AN EVOLVING THREAT

Epidemiologists at the University of Stellenbosch, near Cape Town, South Africa, have assembled a vast collection of tuberculosis strains. With it, they're revealing how drug-resistant strains evolve and spread through human populations. **Charlie Schmidt** reports.

The customers who patronized a small convenience store in the suburbs of Cape Town, South Africa, in 2005 didn't know of the risk they faced by walking through the door. A few would have chatted with the store's owner behind the cash register and waved goodbye to her as they left with their snacks and soft drinks. Although these ordinary encounters might have seemed harmless, they weren't—the owner suffered from an aggressive strain of multidrug-resistant tuberculosis (MDR-TB). And with each cough and sniffle, she was expelling virulent TB bacteria into the air.

By October of that year, four teenagers from a nearby school had come down with the illness, drawing the attention of researchers at the University of Stellenbosch, some 50 kilometers east of Cape Town. Led by professors Tommie Victor and Rob Warren, both molecular epidemiologists from the health sciences faculty, the research team screened sputum samples from the four sick students against the university's database of TB cultures by using DNA analysis. They quickly became troubled by what they saw. All of the sick students carried a TB strain known as Beijing 220, which had previously appeared only in isolated cases but was more recently showing up in clusters.

Beijing 220 is emblematic of the strains that drive fear into the heart of the TB research community—it can pass through the air from one person to another, it competes successfully for survival with drug-susceptible strains and, under certain circumstances, it can morph easily into 'extensively drug-resistant tuberculosis' (XDR-TB), the most terrifying form of the illness, resistant to virtually all known treatments. Investigations by the Stellenbosch team now show that Beijing 220 is spreading quickly in parts of South Africa and accounts for the majority of drug-resistant cases of TB in the country's Western Cape province, which includes Cape Town.

The mountain-fringed city of Stellenbosch, home to the scenic vineyards that produce South Africa's best wine, also falls within the Western Cape province. And TB incidence rates among the 1.4 million people living in the rural districts surrounding the city exceed 1,300 cases per 100,000 (compared to 50 per 100,000 in Europe). From their post at the University of

Stellenbosch, Victor and Warren live and work amidst a global epidemic of drug-resistant TB that shows no signs of slowing.

South Africa ranks fourth on the World Health Organization's (WHO's) list of 22 high-TB-burden countries, just behind Indonesia, China and India. According to the WHO's latest survey, released in March 2008, approximately 9 million new TB cases are detected globally every year. Of these cases, nearly 500,000—or roughly one in 20—are drug resistant.

When tuberculosis is susceptible to drugs, it can be cured with six months of 'directly observed treatment, short course' (DOTS), which involves four antibiotics, including isoniazid and rifampin. Multidrug-resistant strains of TB, on the other hand, require at least two years' treatment with up to six second-line medications, each of them more toxic and less effective than the last.

Since 2000, Stellenbosch scientists have used a genetic approach to tracking TB in four nearby rural districts home to 1.4 million people. As part of that investigation, Victor and Warren have assembled one of the largest collections of TB samples in the world—8,000 drug-sensitive cultures and 3,000 drug-resistant cultures. In doing so, they have created a valuable resource for studies that use DNA fingerprinting to track how drug-resistant strains evolve and spread through populations. "When we're looking for strains to study evolutionary events in TB drug resistance, their archive is the first I turn to. It's a global asset," says Megan Murray, who teaches epidemiology at the Harvard School of Public Health in Boston and collaborates with the Stellenbosch team.

Courtesy of the Victor Lab

The big view: Scientists from Stellenbosch (pictured) monitor TB in nearby regions

A sequence of events

Working with Murray and collaborators at the Broad Institute of the Massachusetts Institute of Technology and Harvard, Victor and Warren have begun to piece together how normal tuberculosis strains mutate to become resistant to antibiotics. By sequencing and comparing the DNA of numerous strains, the scientists identify gene variations that seem to correlate with drug-resistance.

To confirm that a specific sequence change confers drug resistance, the scientists introduced the genetic mutation into a drug-sensitive strain and see how the organism reacts in the presence of antibiotics. Alternatively, scientists can study the protein that a variant gene produces to determine whether it affects a drug resistance pathway. So far the team including the Broad Institute geneticist James Galagan has identified nearly 40 mutations that can either confer or enahnce drug resistance in TB bacteria. The ongoing agenda, Galagan says, is to sequence thousands of TB bacteria to derive the most comprehensive view of drug resistance possible.

Pieter Bauermeister/AFP/Getty Images

Resistance measures: People with extensively drug-resistant TB have defied quarantines

Broadly speaking, people can become sickened by drug-resistant TB in one of two ways: they can either develop 'acquired' MDR-TB by not adhering to treatment—a failure that allows resistant microbes to flourish—or they can catch 'primary' MDR-TB from another person's acquired disease.

For a long time, experts have assumed that drug-resistant strains of tuberculosis would not spread beyond hospitals and other clinical settings. This was based on the idea that such strains are so compromised by resistance mutations that they cannot replicate efficiently.

The scientific evidence now suggests that not all drug-resistant strains suffer from these genetic 'fitness-costs'. Indeed, four types of tuberculosis strains now account for 70% of all multidrug-resistant cases in the Western Cape province, each potentially as fit as its drug-sensitive counterparts, if not more. Of them, Beijing 220 accounts for the largest fraction, followed by a strain described by Victor's team in 2007 and dubbed DRF150—also referred to as low-copy clade, or LCC (*Int. J. Tuberc. Lung Dis.* **11**, 195–201; 2007).

With historical origins in East Asia and documented outbreaks worldwide since the early 1990s, Beijing strains can evade the protective effect of the *Mycobacterium bovis* vaccine; the main inoculation used against TB, given chiefly to children and health care workers.

"The [Beijing] 220 strain seems to have unique characteristics," Victor explains. "It's extremely aggressive, and our database shows

Pieter Bauermeister/AFP/Getty Images

Monitoring progress: Health workers keep tabs to make sure TB patients take their meds

that its expansion in the Western Cape during the last six years has been much greater than that of other Beijing strains. Thus far, none of the 220 infections we've seen have been drug susceptible. We think there must have once been a susceptible counterpart that likely died out because it couldn't spread as well as MDR 220."

All in all, the Stellenbosch research team has also shown that primary transmission cases account for more than 60% of MDR-TB cases in the Western Cape (*Int. J. Tuberc. Lung Dis.* **12**, 99–104; 2008).

Extreme resistance

The emergence of XDR-TB—first detected on a large scale three years ago in a region of South Africa known as Tugela Ferry—make these TB sequencing efforts particularly urgent. Whereas with MDR-TB doctors can achieve cure rates around 80%, according to the Boston-based global service group Partners in Health, the cure rates for XDR-TB fall consistently below that. A new study released this summer reported that among nearly 50 patients with XDR-TB in Peru, daily supervised treatment at community centers achieved a cure rate above 60% (*N. Engl. J. Med.* **359**, 563–574; 2008).

But other data suggest that extensively drug-resistant tuberculosis carries a far greater risk than MDR-TB. Citing results from his survey of 4,000 TB cases across several European countries, Giovanni Battista Migliori, director of the WHO's Collaborating Centre for TB and Lung Diseases in Tradate, Italy, says XDR-TB's mortality rate is up to 5 times greater than that of MDR-TB (*Emerg. Infect. Dis.* **13**, 780–782; 2007).

Migliori points out that very few of the 4,000 Europeans included in the study were co-infected with HIV, which exacerbates TB by blunting the body's defensive immune response. Among patients with HIV—endemic throughout sub-Saharan Africa—XDR–TB fatality rates have approached 100%, he says. Indeed, 52 of the 53 HIV-positive individuals stricken with XDR-TB in 2005 Tugela Ferry died within weeks of developing TB symptoms. In fact, tuberculosis has become the world's number one killer of HIV-infected people.

Victor's analysis of cases near Stellenbosch revealed 17 genetically unique XDR-TB strains. On the basis of this finding, he concludes that the acquisition of new resistance mutations from drug treatment failure still drives XDR-TB's spread in the region (*Int. J. Tuberc. Lung Dis.* **12**, 99–104; 2008). Transmission of XDR-TB strains from person to person, he explains, would have produced more clustering of identical genotypes. What's more, Victor adds, most XDR-TB has been detected in patients who were initially sickened by either the Beijing 220 or LCC strains, a reflection of how those strains are uniquely poised to mutate into an XDR form.

At least 101 'pre–XDR-TB' cases, classified as MDR-TB with additional resistance to at least one second-line drug, have been identified by the Stellenbosch research team. "We predict this large pool of patients will ultimately become XDR by further selection for second-line drug resistance," Victor says. "This is worrisome."

Ted Cohen, an epidemiologist and tuberculosis specialist at Harvard Medical School involved in the effort to track the evolution of various strains, also believes that rare drug resistance mutations that do not exert fitness costs could become increasingly common. "The concern here is that these rare genotypes might be preferentially transmitted," he explains. "Over time, they could predominate in the population."

But others, including Migliori, question the risk of XDR-TB transmission, given that so many resistance mutations—at least one per drug—are required to convert an MDR into an XDR strain. "These mutations affect the bacteria's metabolism and ability to divide," he says. "Hypermutated TB tend grow very slowly in culture; you get the feeling that they're not very aggressive."

Diagnosing the problem

Experts emphasize that long delays in diagnosis are particularly problematic. TB patients who don't respond to first-line treatment because they harbor a drug-resistant form of the disease run the risk of infecting others in the community. Both the WHO and the US National Institute of Allergy and Infectious Diseases, in Bethesda, Maryland, have made shortening diagnostic delays a top priority on the TB agenda.

Clinicians generally diagnose tuberculosis on the basis of cough, weight loss and other

Courtesy of the Victor Lab

Frozen feature: Tommie Victor (pictured left) keeps the labs vast collection of samples on ice

Courtesy of the Victor Lab

Spreading hope: A rural clinic distributes tuberculosis medications to people with the disease

symptoms, in addition to 'smear-positive' findings in sputum, made by looking through a microscope at smears on slides, which can reveal the presence of mycobacteria (a bacterial family that also includes the *Mycobacterium tuberculosis* species that causes TB).

Going beyond smear-positive findings to confirming a TB diagnoses is time consuming, however. Different species of mycobacteria can't be visually distinguished. To diagnose TB accurately, scientists have to grow the sampled bacteria in a Petri dish, which can take up to three weeks.

In developing countries, where smear-positive findings almost always indicate TB (as opposed to wealthier countries where other mycobacterial infections are more common), putative cases are treated immediately with DOTS. MDR-TB is suspected when DOTS therapy fails, but confirming that requires several weeks of drug-sensitivity testing with cultured bacteria. The whole process of diagnosing MDR-TB can take two months or more, and can cost in excess of $100.

Given that, Victor and his Stellenbosch colleagues, including Paul van Helden, have worked hard to enhance the speed of MDR diagnoses with new technology. In April 2008, the team published a new assay that flags resistance to rifampin within three to four hours after a short culture period (*J. Clin. Microbiol.* **46,** 1369–1373; 2008). The assay focuses on slight variations within a *Mycobacterium tuberculosis* gene called *rpoB*—variations that can influence the reaction of tuberculosis to the drug rifampin. Preliminary evidence suggests that the molecular assay has a diagnostic accuracy of 95% at what Victor claims is a cost of $3.

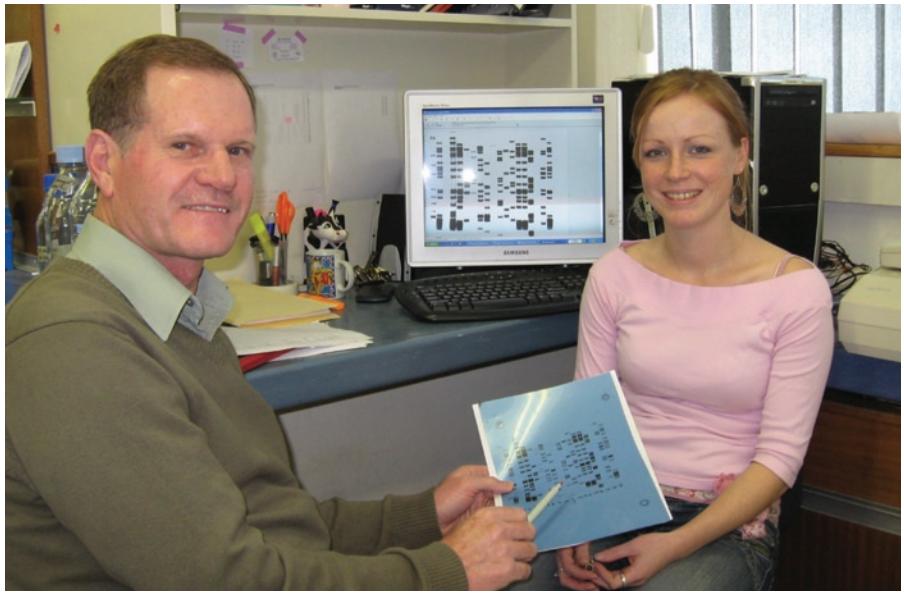
On 30 June 2008, a collaboration between the WHO, the Stop TB Partnership, the Foundation for Innovative New Diagnostics and the Geneva-based drug purchasing organization UNITAID endorsed two rapid MDR diagnostic tests for individuals with sputum-positive tuberculosis. "With the old methods, it takes two to three months to get an MDR diagnosis; these tests do it in a day," says Mario Raviglione, director of the WHO's Stop TB program.

One of the new tests, the Genotype *MTBDRplus* test, developed by Hain Lifescience, in Nehren, Germany, reveals mutations in the *rpoB* gene that confer resistance to rifampin, as well as mutations in the promoter region of the *inhA* gene that confer resistance to isoniazid. The other test—based on a method known as a 'line-probe assay' that compares specific gene regions—detects resistance to rifampin only. Raviglione says the Hain test was priced at $5 a go, whereas the line-probe assay's cost is $8 per test. "But commercialization of these tests is just beginning, and costs could vary by country," he adds. According to Raviglione, developing countries wanting to purchase such tests will receive help from UNITAID and the Global Fund to fight AIDS, Tuberculosis and Malaria, supported chiefly by the Bill & Melinda Gates Foundation.

Ideally, the new PCR assays will limit those costs and allow patients with MDR-TB to be treated appropriately faster. But Victor cautions that both his test and those endorsed by the WHO have certain limitations. None are 100% accurate; they rely on extremely pure DNA that is uncontaminated by blood, smoke and other interferences. And they require DNA 'amplification' by PCR, a technique involving laboratory instruments that might not be readily available and that come with a high price tag of about $5,000. "What we're ultimately looking for is a microchip tool that could allow us to diagnose MDR immediately in the field," he says. Victor's collaborations with Murray aim to make that test a reality, but a prototype is several years away, he says.

The researchers in Stellenbosch and others around the globe tracking tuberculosis strains face an uphill challenge: the bacteria that cause this disease can mutate far faster than health agencies can move treatments and diagnostics off the shelves and into affected communities. So, in Victor's lab and elsewhere, they remain in a race against the clock to pinpoint the mutations that give TB the ability to resist treatment. These same mutations might ultimately give medical workers the power to quickly spot and stop outbreaks. Only then, say researchers, will they be able to breathe easy.

Charlie Schmidt is a science writer based in Portland, Maine.



Courtesy of the Victor Lab

Mutant menace: Victor and his student analyze the DNA fingerprints of drug resistant samples