Evolution of the Extensively Drug-Resistant F15/LAM4/KZN Strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa

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(See the editorial commentary by Iseman on pages 1415–6)

**Background.** Although several hot spots of multidrug-resistant tuberculosis have been identified on the African continent, extensive drug resistance (XDR) has not been reported until recently, when a large number of XDR cases were identified in KwaZulu-Natal. The majority of the patients involved were infected with the same strain of *Mycobacterium tuberculosis* (F15/LAM4/KZN). We report this strain’s development from multidrug resistance to XDR.

**Methods.** We searched databases for studies performed during the period 1994–2005 that involved the resistance patterns of isolates of *M. tuberculosis* with the F15/LAM4/KZN strain fingerprint.

**Results.** As early as 1994, the F15/LAM4/KZN strain was responsible for a number of cases of multidrug-resistant tuberculosis, indicating the ability of the strain to cause cases of primary resistant tuberculosis. Some of the isolates were also resistant to streptomycin. From 1994 onwards, multidrug-resistant isolates with resistance to additional drugs were found, and the first XDR isolate was discovered in 2001.

**Conclusions.** Drug resistance to as many as 7 drugs developed in a local strain of *M. tuberculosis* in slightly more than a decade. This coincided with the introduction of the directly observed therapy–based and directly observed therapy–plus–based tuberculosis-control programs. It is postulated that the introduction of these programs in the absence of susceptibility testing or drug resistance surveillance has been instrumental in the development of XDR in this highly transmissible F15/LAM4/KZN strain. The expanding pool of human immunodeficiency virus–infected, tuberculosis-susceptible individuals has likely contributed to this development.

Antimicrobial drug resistance in infectious agents occurs by means of several mechanisms; these include genetic changes and the acquisition of genes coding for resistance. Mycobacteria differ from most other bacteria by the presence of a cell wall with unique properties. Exchange of genes across this type of cell wall is difficult. Therefore, development of drug resistance in this group of bacteria evolves mainly from mutational events [1]. Resistance only becomes apparent if the resistant subpopulation of bacteria survives during treatment. To avoid this, patients with tuberculosis (TB) are treated with a combination of drugs. Development of resistance to one of the drugs in the regimen is not relevant to the survival of the bacterium in which that occurred, because there will always be ≥1 drug to which it is still susceptible. Selection of resistant mutants only happens when patients are treated with inappropriate regimens or when patients become selectively noncompliant by not taking all of the 3 or 4 drugs they were prescribed. The long duration of treatment, as well as the large amount of drugs and their gastrointestinal adverse effects, contribute to a relatively high rate of noncompliance in patients with TB.

Although the development of drug resistance in other bacteria rapidly resulted in treatment failure, this only became a problem in *Mycobacterium tuberculosis* when resistance to isoniazid and rifampicin emerged in combination. This multidrug resistance (MDR) was believed not to have impacted TB-control programs, because the genes that code for resistance are not transferable, and the MDR organisms were thought to be less transmissible to other humans [2]. However, as early as 1993, an outbreak of MDR-TB occurred in New York City [3], challenging the concept of decreased transmissibility of such strains. In addition, the numbers of individuals who needed treatment with second-line drugs increased worldwide. After these developments, the development of resistance to second-

<table>
<thead>
<tr>
<th>Year</th>
<th>Genotyped isolates</th>
<th>F15/LAM4/KZN isolates</th>
<th>MDR F15/LAM4/KZN isolates</th>
<th>Other isolates that were MDR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1994</td>
<td>322</td>
<td>6 (2)</td>
<td>4</td>
<td>2</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>1995</td>
<td>104</td>
<td>20 (19)</td>
<td>12</td>
<td>8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>1996</td>
<td>140</td>
<td>21 (15)</td>
<td>15</td>
<td>6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>1997</td>
<td>32</td>
<td>10 (31)</td>
<td>8</td>
<td>2</td>
<td>&lt;.001</td>
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<tr>
<td>1998</td>
<td>23</td>
<td>2 (9)</td>
<td>1</td>
<td>1</td>
<td>.16</td>
</tr>
<tr>
<td>1999</td>
<td>47</td>
<td>8 (17)</td>
<td>4</td>
<td>4</td>
<td>.02</td>
</tr>
<tr>
<td>2000</td>
<td>45</td>
<td>14 (31)</td>
<td>8</td>
<td>6</td>
<td>.01</td>
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<tr>
<td>2001</td>
<td>88</td>
<td>15 (17)</td>
<td>13</td>
<td>2</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>2002</td>
<td>165</td>
<td>13 (8)</td>
<td>10</td>
<td>3</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Total</td>
<td>966</td>
<td>109 (11)</td>
<td>75 (69)</td>
<td>34 (4)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

NOTE. MDR, multidrug resistant.
* Determined using the χ² test or Fisher’s exact test; P < .05 was considered to be statistically significant.

The evolution of drug resistance in such a strain in KwaZulu-Natal, South Africa, where there is a steadily increasing number of people with AIDS-related immunocompromise. Recently, a cluster of persons infected with this strain was reported [6]. In all of these patients, the infecting organism was resistant to isoniazid, rifampicin, kanamycin, and the fluoroquinolones. Such strains are now known as extensively drug-resistant (XDR) strains [7]. The strain involved was referred to as “the KwaZulu-Natal strain” [7]. In accordance with a recently published article about the classification of M. tuberculosis strains [8], our strain is classified as F15/LAM4/KZN.

METHODS

Mycobacterial isolates. Until 2006, mycobacterial cultures of specimens obtained from patients in KwaZulu-Natal were performed in 2 laboratories: the TB laboratory associated with the Nelson R. Mandela School of Medicine (KwaZulu-Natal) and the provincial TB laboratory at King George V Hospital (Durban, KwaZulu-Natal), which is the MDR-TB referral center for the province. In February 2006, both laboratories were combined into the KwaZulu-Natal TB Laboratory of the National Health Laboratory Services. Isolates were genotyped in the context of several studies performed during 1993–2006.

Susceptibility testing. Susceptibility testing was done using different methods at the 2 TB culture facilities. The 1% proportion method, with Middlebrook 7H10 agar [9], was used at the Nelson R. Mandela School of Medicine laboratory. The following concentrations were tested: isoniazid, 0.2 and/or 1 mg/L; rifampicin, 1 mg/L; ethambutol, 7.5 mg/L; and streptomycin, 2 and/or 10 mg/L. This 1% proportional method is also used by the combined laboratory. At the King George V Hospital laboratory, Lowenstein-Jensen media [10] were used, with the following concentrations of antibiotics: isoniazid, 0.2 and 1 mg/L; rifampicin, 40 mg/L; ethambutol, 2 mg/L; streptomycin, 4 mg/L; ethionamide, 20 mg/L; kanamycin, 20 mg/L; ciprofloxacin, 5 mg/L; ofloxacin, 2.5 mg/L; and thiacetazone, 2 mg/L.

Restriction fragment—length polymorphism (RFLP) analysis. IS6110 RFLP analysis was performed with the method described by Van Embden et al. [11] RFLP patterns were analyzed using GelCompar software, version 4.0 (Applied Maths), and the results were confirmed visually. Isolates containing <5 copies of IS6110 were further differentiated by AluI digestion and hybridization with a polymorphic GC-rich repetitive sequence [12].

Spoligotyping. Spoligotyping was performed using a kit from Isogen-Life Science in accordance with the manufacturer’s instructions. The presence or absence of 43 spacers in the direct-repeat region of isolates of M. tuberculosis was detected as follows: the direct-repeat region was amplified by primers, one of which was biotinylated; the amplified products were reverse-hybridized to spacer sequence oligonucleotide probes immobilized on a Biodyne C membrane; and detection of spacer sequences was achieved with streptavidin peroxidase and enhanced chemiluminescence.

RESULTS

Proportion of MDR F15/LAM4/KZN strains amongst genotyped M. tuberculosis isolates. During the period 1994–2002,
a total of 966 *M. tuberculosis* isolates were recovered from patients in KwaZulu-Natal and were genotyped by means of IS6110 fingerprinting (table 1). Of these isolates, 109 (11%) belonged to the F15/LAM4/KZN genotype. The proportion per year varied from 2% to 31%, but this rate was highly dependent on the selection of isolates: the proportion was low in studies that included consecutively enrolled patients but high when only drug-resistant isolates were included. The numbers of MDR-TB isolates among the 109 F15/LAM4/KZN isolates and the 857 other isolates were 75 (69%) and 32 (4%), respectively ($P < .001$). During all years, the proportion of MDR-TB isolates was highest among those with the F15/LAM4/KZN genotype. No fingerprinting was performed in 2003 and 2004. In 2005, a group of 102 MDR-TB isolates from one district in KwaZulu-Natal was analyzed, and 60 (59%) of these isolates belonged to the F15/LAM4/KZN family.

**Drug resistance patterns.** Figure 1 shows the chronological evolution of resistance in the F15/LAM4/KZN strain. Genotyping and surveillance for drug resistance commenced in 1994. Already in that year, resistance to isoniazid appeared in combination with either rifampicin (MDR) or ethambutol. In addition, MDR was noted in combination with resistance to streptomycin. MDR combined with resistance to ethambutol was first found in 1995. Resistance to second-line drugs started to emerge in 1997 with ethionamide, followed by capreomycin in 1998, kanamycin in 1999, and fluoroquinolones in 2000. The first XDR isolate with the F15/LAM4/KZN fingerprint was found in 2001 in one patient, followed by the large number of isolates found in Tugela Ferry in 2005. No information is available for the years 2002–2004.

**DNA fingerprint patterns.** The RFLP patterns of the F15/LAM4/KZN family of strains that infected patients during 1995–2000 in KwaZulu-Natal is shown in the dendrogram in figure 2. These strains were found to possess identical spoligopatterns, with spacers 21–24, 33–36, and 40 absent. Other strains were found to have different spoligopatterns. Analysis of the evolution of fingerprint patterns did not reveal a chronological association with the development of resistance (data not shown).

**DISCUSSION**

We report on the development of resistance in a strain of *M. tuberculosis* with a distinct IS6110 RFLP fingerprint pattern. This strain was already present in the KwaZulu-Natal province of South Africa in 1994, when fingerprinting of *M. tuberculosis* isolates commenced. Fingerprinting has been performed from that year onwards on selected isolates that formed part of specific projects. One of these projects was monitoring of primary resistance through fingerprint analysis. As a result of a lack of funding, only small numbers of isolates were included in most years, and no such analysis was performed in 2003 and 2004. However, the data presented in table 1 indicate that the F15/LAM4/KZN strain was consistently around and is responsible for a significant proportion of transmissible MDR-TB.

Although the IS6110 fingerprint of our strain may be unique, the spoligotype has been reported before and is listed in the spoligotyping database as ST 60. This spoligotype has been found in 12 countries on the European and North and South American continents [13]. After the South African classification of *M. tuberculosis* strains, the organism belongs to the F15 family, which forms part of the Latino-American and Mediterranean (LAM) family and corresponds with the LAM4 subgroup [8]. Therefore, the strain is now named F15/LAM4/KZN.

Figure 1 illustrates how the F15/LAM4/KZN strain developed resistance over time. One can only speculate about the reasons...
Figure 2.  

A, Dendrogram depicting restriction fragment–length polymorphism patterns determined using GelCompar, version 4.0 (Applied Maths).

for this development. A general observation in antibacterial therapy is that treatment with fixed combinations of drugs will lead to infections with organisms that are resistant to that combination. This was thought not to be applicable to *M. tuberculosis* for 2 reasons: (1) combination therapy would prevent in vivo survival of mutants that are resistant to one drug, and the chance of mutations leading to resistance to ≥2 drugs in the same cell is extremely small; and (2) the loss of fitness of drug-resistant strains limits their transmission [14, 15]. From this it follows that focusing on optimization of the TB-control programs for drug-susceptible TB will prevent resistance to spread. If the vast majority of patients fully adhere to their treatment regimens, no resistance will develop, and drug-resistant strains that develop in the small minority of persons who are not compliant will not spread.

However, this policy ignores the observations of spreading MDR strains such as the F15/LAM4/KZN strain (table 1). This means that, in the absence of susceptibility testing, a growing proportion of patients started receiving a regimen of isoniazid, rifampicin, pyrazinamide, and ethambutol while infected with an MDR strain. MDR isolates of the F15/LAM4/KZN strain that are resistant to ethambutol were found already in 1995 (figure 1). Pyrazinamide susceptibility has not been tested regularly. However, resistance in a small percentage of isolates was found in a pilot study performed in our laboratory in 1994. Therefore, in the absence of susceptibility test results at the commencement of treatment, patients have been treated unintentionally with 1 or 2 active drugs only. This not only resulted in treatment failures, but also in further selection of drug-resistant strains. Failures are usually only recognized at 3 months of treatment. The process of culture and susceptibility testing that follows takes another 2 months, during which patients usually continue to receive the standard regimen.

South Africa adopted the directly observed therapy–plus strategy for identified MDR cases in 2001. This strategy did not include drug susceptibility testing for the second-line drugs. Depending on the susceptibility to ethambutol, patients commenced blinded treatment with either ethambutol or cycloserine, in combination with pyrazinamide, ethionamide, kanamycin, and ciprofloxacin or ofloxacin (depending on availability). Figure 1 shows that ethionamide resistance was already present in the MDR F15/LAM4/KZN strain in 1997, always in combination with ethambutol resistance. Kanamycin resistance was found in 1999, and fluoroquinolone resistance was found 1 year later. Cycloserine resistance is not reported, because the test for such resistance, like the test for pyrazinamide resistance, is associated with technical difficulties.

The first XDR F15/LAM4/KZN isolate was identified in 2001. This could have resulted in clonal spread of this particular isolate. However, it is obvious that, when the directly observed therapy–plus strategy was implemented in 2001, a proportion of patients again started receiving regimens that contained too few effective drugs. This has likely contributed to the development of XDR TB in different parts of the province, as indicated by its development into a family of strains (figure 2) and the difference in susceptibility between the 2001 XDR isolate and the isolates from Tugela Ferry (figure 1).

Another important observation regards to streptomycin resistance. The South African TB-control program uses streptomycin as a fifth drug in its re-treatment regimen. This is applied in cases in which patients interrupt their treatment. Figure 1 shows that one of the MDR variants of the F15/LAM4/KZN strain was already resistant to streptomycin in 1994. It is that arm out of which the XDR strain developed. It is tempting to postulate that the addition of streptomycin to the standard 4-drug regimen for patients who required re-treatment has assisted in the selection and spread of organisms from the isoniazid-rifampicin-streptomycin-resistant variant.

Although capreomycin had never been used in South Africa, resistance had been found in the F15/LAM4/KZN strain. This might have resulted from the use of aminoglycosides with which capreomycin shows some cross-resistance. Because capreomycin susceptibility testing is not routinely performed, it is presently unknown whether there are F15/LAM4/KZN variant strains that are resistant to that drug in any combination. This is now under investigation.

Although blinded, standardized treatment allowed for selection of increasingly resistant organisms, this happened in the background of an expanding epidemic of HIV infection. As a result, the number of immunocompromised individuals—and, with that, the number of those with increased susceptibility to *M. tuberculosis*—increased. As a result, the pool of patients in whom the F15/LAM4/KZN strain could spread increased as well.

The prominent presence of the F15/LAM4/KZN strain in patients with MDR-TB from 1994 onwards indicates that this strain is one of those that are more effectively transmitted than are other strains. However, another factor that undoubtedly has contributed to this effective transmission is the selective pressure associated with standardized treatment.

In conclusion, as with other bacteria, development of resistance to *M. tuberculosis* results from selection of resistant variants during treatment of patients. The increased pool of patients with HIV-associated immunocompromise in the population aided in this development. Empirical treatment, as applied in TB-control programs, needs to be supported by drug-resistance surveillance programs.

**Acknowledgments**

We thank S. Reddy and T. Kesting, for assisting with IS6110 fingerprinting, and B. Ndimple, for assisting with spoligotyping.

**Financial support.** University of KwaZulu-Natal.

**Potential conflicts of interest.** M.P. and A.W.S.: no conflicts.
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