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Rapid Identification of Laboratory Contamination with *Mycobacterium tuberculosis* Using Variable Number Tandem Repeat Analysis

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Compared with solid media, broth-based mycobacterial culture systems have increased sensitivity but also have higher false-positive rates due to cross-contamination. Systematic strain typing is rarely undertaken because the techniques are technically demanding and the data are difficult to organize. Variable number tandem repeat (VNTR) analysis by PCR is rapid and reproducible. The digital profile is easily manipulated in a database. We undertook a retrospective study of *Mycobacterium tuberculosis* isolates collected over an 18-month period following the introduction of the BACTEC MGIT 960 system. VNTR allele profiles were determined with early positive broth cultures and entered into a database with the specimen processing date and other specimen data. We found 36 distinct VNTR profiles in cultures from 144 patients. Three common VNTR profiles accounted for 45% of true-positive cases. By combining VNTR results with specimen data, we identified nine cross-contamination incidents, six of which were previously unsuspected. These nine incidents resulted in 34 false-positive cultures for 29 patients. False-positive cultures were identified for three patients who had previously been culture positive for tuberculosis and were receiving treatment. Identification of cross-contamination incidents requires careful documentation of specimen data and good communication between clinical and laboratory staff. Automated broth culture systems should be supplemented with molecular analysis to identify cross-contamination events. VNTR analysis is reproducible and provides timely results when applied to early positive broth cultures. This method should ensure that patients are not placed on unnecessary tuberculosis therapy or that cases are not falsely identified as treatment failures. In addition, areas where existing procedures may be improved can be identified.

Broth-based, automated methods for isolating mycobacteria from clinical specimens have significantly increased the sensitivity of and reduced the time to growth detection compared with solid media (7, 11, 16). However, a perennial cause for concern is the occurrence of false-positive cultures of *Mycobacterium tuberculosis* resulting from contamination within the laboratory, which can lead to serious adverse effects on patient management (2, 3, 12, 15). In population-based prospective studies, rates of false-positive cultures have been reported to affect between 0.33 and 4% of culture-positive patients (1, 2, 3, 5, 14, 18). Higher levels of false-positive results have been associated with the use of broth-based systems (5, 10, 15); in one study with an automated system, 6% of all culture-positive isolates were found to be false positive (10).

Typing isolates of *M. tuberculosis* can be both technically demanding and time-consuming and is often available only at specialist centers. Molecular typing methods for the differentiation of strains of *M. tuberculosis* can be divided into those which examine genomic DNA with probes (restriction fragment length polymorphism [RFLP] analysis) and those based on PCR. RFLP analysis is the internationally accepted method of typing *M. tuberculosis*; in this analysis, a Southern blot of restriction endonuclease-digested genomic DNA is hybridized with a molecular probe for IS*6110* (17). Other molecular probes can be used where IS*6110* has limited discrimination (9). Theoretically, methods involving PCR are more convenient, as they are rapid and require lower concentrations of DNA than methods based on RFLPs. However, a recent study has shown that many PCR-based methods have limited reproducibility and are unsuitable for typing strains (9). Of the PCR-based methods examined in this study, only mixed-linker PCR, spoligotyping, and variable number tandem repeat (VNTR) typing were found to be highly reproducible and to retain an acceptable level of discrimination (9).

Unlike mixed-linker PCR and spoligotyping, PCR-based VNTR analysis does not require molecular manipulation techniques in addition to PCR. In VNTR analysis, specific genomic regions containing VNTRs are amplified. The length of amplified DNA is dependent on the number of repeat sequences within the target. Five different loci containing VNTR sequences are amplified, the number of repeats is calculated for each locus, and a digital profile is generated. PCR-based VNTR analysis has been shown to be highly reproducible, and the data generated remain constant over time. The digital profile does not require specialist software for analysis of data and can be stored on a simple spreadsheet or database (6). Studies have shown that VNTR analysis is highly discriminatory, although less so than IS*6110*-RFLP analysis or mixed-linker PCR when the IS*6110* element is present in multiple

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TABLE 1. Primers used for VNTR analysis (6)

Locus	Oligonucleotide sequences
ETR-A	5′-AAA TCG GTC CCA TCA CCT TCT TAT-3′
	5′-CGA AGC CTG GGG TGC CCG CGA TTT-3′
ETR-B	5′-CGC GAA CAC CAG GAC AGC ATC ATG-3′
	5′-CGG TGA TCG AGT GGC TAT ACG CTC-3′
ETR-C	5′-GTG AGT CGC TGC AGA ACC TGC AG-3′
	5′-GGC GTC TTG ACC TCC ACG AGT G-3′
ETR-D	5′-CTC GGG TTG GCC ATC GTC AAA CAC-3′
	5′-CCG GAC CAT CGG TCA CCA CCG TGG-3′
ETR-E	5′-CAT GCC AGG GTT GGC GCG GCC GGT-3′
	5′-TGG CAT GCC GAT CCT GGT GTT CAA-3′

copies (6, 9). However, when few copies of IS*6110* are present, VNTR analysis is more discriminatory than IS*6110*-RFLP analysis (6, 9).

In order to accurately quantify the problem of contamination in the current generation of automated tuberculosis detection systems, we have undertaken a study of isolates of *M. tuberculosis* cultured over an 18-month period in a diagnostic laboratory serving three large hospitals.

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MATERIALS AND METHODS

Clinical specimens. A total of 7,418 clinical specimens were received for processing by the mycobacteriology laboratory at the Leeds General Infirmary between May 1998 and October 1999. These specimens included 4,571 specimens of sputa, bronchial lavage, and other secretions and 1,031 urine specimens. A total of 507 isolates of mycobacteria were recovered; 397 (78%) of these were identified as *M. tuberculosis*. The laboratory serves three large hospitals (Bradford Royal Infirmary, St. Lukes Hospital, Bradford and Leeds General Infirmary), a specialist chest clinic, and primary care centers in the surrounding areas (combined population, approximately 920,000). One of the hospitals serves a large immigrant community from the Indian subcontinent with a high incidence of tuberculosis.

Culturing of specimens. All specimens, with the exception of fluids from normally sterile sites, were decontaminated with the addition of an equal volume of 4% (vol/vol) sodium hydroxide. After 15 min, the specimens were diluted with phosphate-buffered saline to a volume of 20 ml and centrifuged. An aliquot of 19 ml of the supernatant was discarded, and the pellet was resuspended in the remaining supernatant.

All specimens were cultured using the BACTEC MGIT 960 system (Becton Dickinson Microbiology Systems, Oxford, United Kingdom) according to the manufacturer's instructions. Briefly, 0.5 ml of a specimen was added to an MGIT tube containing 7 ml of broth and 0.8 ml of PANTA solution (both supplied by Becton Dickinson). The tube was placed in the predetermined space in the incubator. All specimens were left in the incubator until a positive signal was detected or for 6 weeks if no signal was produced.

In addition, auramine smears were made for all specimens except urine specimens. The deposit from decontaminated specimens was inoculated onto a Lowenstein-Jensen (LJ) slope using a sterile swab. Fluid specimens from normally sterile sites were inoculated onto LJ slopes using a 10-μl bacteriological loop. Slopes were incubated at 37°C for 8 weeks and checked weekly for growth.

Preparation of extracts for PCR. Broths with a positive growth index were examined for the presence of acid-fast bacilli (AFB). Broths positive for AFB that were cultured from specimens either from new patients or from different body sites were used for PCR analysis. Samples of 0.5 ml of culture were removed and placed in sterile 1.5-ml microcentrifuge tubes. The cells were collected by centrifugation, the broth was removed, and 200 μl of InstaGene matrix (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) was added to the pellet. The mixture was incubated at 60°C for 15 min and then transferred to a boiling water bath for 30 min. After brief centrifugation, the supernatant was diluted 1:100 prior to use as a template for PCR. Neat extracts were stored at −70°C for future reference.

PCR-based VNTR. Extracts from broths positively identified as containing *M. tuberculosis* using PCR for the detection of IS*6110* (8) were used as templates for VNTR analysis as described by Frothingham and Meeker-O'Connell (6). Briefly, five PCRs were set up, one for each locus reported to carry variable numbers of exact tandem repeat (ETR) sequences (ETR-A, ETR-B, ETR-C, ETR-D, and ETR-E). The primers are listed in Table 1. A 25-μl PCR mixture contained, in GeneAmp amplification buffer (PE Applied Biosystems, Foster City, Calif.), 25 pmol of forward and reverse VNTR primers; 4% (vol/vol) dimethyl sulfoxide; 1.5 mM magnesium chloride; 0.2 mM each dATP, dCTP, dGTP, and dTTP; and 0.2 U of AmpliTaq Gold (PE Applied Biosystems). The thermocycling reactions were carried out using an Omnigene thermocycler (Hybaid, Teddington, United Kingdom). Following 12 min of activation of AmpliTaq Gold at 95°C, 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C were followed by a final extension for 10 min at 72°C. The amplimers were separated on a 2% MetaPhor agarose gel (Flowgen, Lichfield, United Kingdom) in Tris-borate-EDTA and visualized with ethidium bromide staining. The number of repeats at each locus was calculated from the size of the amplimer generated as described previously (6). The five-digit number generated was entered into an Access database, version 2.0 (Microsoft Corporation), together with specimen details, which included the data on which the specimen was received and processed in the laboratory. The data were sorted according to profile and specimen identification number.

RESULTS

A total of 397 cultures of *M. tuberculosis* were recovered from 243 patients from May 1998 to October 1999. Of these, a total of 155 cultures from 144 patients were typed using VNTR analysis. These specimens exhibited 36 different profile types, of which 18 were unique to a single patient. Also encountered were a further two sets of profiles which were common only to two epidemiologically linked patients (i.e., father and son and nurse and patient). Four profiles were common to more than 10 patients (Table 2). When positive cultures were obtained from multiple body sites, the profiles of the different cultures were identical.

TABLE 2. Distribution of common VNTR profiles among patients and VNTR profiles associated with false-positive cultures in this study

Profile[a]	No. of:			Source included[d]	Adjusted no. of patients[e]	% Distribution
	Cultures[b]	Patients	False positives[b,c]			
02235	18	16			16	13.6
12533	6	6	5	No	**2**	1.7
22433	9	8			8	6.8
22532	3	3	3	No	**1**	0.8
32333	6	6			6	5.1
32433	16	15	1	Yes	14	11.9
32533	6	6	5	Yes	1	0.8
42234	12	12	4	Yes	8	6.8
42235	25	23			23	19.5
42435	7	6			6	5.1
42453	4	4	3	Yes	1	0.8
74265	9	9	7	Yes	2	1.7
Unique	18	18			18	15.2
Uncommon[f]	16	12			12	10.2
Total	155	144	28		118	

[a] Each digit represents the number of exact tandem repeats at each locus. A zero indicates deletion of the ETR-A locus.
[b] Number of cultures which were typed by VNTR. Not all cultures were available for analysis.
[c] Number of patients with presumptive-false positive cultures.
[d] Indication of whether VNTR data were available for the presumed source of contamination.
[e] Number of patients after false-positive data were excluded but with extrapolated source data included (latter data in boldface).
[f] VNTR profiles associated with two patients.

TABLE 3. Retrospective data collected for culture-positive specimens in which contamination occurred during processing on a particular day[a]

Specimen position[b]	VNTR profile[c]	AFB seen[d]	Time to positive MGIT result (wk)[e]	Patient
2	02235	sc	1	r
12	NA	+++	1	s
13	12533[f]	No	2	t
14	12533	No	2	u
16	NA	No	2	u
17	12533	No	2	v
20	NA	No	4	w
22	12533	No	6	x
24	32234	sc	2	y
32	12533	No	4	z

[a] The specimens processed had sequential specimen identification numbers and were processed in the same order. A total of 34 specimens were processed on this day.
[b] Processing position relative to the source specimen.
[c] Each digit represents the number of exact tandem repeats present at each locus. NA, specimens were not available for VNTR analysis.
[d] Seen on auramine staining. sc, scanty AFB seen; +++, presence of many AFB.
[e] Number of weeks between inoculation and positive MGIT result.
[f] The VNTR profile 12533 was found only on one other occasion in this study.

The specimens were sorted numerically and compared with the VNTR profile and the date on which they were processed. An example of the data generated is shown in Table 3. Nine potential incidents of cross-contamination were identified when two or more specimens were processed on the same day and had the same profile. Only three of these incidents were suspected when the results were reported to the clinicians. Incidents of cross-contamination involved up to nine false-positive cultures. A summary of all suspected incidents, together with the associated laboratory activity and culture results, is presented in Table 4. The presumptive false-positive cultures did not necessarily have sequential numbers. Typically, the false-positive cultures and the source specimens were separated by one or two negative cultures. When large numbers of specimens were involved, the negative cultures between false-positive specimens increased in number the further they were away from the source (Table 3). In all potentially false-positive MGIT tubes, there was no growth on the matched LJ slopes.

In the first incident, suspected at about the time of isolation (incident C), cross-contamination of five cultures was suspected due to the large number of culture-positive, smear-negative specimens which followed a smear-positive, culture-positive sputum specimen. Clinically, patients were not thought to have tuberculosis. Following retrospective molecular analysis, a further two cultures processed on the following day were also shown to be implicated. All seven cultures had identical VNTR profiles, a profile which was observed on only one other occasion, 14 months after the original incident. Two additional positive cultures were also isolated during this period. These were duplicates of specimens from two of the other false-positive specimens and were not available for typing. PCR fingerprinting undertaken on crude extracts at the time of the suspected incident using the method of Ross and Dwyer (13) revealed similar fingerprints for the suspected implicated cultures (data not shown). In this incident, the identification number of the last false-positive specimen was 31 specimens after the source.

The second incident involved two specimens processed sequentially (incident D). Both had identical VNTR profiles and were indistinguishable using the PCR fingerprinting method of Ross and Dwyer (13). The first specimen was a smear-positive sputum specimen, and the second specimen was a smear-negative aspirate from an ileopsoas mass. The second patient had neither clinical nor radiological evidence of tuberculosis, and histologic analysis showed evidence of metastatic carcinoma. No granulomas were seen. A second possible incident (incident E), involving the same smear-positive patient, occurred 5 days later. Two further specimens obtained from two patients and processed sequentially from the presumptive source were culture positive and had VNTR profiles identical to those of the presumptive source. This profile was not associated with any other patient in this study. This second incident was not identified at the time of isolation because both of the patients involved had been culture positive for *M. tuberculosis* on other occasions. Unfortunately, none of these other cultures was available for typing.

The third incident (incident F) was suspected when two cases were unexpectedly culture positive. However, the source specimen was not identified at the time. This incident involved three further false-positive specimens, including the detection of *M. tuberculosis* DNA in a culture of *M. avium*. The VNTR profile of the *M. tuberculosis* DNA from this mixed culture was identical to that of the other cultures of *M. tuberculosis* in the batch.

Examination of the typing data revealed a further four incidents where specimens processed on the same day all had identical VNTR patterns. One of these incidents had a profile which was not seen at any other time (incident A), one incident had a profile common to one other patient (incident B), and three incidents involved two profiles common to 8 and 13 other patients (incidents G, H, and I).

DISCUSSION

Automated broth-based culture systems for *M. tuberculosis* are more rapid and sensitive than traditional methods (3, 10). The level of cross-contamination of cultures in our laboratory was higher than expected. Records suggested that there were 34 potential false-positive cultures out of 397 *M. tuberculosis* isolates processed over the 18-month study (8.6%). The MGIT

TABLE 4. Summary of incidents involving cross-contamination

Incident	No. of:			VNTR profile of potential contaminants
	Specimens processed[a]	Positive cultures (patients[b])	False-positive cultures (patients[c])	
A	19	4 (4)	3 (3)	22532
B	34	10 (9)	7 (6)	12533
C	48[d]	11 (8)	9 (7)	74265
D	12	2 (2)	1 (1)	42453
E	16	3 (3)	2 (2)	42453
F	30	9 (7)	5 (5)	32533
G	34	5 (3)	1 (1)	42234
H	21	8 (5)	4 (3)	42234
I	33	5 (2)	2 (1)	32433

[a] During a contamination episode.
[b] Number of patients with positive cultures.
[c] Number of patients with false-positive cultures.
[d] Specimens were processed over two consecutive days.

960 system was introduced into our laboratory in April 1998, and it is not surprising that six of the nine incidents occurred within the first 5 months of this study (incidents A to F).

All of the specimens were inoculated into MGIT broths and onto LJ slopes. However, none of the 34 potentially false-positive specimens was detected on the LJ slopes. This result may have been due to a variety of reasons. A smaller sample volume was inoculated onto the LJ slopes than into the MGIT broths. In addition, the time to detection in broth-based systems is much faster than that on solid media (7, 11, 16). In this study, LJ slopes were incubated and examined for 8 weeks, and it is conceivable that there was insufficient time to detect very small numbers of mycobacteria within this time. Finally, the MGIT system is designed to detect different species of mycobacteria, including species which may be considered more fastidious than *M. tuberculosis* (7, 11, 16). The rich medium used may enhance the recovery of mycobacteria that may have become damaged during transit or decontamination procedures.

The identification of potential cross-contamination by laboratory staff was confounded by the fact that groups of negative isolates were found between the positive isolates. The incidents that were noticed at the time of isolation involved patients from one particular center, where good communication between laboratory and clinic personnel existed. False-positive specimens from patients from one center with a higher-than-average incidence of tuberculosis were not detected. Retrospectively, examination of the clinical notes for patients with suspected false-positive results suggested that the results were inconsistent with the diagnosis in all but one case (incident E; discussed above).

In general, the application of molecular typing methods to cultures in a diagnostic laboratory has many problems. Methods based on RFLPs, such as the standardized RFLP method, which uses IS*6110* as a probe, require the extraction of high-quality genomic DNA at a high concentration. Screening should be secondary to the diagnostic test and ideally should be simple to perform and should generate data that are easy to interpret. Fingerprinting methods based on PCR, while using small amounts of DNA, tend not to be reproducible even within a batch, and so the results are difficult to interpret. Results from protocols requiring band matching are difficult to store for archival purposes and may require suspected similar isolates to be analyzed at the same time. Crude extracts from cultures degrade relatively quickly, reducing further the reproducibility between batches. The amplification of specific loci, as in VNTR analysis, ensures reproducibility within and between batches, as there is no variation in the ability of the primers to anneal to the genomic DNA and so the concentration of DNA in each reaction is less significant. In a recent study to determine the reproducibility of the VNTR method, a set of 20 coded samples of ethanol-treated *M. tuberculosis* was sent from Durham VA Medical Center to the laboratory at Leeds for VNTR analysis. The digital profiles for all of the samples matched exactly those of the referring laboratory, i.e., there was 100% reproducibility for all 100 alleles examined (Frothingham, R., P. L. Strickland, K. A. Davis, A. J. Cobb, D. M. Gascoyne-Binzi, C. Sola, M. A. Behr, and K. Kremer. Abstr. Tuberc. Past Present Future, abstr. 170, p. 75, 2000).

When VNTR analysis was applied to isolates in this study, a wide variety of profiles were generated. This study did not look further into the relationships between strains with identical VNTR profiles. However, VNTR analysis did prove to be useful in screening isolates for potential relatedness. Although some profiles were identified which were common to several patients, it was considered unlikely that the same profile would occur in specimens processed from multiple patients on the same day. The three most common VNTR profiles accounted for cultures from 45% of the patients in this study. Previous studies comparing VNTR analysis with IS*6110* RFLP have shown that when IS*6110* is present in high copy numbers, isolates sharing VNTR profiles might be subdivided by a second typing method (9). In this study, suspected cases of false-positive cultures identified from the VNTR data were confirmed by the lack of clinical evidence of tuberculosis or multiple other culture-negative specimens. Only two of the nine incidents of potential cross-contamination involved common profile types.

The most common cause of laboratory contamination occurs during the initial processing of the specimen (3). Reported causes of contamination include contamination of multiple-use equipment for dispensing reagents, aerosols, splashing, sampling equipment, reprocessing of contaminated specimens, and mislabeling (3, 4, 10, 12, 15, 18). In all incidents identified in this study, it is likely that contamination occurred during initial processing. All sampling of broths with positive growth indicators was performed in a cabinet separate from that used to process the specimens, and specimens were not reprocessed at any time. Following a suggestion that the probable cause of contamination was splashing of equipment or reagents by a positive specimen, an experimental incident was constructed. The neck of a reagent bottle containing water for neutralization was contaminated with a smear-positive specimen, and water was dispensed into sequential MGIT culture tubes. Similarly, the barrel of a pipettor used for adding the processed sample to the broth was experimentally contaminated with a smear-positive specimen and then was used to dispense sterile water into the broth in place of the specimen. Both sets of cultures produced positive results for the tube from which the initial contamination occurred. In the case of the water bottle, the individual cultures became positive in the same order in which they had been contaminated. However, in the case of the contaminated pipettor, the tubes became positive over time in a more random manner, reflecting the pattern of positive tubes seen in our study. Despite previous observations of contamination due to unplugged pipettes (4), there has not been a recommendation to use aerosol-resistant tips for processing of specimens.

In our study, cultures from patients who were previously culture positive for tuberculosis were not sent for further molecular investigations; therefore, the culture from the potential source was not always available for analysis. However, by grouping identical profiles together and identifying other positive cultures processed on the same day, we easily identified potential cross-contamination incidents. When the data were collated, the suspected source specimens were smear positive and showed positive growth within 1 week of incubation. The contaminated cultures became positive between 1 and 5 weeks later, an average incubation time of 3 weeks. The time to positivity increased when false-positive specimens were processed further from the source, as illustrated in Table 3. This

result corresponds to observations in previous studies (1). It was also noted that on five occasions, duplicate specimens of smear-negative sputa had been received for processing in the same batch. In each case, both specimens were contaminated, and so two positive results were returned. Ideally, multiple specimens should be processed in separate batches.

All patients from one center were treated for *M. tuberculosis* infection, despite the fact that the laboratory reports conflicted with the clinical information available at the time. One patient had a urine specimen sent to the laboratory while attending a clinic for a routine checkup. The patient had previously been treated for tuberculosis but was well at the time and had been compliant with her treatment. A further seven specimens were sent for culturing; all showed no growth.

This study was undertaken to determine the false-positive rate in our laboratory and the feasibility of VNTR analysis for its detection. Further study is needed to determine the cost of real-time VNTR analysis in a clinical microbiology laboratory and the cost savings achievable by rapid identification of false-positive results. The reagents and consumable agents required to carry out both PCR identification and VNTR analysis in our laboratory cost approximately $7 (U.S. dollars) per positive MGIT broth. The real costs will be much higher due to labor, PCR licensing, proficiency testing, and other overhead factors. Real-time identification of false-positive cultures will reduce costs by eliminating unnecessary treatment, contact tracing, and further laboratory investigations.

This study was carried out retrospectively, and not all cultures were available for VNTR analysis. However, sufficient data were generated such that patients who potentially had false-positive cultures reported could be identified and the cases could be discussed with the clinicians. Not all positive MGIT broths require VNTR analysis to identify incidents of cross-contamination. Positive broths from all new cases should be analyzed, and the processing dates, identification numbers, and time to positivity of all culture-positive specimens should be documented. In addition, VNTR analysis should also be carried out on positive broths processed close together, even though a patient has previously been culture positive. This practice will alert both laboratories and clinicians to the possibility of false-positive results for this category of patients.

In conclusion, the use of automated broth culture systems should be supplemented with molecular typing of positive broths to identify incidents of cross-contamination. The monitoring of the occurrence of such incidents in the laboratory ensures that careful techniques are used in the laboratory and highlights procedures where problems of cross-contamination are likely to occur. Observation by laboratory staff alone to identify cases of cross-contamination is unreliable. Careful documentation of positive specimens, together with close liaison between clinical and laboratory staff involved in culturing and molecular characterization, is necessary to identify such incidents effectively. Profiling of isolates using VNTR analysis is reproducible and quick, and the results are easy to store and analyze. No specialized equipment other than a thermocycler is required, and sophisticated data-recording facilities are unnecessary. The typing results can be obtained before confirmation of identity and sensitivity results are available. The rapid availability of results will have a significant clinical impact in that patients not clinically thought to have tuberculosis need not be started on treatment. False-positive cultures usually take longer to become positive than source specimens; thus, the VNTR patterns from suspected source specimens should be known before those of possible false-positive specimens. A knowledge of the VNTR profiles of strains present in the community will also aid in the identification of false-positive cultures.

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