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Molecular Strain Typing of *Mycobacterium tuberculosis* To Confirm Cross-Contamination in the Mycobacteriology Laboratory and Modification of Procedures To Minimize Occurrence of False-Positive Cultures

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Molecular strain typing by restriction fragment length polymorphism analysis was used to demonstrate that two clusters of *Mycobacterium tuberculosis* cultures involving six patients resulted from cross-contamination in the mycobacteriology laboratory. Contaminated cultures were processed by the decontamination procedure and were read on the BACTEC instrument following acid-fast bacillus smear-positive specimens from patients with active tuberculosis. Investigation of these episodes suggested opportunities for modification of laboratory procedures to minimize cross-contamination and confirmed the adverse medical and public health consequences of false-positive cultures. Strain-typing results were used in decisions regarding patient care, including the curtailment of unnecessary treatment in one patient. Molecular strain typing appears to be a valuable means of identifying false-positive cultures of *M. tuberculosis* in selected settings.

After decades of steady decline, there has been a dramatic resurgence of tuberculosis in the United States (3, 21). This has resulted in an increase in the number of specimens being processed for mycobacterial culture by clinical laboratories, many of which have limited or diminishing resources. Even transient increases in the volume of testing may overload the capabilities of some facilities, thereby enhancing the likelihood of procedural lapses or equipment malfunction. As more positive specimens are processed there also will be an increased opportunity for the carryover of organisms from positive to negative specimens during batch processing of samples. Episodes of cross-contamination involving conventional (1, 13–15) and radiometric (4, 16, 26, 27) methodologies have been reported previously and may occur more frequently in the current environment.

Laboratory cross-contamination has been detected retrospectively in patients whose cultures yield *Mycobacterium tuberculosis* in the absence of signs or symptoms of tuberculosis and who subsequently have good clinical outcomes without therapy. When these criteria were applied in a recent investigation of a hospital-based outbreak involving multidrug-resistant *M. tuberculosis*, positive cultures for 16% of the 140 patients evaluated were considered to be the result of laboratory contamination (7). This suggests that false-positive cultures may be common in some settings. However, the retrospective recognition of cross-contamination is of little help in the acute management of individual patients. The prospective recognition of false-positive cultures by clinicians is likely to become more problematic as the population of patients who are at risk for *M. tuberculosis* infection increases. The diagnosis of tuberculosis can be particularly complicated in patients who are also infected with human immunodeficiency virus, in whom the presentation of tuberculosis is highly variable (2).

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We report the use of molecular strain typing by restriction fragment length polymorphism (RFLP) analysis to confirm our suspicion that cross-contamination had occurred in our mycobacteriology laboratory. RFLP analysis is a DNA fingerprinting technique that has been used to distinguish strains of many species of bacteria. The RFLP procedure used in the study described here makes use of a repetitive sequence of DNA, IS*6110*, which is found in the genomes of virtually all strains of *M. tuberculosis* (10). Differences in the number and location of IS*6110* within a strain's chromosomal DNA are revealed by RFLP analysis, which yields strain-specific patterns. There appears to be a sufficient diversity of patterns to ensure that epidemiologically unrelated strains have different RFLP patterns, whereas related strains have identical RFLP patterns (25). These results can be available within days when *M. tuberculosis* cultures are used as the source of DNA and, as demonstrated by our experience, may influence patient management decisions.

MATERIALS AND METHODS

Culture methods. Samples from sites that are normally sterile were concentrated by centrifugation (4,000 × *g* for 10 min) when applicable and were inoculated onto a slant of Lowenstein-Jensen medium (Baltimore Biological Laboratory) and into a vial of BACTEC 12B 7H12 medium (Becton Dickinson, Sparks, Md.). Specimens from nonsterile sites were processed by the *N*-acetyl-L cysteine–sodium hydroxide method (12, 18) with centrifugation of 3,800 × *g* for 15 min. Smears were stained with the auramine O fluorescent acid-fast stain (12). Lowenstein-Jensen slants were incubated at 35 to 37°C with 5 to 10% CO_2 and were read weekly. The BACTEC bottles were incubated at 37°C and were sampled on the BACTEC 460, which is equipped with an 82.5-s heating cycle board, three times for the first 2 weeks and then once weekly thereafter for a total of 6 weeks. Vials with a growth index (GI) of >15, those inoculated with a

specimen with an acid-fast bacillus (AFB)-positive smear, or those with specimens from a patient with previously positive cultures were transferred to separate trays, with uninoculated vials placed on either side of these potentially positive cultures. Cultures were sampled daily until the GI reached >100, at which time bottles with an AFB-positive smear were no longer sampled on the BACTEC system. Needles were changed daily, and other measures, as previously recommended in the BACTEC procedure manual (18) for quality control or to minimize cross-contamination, were used. Isolates were identified as being members of the *M. tuberculosis* complex by using AccuProbe (GenProbe, San Diego, Calif.). Approximately 3,600 AFB cultures are submitted to the laboratory each year. At the time of contamination episodes, processing of specimens was done on a rotational basis by a group of four laboratory technicians, each with at least 1 year of prior experience in the mycobacteriology laboratory.

A false-positive culture was defined as a specimen that was submitted from a patient whose clinical course was not likely to have resulted from tuberculosis and that grew *M. tuberculosis* with the same RFLP pattern as that of a true-positive specimen processed concurrently by the laboratory. Two episodes of suspected cross-contamination with *M. tuberculosis* occurred 8 months apart and involved five cultures from four patients in one instance and three cultures from two patients in the other. In each episode, the cultures suspected of being false positives were decontaminated in the same batch and were immediately preceded in processing order by a specimen with a heavily positive AFB smear (true positive). Suspected false-positive cultures also were inadvertently read once on the BACTEC system directly following reading of the smear-positive samples prior to segregation of the vials inoculated with smear-positive specimens. All suspect cultures were positive only in BACTEC bottles and yielded no growth on the accompanying Lowenstein-Jensen slants. In both episodes there were no intervening culture-negative samples between the initial smear-positive sample and the suspected false-positive cultures. BACTEC vials for the specimens with positive AFB smears had GIs of >15 and were smear positive within 3 days of inoculation. The suspected false-positive specimens became positive after 2 to 3 weeks, with the longest times to positivity being observed for samples located the farthest from the smear-positive samples.

RFLP analysis was conducted on isolates from two patients with active tuberculosis who had positive AFB smears (index cases) and the eight isolates suspected of being false-positive cultures. Nine isolates of *M. tuberculosis* recovered from different patients (controls) whose specimens had been processed within the days or weeks before or after each of the episodes of suspected contamination also were tested to confirm that cross-contamination was limited to the cluster of positive samples which were adjacent to one another in reading and processing order.

RFLP analysis. RFLP analysis was performed by the internationally standardized procedure (24). The only significant procedural alteration was the harvesting of cells directly from the Lowenstein-Jensen slants that were obtained from the clinical laboratory and that had a heavy visible growth of mycobacteria. In brief, bacterial cell walls were lysed and whole genomic DNA was extracted and digested with *Pvu*II. The resulting DNA fragments were separated by gel electrophoresis, transferred to nylon membranes, and probed with a horseradish peroxidase-labeled 245-bp sequence of IS*6110* DNA (Amersham).

Clinical impact. The medical records of all six patients were reviewed by one of us (P.M.S.), and in selected cases, the treating physicians were interviewed to determine how the false-positive culture results were integrated into the care of these patients. For those patients with false-positive cultures, evidence of unnecessary resource utilization, therapy, and toxicity was sought. In addition, the physician's willingness to disregard these cultures on the basis of (i) the suspicion of laboratory cross-contamination and (ii) the RFLP documentation of cross-contamination was recorded. County public health tuberculosis control officers were also interviewed to determine the extent of follow-up undertaken for the patients with false-positive cultures.

RESULTS

The RFLP patterns of isolates sequentially processed by the laboratory (Fig. 1) demonstrate that positive cultures recovered before or after each cluster had unique RFLP patterns characteristic of different strains of *M. tuberculosis*. In contrast, isolates suspected of being false positive because they closely followed the smear-positive samples in order of processing and reading on the BACTEC system had the same RFLP fingerprints as the isolates from the respective smear-positive index cases. No epidemiologic relationship between patients was detected either within or outside of the hospital setting. In addition, no evidence of contamination by equipment (such as bronchoscopes) outside of the laboratory could be found, and the false-positive cultures originated from a variety of specimen types. These epidemiologic considerations, together with the clinical information that made the diagnosis of active tuberculosis unlikely, supported the hypothesis that each cluster of positive cultures consisted of one true-positive culture from a patient with active tuberculosis whose strain had contaminated other cultures in the laboratory.

When positive cultures were reported, physicians were made aware that isolation of *M. tuberculosis* from each of the six patients believed to have a false-positive culture could be the result of laboratory cross-contamination. The lack of clinical evidence suggestive of active tuberculosis in three of these patients led to the immediate dismissal of the positive culture result. The false-positive results prompted additional physician consultation or clinic visits and the submission of additional specimens by three patients for microbiologic evaluation. One patient received 2 weeks of therapy for tuberculosis before the result was accepted as spurious. The existence of unrelated pulmonary disease complicated the differential diagnosis in two patients in whom the possibility of tuberculosis could not be excluded, and they were begun on antituberculosis therapy. In one of these patients, treatment was discontinued when RFLP results became available. However, the other patient completed 1 year of antimicrobial therapy. Both patients who received prolonged antimicrobial therapy suffered drug toxicity requiring alterations in their treatment regimens.

Public health officials also were informed of suspicions that cultures of *M. tuberculosis* from these patients might be false-positive results. Nonetheless, at least limited follow-up of most patients was undertaken; this consisted of consultation with physicians, inquiries regarding the patients' subsequent symptoms, and in some cases, skin testing of close contacts. Because of contact between one of the patients and residents of a homeless shelter, some of the occupants of the shelter received skin testing, chest X rays, and isoniazid prophylaxis.

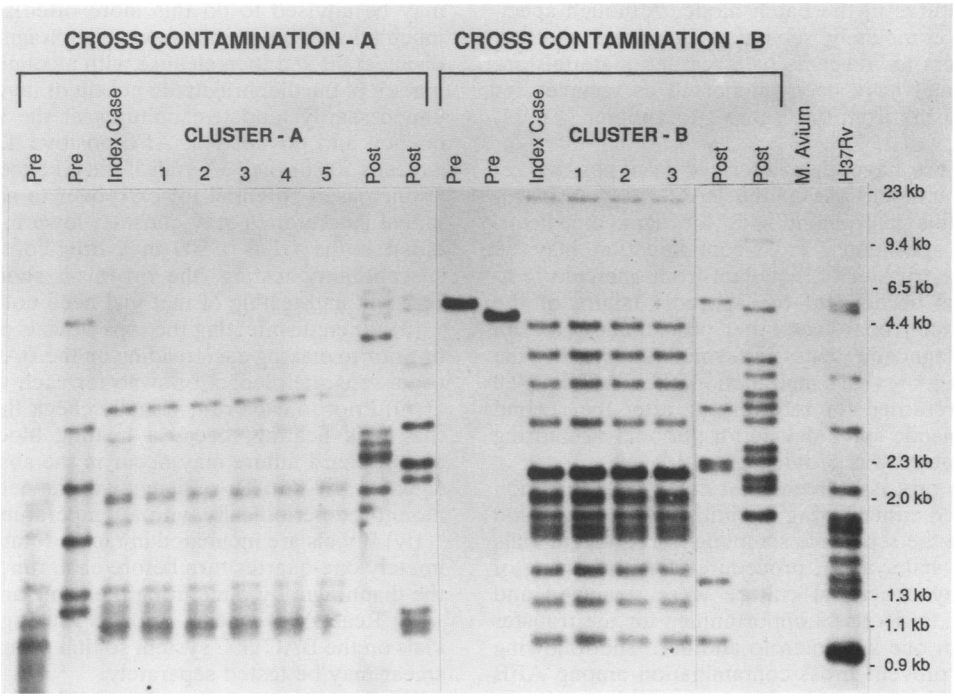

FIG. 1. RFLP patterns of *M. tuberculosis* isolates prior to each episode of cross-contamination (Pre), AFB smear-positive index cases, false-positive cultures (clusters A and B), isolates recovered after each cross-contamination episode (Post), as well as negative (*Mycobacterium avium*) and positive (H37Rv) controls. Molecular sizes are in kilobase pairs.

It is very difficult to determine the precise financial impact of these false-positive cultures. We estimate that the cost of additional testing, treatment, physician consultation, public health follow-up, and laboratory time that resulted from our series of false-positive cultures was approximately $15,000.

DISCUSSION

The isolation of even a single positive culture for *M. tuberculosis* could be the basis of a presumptive diagnosis of tuberculosis. A false-positive culture may have profound consequences on the clinical management of the patient. In our experience, the impact of false-positive cultures was significant, even though it was mitigated by early recognition that these were probably the result of laboratory cross-contamination. Patients with false-positive cultures were nonetheless subjected to additional physician consultations, radiographic studies, and acquisition of samples for culture. Another disturbing consequence of these spurious results was the unnecessary administration of antimicrobial therapy to patients and contacts, resulting in adverse drug reactions in two patients who were unnecessarily treated. In some cases there was extensive public health follow-up of contacts.

Cross-contamination in the mycobacteriology laboratory has been previously demonstrated on the basis of unusual antimicrobial susceptibility patterns (1, 15), unique biochemical characteristics (20), and phage typing (11, 14). However, most strains of *M. tuberculosis* have the same biochemical and drug susceptibility phenotypes. The high incidence of multidrug-resistant strains in certain hospitals (19) also limits the utility of susceptibility profiles for identifying clusters on the basis of unusual antimicrobial susceptibility patterns. Phage typing has been used successfully (11) to confirm instances of laboratory cross-contamination by the Centers for Disease Control and Prevention (CDC), Atlanta, Ga. However, this service is no longer offered by the CDC.

In contrast to these strain-typing techniques, the RFLP method used in the present study can be used for all isolates of *M. tuberculosis*, has an excellent ability to discriminate between strains (25), and can be completed within days after mycobacterial colonies are present on the solid media. In the investigation reported here, RFLP fingerprint analysis of *M. tuberculosis* isolates provided persuasive additional evidence that two clusters of positive cultures were due to cross-contamination of the samples in the laboratory.

Outbreaks involving the transmission of tuberculosis have occurred in hospitals, prisons, and homeless shelters (6). In such a context, when true culture positivity rates are high, false-positive cultures also may have a higher probability of occurring, but they may be difficult to detect on the basis of either laboratory or clinical criteria. The possibility of transmission of a common strain or strains in such settings will reduce the power of RFLP or other strain-typing techniques to ascertain whether an outbreak of infections has occurred or whether positive cultures have resulted from cross-contamination in the laboratory. In such settings, the results of RFLP analysis must be interpreted in light of the relevant clinical and epidemiologic information.

Although the exact cause(s) of cross-contamination in our laboratory was not identified, investigation suggested that it resulted from the carryover of organisms from one sample to another during the decontamination procedure or during reading on the BACTEC system. False-positive results for mycobacteria have been reported to result from specimen mix-up (1), contamination of specimens or reagents with environmental mycobacteria in water (5, 8, 9, 23), or transfer of organisms from one sample to another. Most laboratories

process AFB cultures in the batch mode. Although specimens should be completely separated from one another, common containers for reagents or discarded materials are frequently used and have been implicated as vehicles for transfer of organisms from one sample to another (13, 14, 20).

Other laboratories have also described false-positive results associated with the use of the BACTEC 460 instrument. Because this instrument uses the same needle to sample multiple specimens, cross-contamination may be attributable to the transfer of inoculum from one culture to the next, perhaps because of the sporadic failure of the needle heater, circuit board, or other problems (4, 16, 26, 27). Although diagnostic tests performed on-site by the manufacturer did not reveal a malfunction, the BACTEC 460 instrument was returned for refurbishing after the second contamination episode and a device for periodic monitoring of needle temperature was provided.

In response to our assessment that cross-contamination may have occurred either during the initial decontamination process or during the repetitive sampling of sequential vials by the BACTEC 460 system, procedures for processing of specimens for mycobacterial culture were reviewed and modified in order to decrease opportunities for the transfer of organisms from one specimen to another. The following procedures may prevent cross-contamination among AFB cultures and enable recognition of false-positive cultures should they occur.

(Additional procedures are required to avoid specimen mix-up, false-positive AFB smears (5, 22), or contamination of specimens [9] or reagents [8, 23] with mycobacteria that are often found in water.)

Decontamination procedure. Isolate each specimen completely so that there are no opportunities to transfer an inoculum from one sample to another via pipets, the lips or caps of tubes, splashes, or common reservoirs of reagents or containers used for discarded materials.

(i) When using pipets to deliver reagents, use a separate pipet for each specimen *and* each time that the reagent bottle is entered.

(ii) Predivide into aliquots reagents such as phosphate buffer which are used in volumes that are awkward to transfer via a pipet into individual sterile tubes. Use a separate tube to add reagents to each sample rather than dispensing them from a common container.

(iii) Remove and replace the cap from each specimen tube sequentially during the addition of specimens and reagents, so that only one tube is open at a time and so that tube caps do not become interchanged.

(iv) After centrifugation, pour off the supernatant from each specimen into a separate disposable discard tube instead of using a common discard container.

(v) Ensure that written procedures for the processing of cultures include detailed instructions for problem-prone steps and that staff have a very good understanding of the rationale for all aspects of the procedure.

BACTEC cultures. Ensure that equipment is properly maintained and monitored, avoid contamination of vial tops, and isolate positive cultures from other samples.

(i) Adhere strictly to the manufacturer's recommendations for maintenance and quality control (18) which include the following: change needles daily (install the clean needle prior to running uninoculated vials to be gassed) (4); carefully inspect needles when cleaning and replace ones which appear dull, bent, or damaged; change the needle heater (82.5-s cycle) three times per year (individual laboratories may be advised to do this more often); immediately after inoculation, decontaminate vial diaphragms with appropriate disinfectant and then cleanse with alcohol; to avoid contamination of the diaphragm, do not tilt or invert inoculated vials unnecessarily; and test cultures at the recommended frequency and do not test AFB-positive 12B vials if the GI exceeds 500 or the 13A vials if the GI exceeds 20, because of an increased potential for carryover to adjacent vials (individual laboratories may consider lowering these GI thresholds); if the GI is $\geq$500 in a drug-containing vial during susceptibility testing, the organism should be considered resistant and testing of that vial need not continue.

(ii) When disinfecting the tops of vials prior to inoculation or prior to making each reading on the BACTEC instrument, use a separate pledget or swab for each vial.

(iii) Prior to each run, visually check the needle to ensure that it is heating, because heating block malfunction or circuit board failure may occur in the absence of a warning signal (4, 16, 26). The efficiency of the needle heater may be monitored periodically with a temperature probe.

(iv) If vials are incubated in racks, rotate the vials approximately one-quarter turn before each run to avoid damaging the diaphragm by puncturing the same area too often (4).

(v) Read AFB smears prior to running newly inoculated vials on the BACTEC system so that samples with a positive smear may be tested separately.

(vi) Separate specimens that are likely to be positive (e.g., those with a positive smear, those exceeding your laboratory's GI threshold, those from patients with previous positive smears or cultures) from those that are likely to be negative. Isolate these cultures from one another with an intervening uninoculated vial. Ensure that intervening negative vials are incubated for an adequate amount of time to detect contamination. (Use of intervening negative vials between testing of specimens from patients positive for *M. tuberculosis* is particularly recommended for susceptibility testing on possible drug-resistant isolates because of the increased potential for carryover when vials with high numbers of organisms are repeatedly sampled.)

(vii) Sample vials in order of increasing probability of being positive, as follows (4, 26): uninoculated vials to be gassed, negative patient vials, vials exceeding the laboratory's GI threshold, and those with a positive smear or those being incubated for NAP (*p*-nitro-α-acetylamino-β-hydroxypropiophenone) susceptibilities, or other testing.

Recognition of potential false-positive cultures. Establish procedures which enable early recognition of potentially false-positive cultures and policies which encourage communication with physicians and public health authorities.

(i) Process and read cultures in a consistent and orderly manner. Record the relevant information, including the date of processing and the location of the vial, on a log sheet of positive cultures. This should enable routine notice of the location of positive cultures with respect to one another. Investigate possible contamination when specimens in proximity to one another become positive. Cross-contamination may not occur with all samples in sequence (4, 16), such that negative cultures occasionally may be found between positive cultures.

(ii) Read cultures on the BACTEC instrument in the reverse order in which they were processed. This procedure may help in localizing the source of problems if the true-positive culture suspected of being the source of cross-contamination is known.

(iii) Track positivity rates prospectively and establish thresholds which provoke investigation when these thresh-

olds are exceeded (for example, when the number of positive cultures exceeds the usual positivity rates or when isolates that are of an infrequently encountered species or that have an unusual resistance pattern are recovered sequentially or more frequently than usual).

(iv) Communicate with physicians and public health authorities when laboratory evidence of false-positive cultures is found and encourage them to notify the laboratory of results which appear to be inconsistent with clinical findings.

(v) If epidemiologic information demonstrates that it is unlikely that patients could be infected by the same strain, suspected false-positive as well as control isolates may be submitted to reference laboratories (through the CDC) for strain typing.

These procedures are intended to allow for the isolation of specimens so that there is no opportunity to transfer an inoculum between samples and to ensure that equipment is properly maintained and monitored. The techniques used by individuals who process specimens also may affect contamination rates (1). Therefore, it is crucial that staff be well trained in these procedures and have a thorough understanding of the rationale for all procedural steps. Since the procedures described above were adopted and the BACTEC instrument was refurbished, no instances of cross-contamination have been identified in our laboratory during the subsequent 15 months. The marginal cost of implementing these additional measures resulted in an increase of approximately $0.75 for supplies and 1 to 3 min of technician time for each AFB culture done in our laboratory. This cost is minor compared with the resources that may be consumed as a result of false-positive cultures.

This report demonstrates the utility of a rapid molecular strain-typing method for *M. tuberculosis* for identifying problematic laboratory procedures and altering clinical care. There is no indication for fingerprinting of strains of *M. tuberculosis* or other mycobacteria on a routine basis; however, the ultimate role of such procedures in clinical laboratory practice for quality control purposes remains to be determined. Currently, strain-typing analysis is expensive and time-consuming and can be performed only by CDC and a few reference laboratories. However, the benefits of rapidly recognizing false-positive cultures of *M. tuberculosis* may justify the use of strain typing in selected situations.

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