Role of the Laboratory
for the Diagnosis and Control of Tuberculosis

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When preparing this talk, I have tried to focus on the activities of the California State Public Health Laboratory, to which I belong. As a state laboratory, we are not concerned merely with the activities of our own laboratory, but try to assume responsibility for the overall performance of TB laboratory testing in the State of California.

I. TB Testing in California

1. Role of the State Public Health Laboratory

A state public health laboratory has several roles. The first is to support the tuberculosis control program. We thus provide reference laboratory services, including some specialized testing. Second, we develop, evaluate, and more generally, assume responsibility for establishing new testing methods. Third, we take part in the training of new laboratory staff, as well as the training of personnel from other laboratories. Finally, we consult on the development of regulations that pertain to TB laboratories. As I have noted, our overall goal is to promote an effective TB laboratory system in the entire state.

2. TB Laboratories in California

There are about 250 laboratories conducting TB laboratory testing in the state of California. 25 of them are public health laboratories, and the remainder are either hospital or large commercial laboratories.

Of all the specimens obtained from patients who are suspected of having tuberculosis, about 20% are tested in county public health laboratories; 40% are tested in hospital laboratories; and 40% are tested in large commercial laboratories, such as Quest or Laboratory Corporation of America.

The goal of the state laboratory is to develop an effective TB laboratory system out of this mixture of laboratories. For that purpose, we have defined several goals. First, we would like TB laboratories to perform culture and drug susceptibility testing, as well as acid-fast microscopy. Second, we would like them to use up-to-date, accurate methods. Finally, we want to ensure that positive results from all TB laboratories are reported to state TB control program.

3. Regulation

California has established a number of regulations pertaining to TB testing. Since conducting culture and drug susceptibility test is the standard of practice, California regulation requires that a culture be performed, whenever a patient has a positive acid-fast smear. Thus, during the training conducted by the State laboratory, we recommend conducting a culture of three specimens for each suspected TB patient. In addition, if a new TB patient is culture-positive
for *Mycobacterium tuberculosis*, a second regulation requires that drug susceptibility testing be performed.

TB testing regulations are not created directly by the government. Rather, the State government passes a law that allows the Department of Public Health (DPH) to create regulations. Generally, the regulations are written by DPH employees. However, before a regulation can be set, the law requires that the DPH listen and respond to advice from all the organisations and people who would be affected by the regulation. If a doctor or a laboratory employee do not follow a regulation, they may be obliged to pay a fine, and could even lose their license to work.

II. TB Testing Methods

1. Inadequate TB Testing Methods

As a state laboratory, we would like ensure that all laboratories are using up-to-date and accurate TB testing methods. Unfortunately, some methods that we consider to be inadequate are still used in US TB laboratories.

Allow me to provide several examples. First, some laboratories use an inadequate culture media when conducting culture tests. In the US, we strongly recommend using a liquid culture medium. A second problem is false positive results due to cross-contamination in the laboratory, which happens rather frequently. A third problem is inaccurate drug susceptibility testing results, due to poor quality control. Yet another is false negative AFB smears, which are due to poor stain quality, or to the fact that the laboratory microscopist did not devote sufficient time to reading the slides.

2. Liquid versus Solid Cultures

For culture media, we use the Middlebrook agar plates, as well as the Lowenstein Jensen egg-based medium. We also use a liquid media, called the Mycobacterial Growth Indicator Tube (MGIT), which is manufactured by Becton Dickinson. The reason we recommend the liquid medium is based on a review article, published in 1995. The review covered five articles, in which the sensitivity of the liquid media was compared to that of solid medium in recovering mycobacteria. In all five studies, the liquid mediums recovered more mycobacteria than the solid ones.

The liquid culture medium offers several advantages. First, it utilises selective antibiotics, which inhibit other contaminants, including fungi, but not the growth of mycobacteria. Second, the Becton Dickinson medium offers consistent lot-to-lot drug activity in each batch of media, since the company performs pre-market testing of the medium ingredients. Third, the liquid medium provides quick detection of growth, and a higher number of positives.

Nevertheless, we still use the solid medium cultures as well, since they also offer several advantages. First, they can be produced locally, and are inexpensive, as in the example of the Lowenstein (LJ) Jensen egg-based medium. Another feature we like about the LJ cultures is that they can be stored long-term in the refrigerator, and easily shipped between laboratories, when genotyping or drug susceptibility testing is required.
3. Cross-Contamination

Cross-contamination in the laboratory can be a serious problem. When we decontaminate a sputum sample, we treat it with sodium hydroxide, in order to kill contaminating bacteria and fungi. We then neutralise the sodium hydroxide using a phosphate buffer.

The problem is that some laboratories have been using a common buffer flask to pour into different tubes one after the other. Naturally, this practice increases the chance of obtaining false positive results. This phenomenon can be easily prevented by putting the buffer in individual glass tubes. Since these tubes can be washed and reused, this method is not more costly.

III. Activities of State Public Health Laboratories

There are several ways in which a state public health laboratory can improve the TB laboratory work conducted in other laboratories. First, we collaborate on training initiatives with the National Laboratory Training Network (NLTN). In addition, different US states use various methods to try and improve the practice of mycobacteriology within their state. I will briefly review three models: the Wisconsin networking model; the New York regulation and a ‘fast track model; and the California ‘MGITS by mail’ model.

1. Training

The National Laboratory Training Network (NLTN) is a non-governmental organization, which means they have fewer rules, and can provide training more efficiently. It is funded by grants from government, as well as by registration fees charged for meetings. Besides TB laboratory work, they NLTN provides training on various topics, including influenza, HIV, and bio-terrorism.

Lately, the NLTN has been organising web-based conferences, in which laboratories involved in TB work are able to participate. The participants view PowerPoint slides on a website, and listen to a speaker through the phone or the Internet. The NLTN also holds face to face conferences, and is involved in producing various training materials, such as training videos.

2. The Wisconsin Model

The Wisconsin State Public Health Laboratory uses laboratory networking. This model has proven to be an efficient way for encouraging laboratories to perform their work appropriately, and is also inexpensive. In fact, I believe all the laboratories conducting TB testing in Wisconsin are using adequate methods.

As part of the model, the Wisconsin State laboratory sponsors an annual meeting for all the laboratories that conduct TB laboratory tests in the state. During the meeting, the state laboratory and other laboratories give talks about new methods and problems. At the meeting, people are gathered face-to-face, and are thus under ‘peer pressure’ to use up-to-date methods.
3. The New York Model

The states of New York makes extensive use of regulation. New York State regulations state, for example, that liquid culture medium must be used, and that drug susceptibility testing must include Pyrazinamide.

In addition, the New York State laboratory offers a ‘fast track’ service. Thus, whenever another laboratory has an acid-fast smear-positive specimen, it can collect a second specimen from the same patient, and send it to the state laboratory for free testing, which includes nucleic acid amplification testing.

4. The California Model

a. Organisation of TB Testing Laboratories in California

In California, the situation is slightly different. Since it is a huge state, there are 35 county and city public health laboratories. Some of these laboratories employ merely one person, while others employ up to 80 people. Several of the larger county public health laboratories offer nucleic acid amplification testing for smear positive specimens.

In addition, there is one state laboratory, situated near San Francisco. It is noteworthy that the local public health laboratories are not controlled by the State, but directly by the county. Thus, we, at the state laboratory, cannot control their activities. However, the local public health laboratory directors meets with the state laboratory directors twice per year, so we manage to maintain efficient communication.

b. MGIT by Mail

California also operates the ‘MGIT by mail’ programme. MGIT stands for Mycobacterial Growth Indicator System. The goal of the system is to allow small public health laboratories to use the latest culture technology. In order to participate in the programme, laboratories are required to have TB expertise, a biological safety cabinet, and safe work practices.

Under the model, The California State laboratory purchases the MGIT medium from Becton Dickinson and sends it out to nine local public health laboratories. Each local laboratory decontaminates and concentrates the specimens. Since they conduct the microscopy stage locally, these laboratories are able to obtain quick acid-fast microscopy results.

Then, the local laboratory mails the unincubated MGIT cultures to the state laboratory, where incubate them and treat them like our own specimens. We will then attempt to identify any mycobacteria by using a DNA probe, and conduct a quick drug susceptibility testing.

5. Additional Services Provided By the State Laboratory

The California state laboratory provides several other unique services. First, we perform a rapid detection of drug resistant *M. tuberculosis* in clinical specimens using real-time PCR. Second, we test for susceptibility to second line drugs using the rapid MGIT system. Third, we identify non-TB mycobacteria by using HPLC and biochemical testing. These methods identify mycobacteria by their cell-wall composition. Finally, we conduct genotyping using PCR-based methods.
a. Rapid Detection of Drug Resistant TB

We routinely offer a rapid detection service of drug resistance *M. tuberculosis* in clinical specimens, using real-time PCR. We work with specimens or cultures from patients who are suspected to have drug-resistant tuberculosis. We are able to work with acid-fast smear positive sputum sediment before the culture grows. We are able to detect *M. tuberculosis*, as well as mutations associated with resistance to Isoniazid or Rifampin within a few hours.

b. Susceptibility Testing in MGIT

We also perform susceptibility testing in MGIT, in which we test for susceptibility to our primary drugs, Isoniazid, Rifampin, Ethambutol, and Pyrazinamide. When we detect resistance to Rifampin or any two primary drugs, we test for susceptibility to the second-line drugs we use, Ethionamide, Capreomycin, Amikacin, and Levofloxacain. We have also developed and published new testing methods.

We conducted a multi-centre study to validate the use of the MGIT system to test second-line drug susceptibility. First, we collected some susceptible cultures from patients who were never exposed to the drug. We then collected resistant cultures, which are cultures from patients who have been treated with the drug, but are still positive. In the case of Levofloxacain, some of the resistant cultures we used had known gyrA mutations of several different kinds.

We then determined the minimal inhibitory concentration (MIC) for both the susceptible and resistant cultures. The susceptible cultures have had a lower MIC than the clinically resistant ones. We selected a test concentration that inhibits the growth of the susceptible strains, but not the resistant ones. For example, in the case of levofloxacain, we chose a test concentration of 1.5 micrograms per millilitre. Following the study, we began using MGIT to test second-line drugs routinely.

IV. Universal Genotyping

Another service we offer in the state laboratory is universal genotyping, which sometimes enables us to detect previously unsuspected transmission. We are also able to detect occurrence of false positive cultures, and to confirm suspected links between two or more cases. Our laboratory performs genotyping tasks for the entire Western half of the US, while the Michigan laboratory does it for the Eastern half. We thus genotype about 5 000 cultures per year.

1. Genotyping Examples

I would like to offer a couple of examples of how genotyping can be helpful. In one case, several TB patients were all residing at the same homeless shelter. However, we discovered that their isolates do not indicate matching genotypes. Since our genotype results did not support the hypothesis that recent transmission occurred within the homeless shelter, the TB control programme was not required to hold an outbreak investigation. We thus saved them time and resources.

In a second example, isolates from multiple TB patients have displayed matching genotypes. However, during the contact investigations, no suspected transmission links was revealed between the patients. Then, a review of the epi data revealed that the patients resided in the
same zip code have reported cocaine use. We later discovered that the patients used to smoke the drug, and then exhale it directly into the mouth of another user, so that the smoke would not be wasted. Needless to say, this habit was also an efficient way of transmitting tuberculosis.

At the beginning, the patients refused to provide information on the people they might have infected, or were infected by. However, the genotyping revealed that it was a large outbreak; one of the largest to occur in the US in recent year. Finally, a cluster investigation was initiated, and the outbreak was brought under control.

2. Genotyping Methods

The genotyping methods we use are Spoligotyping, MIRU, and RFLP. We like the Spoligotyping method, since it requires merely a small amount of DNA from a TB culture. It also provides the results as a number, so it is possible to see compare two strains easily. If two strains are different, so would their numbers be. Unfortunately, Spoligotyping is not extremely powerful at discriminating between different strains. Thus, sometimes strains that are not part of the same outbreak will exhibit the same spoligotype. For example, all the Beijing strains have the same spoligotype.

Other speakers have already described the MIRU system. Beginning in late 2008, a new more powerful 24 locus MIRU will be used, and we hope it would offer a greater discriminatory power for Beijing strains. Until today, about half of the Beijing strains in California has exhibited the same MIRU pattern.

Thus, our PCR-based tests, Spoligotyping and MIRU, did not offer high quality discrimination. For that reason, MIRU and Spoligotyping are used together. They both require merely a small amount of DNA, and it is unlikely that two unrelated M. tuberculosis strains would have both the same spoligotype and the same MIRU type.

Unfortunately, that does not hold true for the Manila and Beijing strains. Thus, when an epidemiologic question arises with respect to these two strains, it is necessary to use RFLP, which offers a more powerful discrimination. Usually, we prefer not to use this method, since it requires significant work, and it takes about a week to obtain the results. In addition, since the results are displayed as a visual pattern, it is difficult to create a precise database based on them.

Our universal genotyping approach requires that all isolates undergo PCR-based genotyping, which takes about two weeks. A follow-up request for RFLP would require an additional three weeks.
V. Question and Answer Session

Participant

What is the difference between the stability of the liquid and solid media tests for second line drugs?

Ed DESMOND

We have sent some cultures to the US CDC in Atlanta, where they performed agar proportion testing. We compared the results obtained with solid and liquid media, and there seems to be a high degree of correlation between the two.

Participant

Can the MGIT be used by physicians to determine a second-line drug regimen?

Ed DESMOND

For the first-line drugs, MGIT is an FDA-cleared product, so it can clearly be used for patient care decisions. However, in the US, in-house developments can also be used for patient care decisions, provided that a careful validation study has been performed. Since we have conducted a multi-centre study relating to the use of MGIT in second-line drugs, we are allowed to use it.

Qian GAO

Do you culture the smear-negative cases as well?

Ed DESMOND

Yes, we culture every specimen.

Brigitte GICQUEL

When do you use RFLP?

Ed DESMOND

In most circumstances, the MIRU and Spoligotyping methods provide usable results. RFLP is required, for example, when the epidemiological connection is questionable, and the two patients have either Beijing or Manila strains. However, if two patients who have no connection exhibit identical MIRU and Spoligotyping results, I believe there would be no point in performing an RFLP test.

Brigitte GICQUEL

How do you score the results of the RFLP?

Ed DESMOND

We use BioNumerics.
Ying ZHANG
What is your RTPCR assay for detecting drug resistance?

Ed DESMOND
Molecular Beacons, which we develop in-house. For example, for rpoB mutations, we use three molecular beacons, which enable us to detect about 98% of Rifampicin resistance.

Ying ZHANG
How about PZA?

Ed DESMOND
Unfortunately, that test would be more complicated, since the mutations are more spread out. We would therefore require a large number of molecular beacons. Thus, I do not believe molecular beacons are the appropriate platform for identifying PZA resistance.

Pricille BRODY
Did you test the stability of the second line drugs before beginning to use the MGIT system?

Ed DESMOND
Yes, before we begin using a new test method, we would like to ensure that the drugs are stable at different temperatures. We thus prepare several test media that contain the drug, and store them for a week in a refrigerator, an incubator, and at room temperature. Then, we prepare a fresh batch of the media, and conduct the susceptibility testing. If the drugs are stable, the detection time of the different cultures should be similar, regardless of whether the culture is fresh or one week old.

Ying ZHANG
Does the MGIT provide repeatable results?

Ed DESMOND
Some laboratories have experienced problems with the reproducibility of the susceptibility testing using MGIT. However, we tend to obtain repeatable results. We decided to deviate from the manufacturer’s inoculation protocol, and have devised our own protocol, based on the turbidity standard.

Ying ZHANG
Do you believe the MGIT could be used to conduct PZA susceptibility testing?

Ed DESMOND
(Inaudible). I also believe it is necessary to be careful when interpreting the pncA mutations, since not every mutation necessarily leads to drug resistance.
Ying ZHANG

Some data indicates that a test concentration of 200 might be better than 100.

Ed DESMOND

Even if you are correct, changing the drug concentration would cost the manufacturer, Becton Dickinson, millions of dollars, since it would require FDA approval. Unfortunately, that is one of the problems with the manner in which biotechnology business is conducted in the US.