1 Susceptibility testing of extensively drug resistant and pre-extensively drug resistant
2 *Mycobacterium tuberculosis* against levofloxacin, linezolid, and amoxicillin/clavulanate
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Abstract

Background: Pakistan is a high burden country for tuberculosis (TB). Emergence and increasing incidence of extensively drug resistant (XDR) TB has been reported from Pakistan. Similarly, rates of MDR-TB with fluoroquinolone resistance (pre-XDR) are also increasing. To treat these infections, local drug susceptibility pattern of alternate anti-tuberculosis agents including levofloxacin (LVX), linezolid (LZD) & amoxicillin/clavulanate (AMC) is urgently needed.

Objective: To determine the susceptibility frequencies of drug resistant Mycobacterium tuberculosis (DR-MTB) against levofloxacin, linezolid and amoxicillin/clavulanate.

Methods: All susceptibilities were performed on Middlebrook 7H10 agar. Critical concentration was used for LVX (1µg/ml) whereas minimum inhibitory concentrations (MICs) were performed for LZD and AMC. Mycobacterium tuberculosis H37Rv was used as a control strain.

Results: A total of 102 MTB isolates (XDR: 59; pre-XDR: 43) were tested. Resistance to LVX was observed in 91.2% (93/102). Using an MIC value of 0.5 µg/ml as cutoff, resistance to LZD (MIC ≥1 µg/ml) was noted in 5.9% (6/102). Although sensitivity breakpoints are not established for AMC, the MIC values were high (>16µg/ml) in 97.1% (99/102).

Conclusion: Our results demonstrate that LZD may be effective for treatment of XDR and pre-XDR cases from Pakistan. High resistance rates against LVX in our study suggest 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.
INTRODUCTION

Drug resistant tuberculosis (DR-TB), 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 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 rifampicin). 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, rifampicin, with additional resistance to any of the fluoroquinolone and any one of the 2nd 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). Presence of pre-XDR TB (MDR-TB with additional resistance to any fluoroquinolone ‘or’ to any one of the three 2nd line injectables) has also been reported as a prognostic factor for poor outcome (5).

Desperate for newer options, drugs that have been used for the treatment of DR-TB include later generation fluoroquinolones (levofloxacin, moxifloxacin), linezolid, amoxicillin-clavulanate, clarithromycin, thioridazine and clofazimine.
Fluoroquinolones are a determining factor in XDR definition. A comparative study of levofloxacin and ofloxacin in MDR-TB patients demonstrated better outcomes in patients treated with levofloxacin 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).

Linezolid, the first oxazolidinone shown to have anti-mycobacterial activity in vitro has been used to treat DR-TB patients including XDR-TB, with favorable outcomes (8).

The WHO has classified Amoxicillin/clavulanate in category-V antituberculosis drugs. 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 susceptibility of DR-TB isolates to these agents, 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 Clinical Laboratory, Aga Khan University Hospital (AKUH), Karachi, Pakistan, from February 2010 to May 2011. The hospital and its clinical laboratory are accredited by the Joint Commission International Accreditation (JCIA). All the strains used in this study were collected prospectively. Clinical information was obtained as routine laboratory practice and not specifically as part of this study. For the purpose of this study, drug resistant *M. tuberculosis* strains used include: 1. XDR strains and 2. MDR strains which are also resistant to ofloxacin (MDR-OFX or pre-XDR: MDR-TB also resistant to...
any fluoroquinolone). The study included all of the XDR strains isolated in our laboratory during
the study period (n=59) of which 32 had previous TB treatment, one was untreated and treatment
history was not available for 26 cases. Pre-XDR strains (n=43) were also included, of these 35
had received treatment for TB, 7 were untreated while for one patient treatment history was not
available. Duplicate specimens from the same patients were excluded.

TB culture was performed using LJ, MGIT and Middlebrook 7H10 agar for all the specimens.
MTB was isolated from clinical specimens using standard microbiological procedures and MTB
was identified using nitrate reduction, niacin accumulation and PNB (para-nitrobenzoic acid)
sensitivity (10, 11). Furthermore colonial morphology, pigmentation and rate of growth were
also observed to ensure mixed cultures of non-tuberculous mycobacteria (NTM) were not
included.

Antibiotics

Antibiotics were procured from their manufacturers in pure form (Levofloxacin, amoxicillin and
clavulanate from Sigma; linezolid 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
Agar proportion method was used for determining susceptibility of the study strains to all tested antibiotics. Middlebrook 7H10 agar supplemented with 10% OADC was used for all susceptibility testing. *Mycobacterium 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 ≤1.0 µg/ml and resistant ≥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 media system (13). More recently wild type MIC distributions were described and 0.5 µg/ml was defined as an epidemiological cut-off value for LZD against MTB in Middlebrook 7H10 agar (14). In this study, therefore, MIC values were determined for both LZD and AMC that were tested at 0.06-16 µg/ml and 0.25-16 µg/ml respectively. Resistance to LZD was determined using an MIC value of 0.5 µg/ml as cutoff (susceptible ≤0.5 µg/ml and resistant ≥1.0 µg/ml). In the absence of any recommendation for cut-off for amoxicillin-clavulanate, MIC values were used as such to show MIC distribution amongst these strains.

**Data management and analysis plan**

Data obtained was entered into the statistical software SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Frequencies of susceptibility results were calculated for all the three drugs tested.

**RESULTS**

A total of 102 MTB isolates were tested against LVX, LZD and AMC. Of these, 59 (58%) were XDRs and 43 (42%) were pre-XDR isolates. Among these 102 cases, only one DR-TB was isolated from extrapulmonary site, remaining were pulmonary isolates. Amongst the XDR patients, one was untreated, 32 had received prior treatment while treatment history was not...
available for 26 patients. The untreated XDR patient had a strong contact history; two family
members had previously succumbed due to XDR tuberculosis. Within the pre-XDR group, 7
patients were untreated and 35 had received treatment for tuberculosis.

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

Minimum inhibitory concentration values for linezolid and amoxicillin-clavulanate are shown in
table 1. Based on the MIC cut off (0.5 µg/ml) used for LZD in this 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). Most (96/102, 94.1%) of the
MTB isolates showed an MIC value of ≤0.5 µg/ml for LZD and therefore were considered
susceptible to LZD. Only one XDR isolate was found to have an MIC of 2 µg/ml. Amoxicillin-
clavulanate MIC values were ≥16 µg/ml for 98% (100/102) of the DR-TB isolates.

**DISCUSSION**

Drug resistance in *Mycobacterium tuberculosis* does not recognize international boundaries.

MDR and XDR-TB cases are slowing efforts towards TB eradication. Drug options to treat these
patients are very limited. In this study we have demonstrated susceptibilities of drug resistant
*Mycobacterium tuberculosis* strains against 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, majority (89.3%, 67/75) of DR-
TB cases had received ATT. These findings emphasize the contribution of prior ATT to
emergence of DR-TB in this setting. The one XDR-TB patient (1/33) who had not received
previous TB treatment came from a family where 2 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 in this study. This is in contrast to earlier
studies reporting 54.3% (19/35) LVX resistance in XDR isolates from Peru (9), and 28%
resistance amongst MDR-TB cases from Taiwan (15). Both of these studies have described using
1 µg/ml as a cut off for defining resistance to levofloxacin. World Health Organization
recommends a critical concentration of 2 µg/ml for levofloxacin in Middlebrook 7H10 agar (16).
For the present study levofloxacin critical concentration of 1 µg/ml has been used which is
recommended by the Clinical and Laboratory Standards Institute (12). This lower cut off value is
also supported by a recent study describing epidemiological cut-off (ECOFF) value for LVX in
Middlebrook 7H10 agar by proportion method as 0.5 µg/ml and suggest that ECOFF could be
adjusted to 1 µg/ml if a large number of isolates were tested in several laboratories (17).

Increasing trend of fluoroquinolone resistance in MTB has also been reported from India (18). In
Pakistan fluoroquinolone resistance amongst patients with no prior history of TB treatment has
been reported at 5.6% (19). High rate of LVX resistance in the current study is not surprising in
a setting where antibiotics are available over the counter (20) and antimicrobial resistance in
other bacteria is very high in the community as well (98% of Neisseria gonorrhoeae isolates and
~30% of Salmonella enterica serovar Typhi isolates resistant to fluoroquinolones) (21, 22).

Using ≤0.5 µg/ml as cut-off for susceptibility, 94% of our MTB isolates were noted to be
susceptible to LZD. In Pakistan linezolid is used mainly to treat infections caused by
vancomycin resistant Enterococcus species (VRE) and methicillin resistant Staphylococcus
aureus (MRSA) particularly in cases of an adverse reaction to vancomycin or as an oral option.
Linezolid MIC of ≥1 µg/ml was noted in 5.9% of the MTB isolates tested. This level of LZD
resistance in MTB 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 LZD MIC of >4µg/ml in 1.9-5.2 % of their MDR strains (23, 24). The fact
that in this study LZD MIC of 2µg/ml 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. Linezolid has been
used successfully for the management of XDR and pre-XDR TB patients. While 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 MIC of ≥16µg/ml for AMC. This finding is consistent
with a study from Iran, reporting an MIC of >32µg/ml in all their 90 MTB isolates tested against
amoxicillin/clavulanate (26). Given the achievable peak serum concentration for AMC of 7.2
µg/ml and 11.6 µg/ml after 500/125 mg and 875/125 mg dose respectively (27), our findings
exclude AMC from use against DR-TB strains in this setting. Early bactericidal activity (EBA)
studies show conflicting results for amoxicillin-clavulanate. EBA of AMC against MTB 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 (MIC of ≤1 µg/ml was described against AMC for both isolates by
broth dilution method) (30).

Limitations of this study include the fact that complete treatment histories/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-tuberculosis drugs has been questioned due to its limited reproducibility and lack of correlation 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 endemic regions. Present study describes susceptibilities of XDR and pre-XDR MTB clinical isolates to levofloxacin, linezolid and amoxicillin/clavulanate from Pakistan. Results demonstrate that linezolid is a good option in these cases. Current evidence suggests using newer generation fluoroquinolones for DR-TB treatment despite in vitro resistance. However, high resistance rates against LVX in our study suggest 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.

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**Conflict of interest:** All authors declare no conflict of interest.
References:


Table 1: Distribution of minimum inhibitory concentrations of MTB isolates against linezolid and amoxicillin-clavulanate

<table>
<thead>
<tr>
<th>Strain tested*</th>
<th>Linezolid MIC (µg/ml)†#</th>
<th>Amoxicillin/clavulanate MIC (µg/ml)†#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>XDR</td>
<td>6 (10.2)</td>
<td>50 (84.7)</td>
</tr>
<tr>
<td>Pre-XDR</td>
<td>5 (11.6)</td>
<td>35 (81.4)</td>
</tr>
<tr>
<td>Total</td>
<td>11 (10.7)</td>
<td>85 (83.3)</td>
</tr>
</tbody>
</table>

*Total number of strains tested: 102 (XDR: 59 and Pre-XDR: 43)
†Number of strains N (%) at each MIC value is shown
*MIC values of *Mycobacterium tuberculosis* H37Rv for linezolid and amoxicillin/clavulanate were 0.25 & 0.5 µg/ml respectively