102 cases, only one DR-TB was isolated from the extrapulmonary site; the rest were pulmonary isolates. Among the XDR patients, 1 was untreated, and 32 had received prior treatment, while a treatment history was not available for 26 patients. The untreated XDR patient had a strong contact history; two family members had previously succumbed to XDR-TB. Within the pre-XDR group, 7 patients were untreated and 35 had received treatment for TB.

Using 1 μg/ml as a critical concentration for LVX, 93/102 (91.2%) of the strains tested were found to be resistant to LVX (susceptible, ≤1 μg/ml; resistant, ≥2 μg/ml). These included 53/59 XDR strains and 40/43 pre-XDR strains.

The MIC values for LZD and AMC are shown in Table 1. Based on the MIC cutoff (0.5 μg/ml) used for LZD in the present study, 5.9% (6/102) of the strains tested were found to be resistant (i.e., LZD MIC ≥ 1.0 μg/ml). These LZD-resistant isolates belonged to both XDR and pre-XDR groups (3 from each group). For most (96/102 [94.1%]) of the *M. tuberculosis* isolates, the LZD MIC was ≤0.5 μg/ml, and therefore these strains were considered susceptible to LZD. Only for one XDR isolate was the LZD MIC found to be 2 μg/ml. The AMC MIC values were ≥16 μg/ml for 98% (100/102) of the DR-TB isolates.

DISCUSSION

Drug resistance in *M. tuberculosis* does not recognize international boundaries. MDR- and XDR-TB cases are slowing efforts toward TB eradication. The drug options for treating these patients are very limited. In the present study we demonstrated the susceptibilities of drug-resistant *M. tuberculosis* strains to LVX, LZD, and AMC. To our knowledge, this is the first study reporting susceptibilities to these agents for DR-TB from Pakistan.

Among DR-TB cases where treatment histories were available, the majority (89.3% [67/75]) of DR-TB cases had received antituberculosis treatment (ATT). These findings emphasize the contribution of prior ATT to the emergence of DR-TB in this setting. The one XDR-TB patient (1/33) who had not received previous treatment for TB came from a family where two members had died of XDR-TB, highlighting the transmission of XDR-TB and importance of infection control measures in such cases.

A very high rate of LVX resistance (91.2%) is reported here. This is in contrast to earlier studies reporting 54.3% (19/35) LVX resistance in XDR isolates from Peru (9) and 28% resistance among MDR-TB cases from Taiwan (15). Both of these studies have described using 1 μg/ml as a cutoff for defining resistance to LVX. The WHO recommends a critical concentration of 2 μg/ml for LVX in Middlebrook 7H10 agar (16). For the present study, an LVX critical concentration of 1 μg/ml has been used, as recommended by the Clinical and Laboratory Standards Institute (12).

This lower cutoff concentration is also supported by a recent study which describes the epidemiological cutoff (ECOFF) value for LVX in Middlebrook 7H10 agar determined by the proportion method as 0.5 µg/ml and suggests that ECOFF could be adjusted to 1 µg/ml if a large number of isolates were tested in several laboratories (17). An increasing resistance to fluoroquinolone in *M. tuberculosis* has also been reported from India (18). In Pakistan, fluoroquinolone resistance among patients with no history of TB treatment has been reported to be 5.6% (19). The high rate of LVX resistance observed in the present study is not surprising in a setting where antibiotics are available over the counter (20), and antimicrobial resistance among other bacteria is very high in the community as well (98% of *Neisseria gonorrhoeae* isolates and ~30% of *Salmonella enterica* serovar Typhi isolates are resistant to fluoroquinolones) (21, 22).

Using ≤0.5 µg/ml as a cutoff for susceptibility, 94% of our *M. tuberculosis* isolates were noted to be susceptible to LZD. In Pakistan, LZD is used mainly to treat infections caused by vancomycin-resistant *Enterococcus* species and methicillin-resistant *Staphylococcus aureus*, particularly in cases of an adverse reaction to vancomycin or as an oral option. An LZD MIC of ≥1 µg/ml was noted in 5.9% of the *M. tuberculosis* isolates tested. This level of LZD resistance in *M. tuberculosis* may be partly explained by its clinical and/or uncontrolled usage because of the availability of all antibiotics as over-the-counter medications in the country. A number of studies have reported an LZD MIC of >4 µg/ml among 1.9 to 5.2% of their MDR strains (23, 24). The fact that the LZD MIC of 2 µg/ml observed here was seen in only one XDR strain suggests that LZD may be considered as part of second-line therapy for DR-TB in this population. LZD has been used successfully for the management of XDR and pre-XDR TB patients. Although long-term LZD usage is associated with bone marrow suppression and neuropathy, treating patients with a lower LZD dose has been reported to be associated with fewer adverse effects and successful outcomes (25).

Almost all of the strains tested showed an AMC MIC of ≥16 µg/ml. This finding is consistent with a study from Iran, reporting an AMC MIC of >32 µg/ml for all 90 of their *M. tuberculosis* isolates (26). Given the achievable peak serum concentrations for AMC of 7.2 and 11.6 µg/ml after the administration of 500/125-mg and 875/125-mg doses, respectively (27), our findings exclude AMC from use against DR-TB strains in this setting. Early bactericidal activity (EBA) studies show conflicting results for AMC. The EBA of AMC against *M. tuberculosis* is reported to be comparable to ofloxacin but lower than isoniazid (28); other studies, however, do not confirm these findings (29). Despite these conflicting reports, clinical studies describe successful outcomes with AMC used in combination with other antimycobacterial agents for the treatment of DR-TB. Two patients with MDR-TB were reported to be successfully treated with regimens containing AMC (an AMC MIC of ≤1 µg/ml was determined for both isolates by the broth dilution method) (30).

The limitations of the present study include the fact that complete treatment histories or outcomes were not available for most of the patients and previous individual treatment regimens could not be ascertained. We were also unable to test other later-generation fluoroquinolones, e.g., moxifloxacin and gatifloxacin, and therefore could not compare results of different fluoroquinolones.

The reliability of DST for second-line anti-TB drugs has been questioned due to its limited reproducibility and the lack of cor-

relation of susceptibility results with clinical outcomes (16, 31). However, in the absence of better susceptibility methods, it may be valuable to evaluate the potential value of second-line antimicrobials in regions where TB is endemic. We describe here the susceptibilities of XDR and pre-XDR *M. tuberculosis* clinical isolates from Pakistan to LVX, LZD, and AMC. The results demonstrate that LZD is a good treatment option in these cases. Current evidence suggests using newer-generation fluoroquinolones for DR-TB treatment despite *in vitro* resistance. However, the high resistance rates against LVX noted in our study suggest caution in the use of this drug for DR-TB cases from this area. Drug susceptibility testing against LVX and AMC may be helpful in complicated and difficult-to-manage cases.

ACKNOWLEDGMENTS

Linezolid was kindly provided by Continental Pharmaceuticals, Karachi, Pakistan.

This study was supported by a seed money grant from the Department of Pathology and Microbiology, Aga Khan University, Karachi, Pakistan.

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