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Performance of an Automated Q-Beta Replicase Amplification Assay for *Mycobacterium tuberculosis* in a Clinical Trial

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We present data from a clinical trial study in which an automated version (Galileo) of a previously described Q-Beta replicase-amplified probe assay (J. S. Shah et al., J. Clin. Microbiol. 33:1435–1441, 1995) was used for the direct detection of *Mycobacterium tuberculosis* complex in sputum. The assay was designed to target specific regions of 23S rRNA found in *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, and *Mycobacterium microti* and had a sensitivity ranging from approximately <10 to 300 CFU. The assay was tested for cross-hybridization by using large numbers (e.g., 10^5 to 10^{10} CFU/assay) of 133 other organisms commonly found in respiratory tract samples, including non-*M. tuberculosis Mycobacterium* spp., other bacteria, fungi, and viruses. All of these competitors tested negative by the assay. Automated assay results for 780 respiratory tract samples (sputum or bronchoalveolar lavage specimens) collected and tested at three trial sites in the United States) were compared with the results of culture and acid-fast microscopy. Aliquots of conventionally digested and decontaminated sputum pellets were heated at 100°C and mechanically disrupted prior to hybridization and background reduction, amplification, and detection in a closed disposable test pack. Pertinent elements of individual patient histories relating to tuberculosis exposure, previous active disease, antituberculosis therapy status, etc., were considered in the resolution of discrepant results for 48 (assay false-positive) samples. Seventy-one of 90 (78.9%) culture-positive samples were positive when tested in the Galileo assay, while 7% of culture-negative samples were assay positive, corresponding to a sensitivity of 79% and a specificity of 93%. Following resolution of discrepant results by chart review, the sensitivity and specificity for the Q-Beta replicase amplification assay with the Galileo analyzer were 84 and 97%, respectively. A total of 69.2% of smear-negative (culture positive) samples were detected by the assay. Ten test packs at a time were automatically processed by the Galileo analyzer without operator intervention following loading of samples. The first result was reported in approximately 3 h, and the last result was available in 6.5 h. To our knowledge, this is the first report of a clinical study with a fully automated amplification probe hybridization assay for the detection of pathogens directly from a clinical specimen.

The recent worldwide increase in the occurrence of tuberculosis (7, 21) and the appearance of multidrug-resistant strains have heightened interest in rapid, sensitive methods for the identification of *Mycobacterium tuberculosis*. Simplification of procedures and automation of testing would be particularly desirable in the mycobacteriology laboratory. Potential benefits of such new methods include enhancement of overall patient care, more efficient use of often limited isolation facilities, improved and faster intervention to control the spread of the disease, streamlined laboratory work flow, and net reduction of health care costs at several levels (26).

The "gold standard" and most extensively used methods for the detection of *M. tuberculosis* continue to be the relatively sensitive (but slow) culture methods, requiring up to 6 to 8 weeks for culture on solid media and up to 2 to 3 weeks for culture in liquid media. For some time, nucleic acid probe tests have been used for the definitive identification of several mycobacterial species following culture (13, 33), and amplification methods have recently been developed for testing for pathogens directly from a clinical specimen. The PCR for the detection of *M. tuberculosis* has been directed at both genomic DNA (46) and species-specific regions of 16S rRNA (Roche Amplicor) (13, 14, 34). Recently, several investigators have evaluated the Mycobacterium Tuberculosis Direct test (MTD; Gen-Probe, Inc., San Diego, Calif.), which couples transcription-mediated amplification to Accuprobe detection technology (8, 22, 23, 35). The U.S. Food and Drug Administration (FDA) has recently approved both Amplicor and MTD for testing of smear-positive specimens. Both Amplicor and MTD use several manipulations of sample and precision transfer of microliter volumes of reagents, which may contribute to human error or cross-contamination of samples. Additionally, because these amplification techniques use enzymatic steps early in the procedures, they may also be susceptible to inhibition by constituents found in some samples, resulting in false-negative results (2, 12).

We have developed a rapid nucleic acid probe assay for *M. tuberculosis* directed at 23S rRNA. This test is based on reversible target capture (RTC) technology (32) and the Q-Beta replicase amplification and detection system (37). RTC is used to impart high specificity in capturing the target from the sample and simultaneously removing potential inhibitory clinical matrix components prior to amplification and detection. Capture and detector probe pairs can be designed and hybridization and elution conditions can be manipulated to provide the desired specificity for each target organism or organism group. We have previously described RTC and basic Q-Beta replicase amplification for several infectious disease agents (see the references cited in references 2, 9, 26, 32, 37, 38, 40,

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44, and 51). A prototype instrument platform (Galileo) has been developed to totally automate all RTC and amplification and detection steps inside a closed disposable test pack requiring minimal operator intervention or specialized training (30, 40). Following sample processing, all assay steps were performed in the disposable test pack, which was designed to limit contamination from replicable RNA, to provide convenient packaging to store all reagents, and to control potential biohazards. We used a simple sample processing protocol to concentrate and release RNA targets from *M. tuberculosis* cells in sputum after a standard sodium *N*-acetyl L-cysteine (Nalc)–NaOH (Nalc-NaOH) digestion and decontamination step. Up to 10 samples were processed per run in the Galileo analyzer. The time to obtain the first result was approximately 3 h, and the last result was reported in 6.5 h. Results were reported as positive or negative for the presence of *M. tuberculosis* complex organisms.

Here we present data from three geographically distinct U.S. trial sites at which the automated assay was compared to acid-fast staining and culture for the direct detection of *M. tuberculosis* in respiratory tract samples submitted for mycobacterial culture.

MATERIALS AND METHODS

Reagents. Oligo$(dT)_{14}$- and oligo$(dC)_{25}$-derivatized magnetic particles, capture probes, and RNA detector probes were prepared as described elsewhere (38–40). All reagents for testing a single sample (RTC, amplification, and detection) were packaged in a closed disposable Surlyn test pack, labeled in bar code form to include information concerning reagent performance characteristics (40).

Mycobacteria and competitor microorganisms. Nine clinical isolates of *M. tuberculosis* were characterized by restriction fragment length polymorphism analysis to confirm their clonal nature (28); these strains were quantified and kindly supplied by B. Kreisworth, Public Health Research Institute, New York, N.Y. Other bacterial strains were obtained from the American Type Culture Collection (ATCC; Rockville, Md.) and were grown by standard microbiological procedures. The viruses used in this study included adenovirus type 5, human cytomegalovirus strain AD 169, echovirus type 11, herpes simplex virus type 1 (MacIntyre strain), rhinovirus type 2, and respiratory syncytial virus (Long strain). Viruses were supplied as high-titer (6.3×10^8 to 1.5×10^{10} particles/μl) quantitated stocks by Advanced Biotechnologies, Inc., Columbia, Md. *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Mycoplasma pneumoniae* were obtained from ATCC and were cultivated by K. D. Stottmeier, Inc., Norwood, Mass.

Sputum samples and sample processing. Fresh sputum or bronchoalveolar lavage samples submitted for mycobacteriologic testing were included in this study. Other than being appropriate for diagnostic workup for pulmonary mycobacterial disease as defined by existing protocols at the individual test sites, specific patients or patient groups were not selected to provide samples for this study. Samples were excluded from the study when volumes were inadequate for the sample to be tested by all tests or when complete patient information was not available. Data for 780 samples are included in this report. Samples were processed at the test sites immediately upon receipt or were stored for up to 48 h at 2 to 8°C, and were then digested and decontaminated by standard Nalc-NaOH procedures. Concentrated sediments were then split by using one aliquot for standard culture and the second aliquot for acid-fast microscopy and subsequent testing by Q-Beta replicase amplification. After sampling for acid-fast microscopy, this second aliquot was heated at 100°C for 15 min and was then mechanically disrupted in a proprietary sample processing device. These steps took approximately 21 min and are described elsewhere (39, 40, 51). Previously cultured (negative) sputum aliquots were treated as described above and were then spiked with serial dilutions of *M. tuberculosis* cells for use in establishing the sensitivity of the assay.

Five hundred-microliter volumes of processed clinical samples, spiked sputum, positive or negative controls, or suspensions of organisms for competitor studies were added to totally closed disposable test packs for processing in the Galileo instrument. All hybridization, background reduction, and amplification steps were done automatically in the Galileo instrument without operator intervention (30). The temperature for all operations within the Galileo instrument was controlled at 38°C. The prototype instruments were manufactured by Wilj Division, Integrated Technologies, Ltd., Ashford, United Kingdom. The Galileo instrument platform is described in greater detail elsewhere (30, 40).

Amplification data analysis. The Galileo instrument processed each test pack through four rounds of RTC, after which the probe amplification reaction was initiated by the addition of Q-Beta replicase enzyme and nucleoside triphosphates in an appropriate reaction mixture containing propidium iodide (40). After the time and relative fluorescence for the amplification cycle were measured, they were analyzed with a curve-fitting software algorithm to calculate the slope of the dye-binding curve and the response time (RT) (9). Slope value limits were statistically determined and aided in the identification of contaminating RNAs (9), which were infrequently observed in the Q-Beta replicase amplification reactions. If there was no amplification of detector probe as indicated by no measurable increase in fluorescence, the result was reported as negative. If there was a detectable increase in fluorescence, the slope of the dye binding curve (i.e., the rate of fluorescence increase) was evaluated. If the slope was >160 or <45 relative fluorescence units/min, the result was reported as fail. Finally, if slope values were between 45 and 160, RTs were calculated and examined as part of the software algorithm of the instrument. The RT for the sample was compared to the RT cutoff, i.e., a value determined empirically from an analysis of samples from patients with and without tuberculosis (40). By using this information, results were reported as fail (data not evaluable), negative (i.e., *M. tuberculosis* was not present in the sample at detectable levels), or positive (i.e., *M. tuberculosis* was present).

A cutoff time was determined for each lot of reagents and was based on the RT for a reaction mixture containing approximately 11 or fewer detector probe molecules. Because the doubling time for the detector probe varied slightly between reagent lots (9), cutoff values were determined and assigned for each lot of manufactured disposable test packs and were imprinted in the bar code on the disposable test pack to be used in data analysis for each individual sample processed by the Galileo instrument. RTs earlier than the RT cutoff yielded positive results; those later than the RT cutoff were negative. Pertinent patient and sample information, interim reports on the progress of individual samples during the test procedure, and final results were displayed on an external video monitor. RTs per se were not reported to the operator by this software version.

Study protocol. Amplification results were compared to confirmed culture results. Samples positive by the Galileo assay and by culture were categorized as established positive results. Culture and acid-fast smear procedures were those recommended by the U.S. Public Health Service (24). Comprehensive clinical data were available for all patients providing samples for this study. Clinical data relevant to the resolution of discrepant results included a past history of exposure to active tuberculosis, diagnosed tuberculosis, specific therapy history, chest X-ray evaluation, skin test reactivity, acid-fast smear results, overall clinical evaluation, and classification according to the American Thoracic Society system for grouping patients with known or suspected pulmonary mycobacterial infections (1). Samples which were Galileo assay negative and culture negative were designated as having established negative results. Most discrepant results were resolved and classified as established positive or negative results by reference to appropriate elements of the patient history which were consistent with confirmation of *M. tuberculosis* infection or tuberculosis disease. That is, positive amplification results (for specimens reported as culture negative) in conjunction with specific therapy for previously diagnosed tuberculosis (usually within the previous 6 months) and/or contemporaneous positive cultures, X-ray, or skin test results yielding an independent diagnosis of tuberculosis were reclassified from false positive to established positive. Given the sensitivity of the assay (approximately 5×10^4 target rRNA molecules per test), a false-negative result may be explained by insufficient target levels in the original sample or may be an artifact of splitting the sample. If the results were not resolved, samples retained designations as false positive or false negative by the Galileo assay.

RESULTS

Inclusivity and exclusivity. The inclusivity and exclusivity of the assay were examined by testing aliquots of 66 dense cultures of *Mycobacterium* species including *M. tuberculosis* complex and 57 other *Mycobacterium* species (some species were tested in replicate). Additionally, we tested 83 cultures representing 76 other bacteria, fungi, and viruses which might be found in sputum samples (Table 1). All species of the *M. tuberculosis* complex (including *M. microti*) produced RTs of from 4.21 to 5.13 min, indicating that similar (high) levels of target had hybridized specifically, were captured, and were available for amplification and that all samples were equally detected (RT data are not presented). *M. tuberculosis* ATCC 25618 and ATCC 25177 (virulent and avirulent strains, respectively), *M. bovis* ATCC 19210, *M. bovis* BCG ATCC 27290, and *M. microti* ATCC 19422 were positive, as determined by the RT data analysis software; however, a single *M. microti* sample yielded a FAIL result due to a slope measurement which was outside the acceptable limits. Analysis of the raw data for this sample indicated that the slope of the dye binding curve was marginally above the statistically preestablished upper limit of 160. All 62 other non-*M. tuberculosis* complex *Mycobacterium*

TABLE 1. Inclusivity and exclusivity testing of the Q-Beta replicase amplification assay for *M. tuberculosis* with the Galileo analyzer

Species
Mycobacteria
M. tuberculosis complex[a]
M. bovis[b]
M. microti
M. tuberculosis[b]
Non-*M. tuberculosis* complex[c]
M. abscessus
M. acapulcensis
M. agri
M. aichiense
M. asiaticum
M. aurum
M. austroafricanum
M. avium
M. brumae
M. brunense
M. celatum[b]
M. chelonae
M. chitae
M. chubuense
M. confluentis
M. diernhoferi
M. duvalli
M. engbaekii
M. flavescens
M. fortuitum
M. gadium
M. gastri
M. genovense
M. gilvum
M. gordonae
M. intracellulare
M. kansasii
M. lactis
M. malmoense
M. marinum
M. moriokaense
M. neoaurum
M. novum
M. nonchromogenicum
M. obuense
M. parafortuitum
M. peregrinum
M. petroleophilum
M. phlei
M. porcinum
M. poriferae
M. pulveris
M. rhodesiae
M. scrofulaceum
M. senegalense
M. shimoidei
M. simiae
M. smegmatis
M. sphagni
M. szulgai
M. terrae
M. thermoresistable
M. triviale
M. ulcerans
M. vaccae
M. valentiae
M. xenopi
Nonmycobacteria[d]
Bacteria
Acinetobacter calcoaceticus
Aeromonas hydrophila
Actinomyces israelii
Bifidobacterium bifidum
Bordetella pertussis
Chlamydia trachomatis
Citrobacter freundii
Corynebacterium bovis
Corynebacterium diphtheriae
Corynebacterium jeikeium
Corynebacterium renale
Corynebacterium xerosis
Eikenella corrodens
Enterobacter aerogenes
Enterobacter cloacae
Enterococcus faecalis
Enterococcus faecium
Escherichia coli
Fusobacterium necrophorum
Fusobacterium nucleatum
Hemophilus influenzae
Hemophilus parainfluenzae
Klebsiella ozaenae
Klebsiella pneumoniae
Lactobacillus acidophilus
Lactobacillus casei
Legionella micdadei
Legionella pneumophila
Moraxella catarrhalis
Mycoplasma pneumoniae
Neisseria gonorrhoeae
Neisseria meningitidis
Nocardia asteroides[b]
Nocardia brasiliensis
Nocardia otidiscaviarum
Peptococcus asaccharolyticus
Peptostreptococcus prevotti
Porphyromonas asaccharolytica
Porphyromonas gingivalis
Prevotella corporis
Prevotella melaninogenica
Proprionibacterium acnes
Pseudomonas aeruginosa
Rhodococcus bronchialis
Rhodococcus equi
Rhodococcus rhodochrous
Rhodococcus sputi
Salmonella enteritidis
Salmonella typhi
Serratia marcescens
Staphylococcus aureus[b]
Staphylococcus epidermidis
Streptococcus agalactiae
Streptococcus bovis
Streptococcus mutans
Streptococcus pneumoniae
Streptococcus pyogenes
Streptococcus salivarius
Streptococcus sanguis
Streptomyces griseus
Tsukamurella paurometabolum
Veillonella atypica
Veillonella parvula
Xanthomonas maltophilia
Fungi
Aspergillus fumigatis
Blastomyces dermatitidis
Candida albicans
Cryptococcus neoformans
Histoplasma capsulatum[b]

Continued

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TABLE 1—*Continued*

Species
Viruses
Adenovirus type 5
Cytomegalovirus strain AD 169
Echovirus type 11
Herpes simplex virus type 1 strain MacIntyre
Rhinovirus type 2
Respiratory syncytial virus strain Long

[a] All listed *M. tuberculosis* complex bacteria except *M. microti* tested positive in the assay; *M. microti* failed to provide a result (positive or negative) due to a fluorescence reading outside of the specified limits.
[b] Multiple strains were tested.
[c] All listed non-*M. tuberculosis* complex mycobacteria tested negative in the assay.
[d] All listed nonmycobacteria except one of five strains of *H. capsulatum* tested negative in the assay; the one *H. capsulatum* strain tested positive (see text).

samples were tested at high input levels (i.e., 10^5 to 10^7 CFU/test) and produced negative results. Equivalently high levels of other competitor organisms (Table 1) were also tested. All of these organisms with the exception of one sample of *H. capsulatum* gave negative results. Four subsequently tested *H. capsulatum* samples all gave negative results, indicating the first sample had a false-positive result and that the result was independent of the target added.

Dose-response. As an extension of the inclusivity study, we determined RTs for serial dilutions of several clinical *M. tuberculosis* isolates. Dense cultures of nine distinct clinical *M. tuberculosis* isolates (averaging approximately 10^7 CFU/ml) were diluted in a 10-fold dilution series, processed, and tested in four replicates per dilution in the assay. Colony counts were approximately 8 to 100 CFU/assay point for the highest dilutions tested. Average RT versus the numbers of CFU/assay point are plotted in Fig. 1, and in most instances they were similar among the strains tested. These data suggest a detection limit ranging from <10 to approximately 300 CFU; however, determination of actual analytical sensitivity will require repeated testing of a larger number of strains.

Clinical study results. Automated assay data collected in early 1995 from three clinical sites in the United States are included in this report. Sites included university teaching hospitals in the Northeast (site A; 267 samples from 155 patients; 34 true-positive samples from 9 patients) and Southeast (site C; 356 samples from 254 patients; 31 true-positive samples

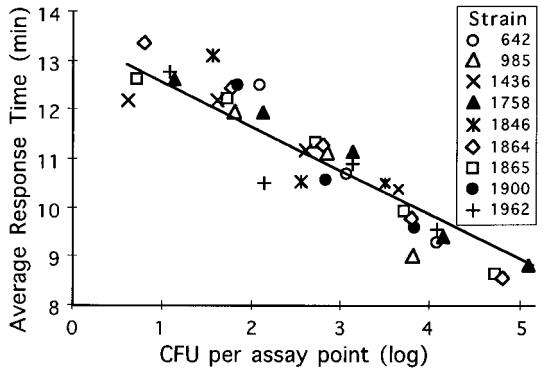

FIG. 1. Dose-response of the Galileo assay for clinically isolated strains of *M. tuberculosis*. The indicated dilutions of cultures of nine clinical isolates were assayed in the Galileo analyzer. Each symbol corresponds to a distinct, numbered strain.

TABLE 2. Site-by-site performance of the Galileo assay for *M. tuberculosis*

Result and site	*M. tuberculosis* culture result (no. of specimens)		Galileo assay result (no. of specimens)[a]				Sensitivity (%)	Specificity (%)
	Positive	Negative	TP	FN	TN	FP		
Unresolved								
A	35	232	32	3	216	16	91	93
B	20	137	13	7	117	20	65	85
C	35	321	26	9	309	12	74	96
Total	90	690	71	19	642	48	79	93
Resolved[b]								
A	37	230	34	3	216	14	92	94
B	40	117	33	7	117	0	83	100
C	40	316	31	9	309	7	78	98
Total	117	663	98	19	642	21	84	97

[a] TP, true positive; FN, false negative; TN, true negative; FP, false positive.
[b] Resolved versus culture results and patient history (clinical status).

from 20 patients) and one midwestern state health department laboratory (site B; 157 samples from 113 patients; 33 true-positive results from 31 patients). The study was designed to comply with FDA guidance documents for validating amplified nucleic acid hybridization tests generally (48), and for specific *M. tuberculosis* diagnostic products (49). Standard high-sensitivity techniques for auramine fluorochrome microscopy and culture were used as reference methods; two of the three sites used the BACTEC system to obtain accelerated culture results. All of the sites confirmed the identities of the isolates by using Gen-Probe confirmation tests. The three sites had prevalence rates of *M. tuberculosis* in respiratory tract specimens (determined by culture) of 10 to 13%. Investigators at the sites were trained and then monitored for proficiency in precision and reproducibility trials with mock samples (negative samples and spiked samples defined as positive) which were also assayed on Galileo instruments in our laboratories. After this training phase, investigators began testing clinical samples by following the study protocol. Samples included induced sputum, bronchoalveolar lavage, or expectorated sputum. Assays were run with fully documented user software, and the results were reported as positive, fail, or negative. After the assays were completed, the used sample test packs were examined for evidence of leakage, failure of the reagent blisters to burst, or unusual patterns of separation or partitioning of magnetic particles to ensure that no pack or instrument failure had occurred. Data obtained from tests with the failed packs were not included in the analysis. If sufficient sample was available, the test was repeated.

Preliminary, unresolved results for the 780 samples tested are presented in Table 2. Seventy-one (78.9%) of the 90 *M. tuberculosis* culture-positive samples were positive with the Galileo instrument. Forty-eight (7.0%) of 690 culture-negative samples were also positive with the instrument. These raw data correspond to approximately 79% sensitivity and 93% specificity compared to culture results. Specificity was greater than 90% at two of the trial sites and 85% at the third site. Sensitivity calculated from unresolved data was more variable; i.e., it ranged from 65 to 91%.

Resolved clinical study results. To resolve discrepancies between culture and Galileo assay results, clinical histories and results for other recent cultures were reviewed for all specimens yielding apparently false-positive assay results (48 specimens). By using standard clinical classification criteria (1) and protocols for the resolution of discrepant results suggested by

TABLE 3. Resolution of presumed false-positive samples by amplification following patient chart review

Site	Sample no.	Acid-fast smear result[a]	Clinical resolution[b]
A	T62537	neg	Other positive cultures, on therapy
A	W60576	neg	Recent history, on therapy
B	6301	neg	Previous history, 1992
B	6305	neg	Previous history
B	6314	neg	Previous history, 1994
B	6317	neg	Previous history, 1994
B	6356	neg	Exposure history
B	6358	neg	Previous history, unknown date
B	6366	neg	Previous history, 1994
B	6388	neg	Previous history, unknown date
B	6430	neg	Recent positive culture
B	6547	neg	Previous history, 1993
B	6548	neg	Previous history, 1992
B	6564	neg	Previous history, unknown date
B	6764	neg	Previous exposure
B	6834	neg	Previous exposure
B	6840	neg	Previous history, 1994
B	6888	neg	Previous positive cultures
B	6893	pos	Recent positive cultures, 1995
B	6894	neg	Recent positive cultures, 1995
B	6895	neg	Recent positive cultures, 1995
B	6911	neg	Previous history, unknown date
C	10.342	neg	Recent history and positive culture, on therapy
C	83.191	neg	Previous history, 1953, on therapy
C	88.259	neg	Previous history, 1993, classified as having tuberculosis, but not clinically active
C	89.167	pos	Other positive cultures, on therapy
C	97.151	pos	Other positive cultures, on therapy

[a] neg, negative; pos, positive.
[b] Indicates results of culture or clinical history of diagnosed tuberculosis. The 27 samples with resolved results were from 24 patients. Three samples from site B were from a single patient. Two samples from site C were from a single patient. "Previous history" indicates a previous history of tuberculosis.

FDA (48, 49), trial site investigators reclassified 27 false-positive results (Table 3). Figure 2A presents the results of the final analysis after the resolution of discrepant results. At one site, the results for all 20 samples with false-positive results were resolved. The resultant sensitivity at this site rose from 65 to 83% and the specificity rose from 85 to 100% (Table 2). By using the resolved data (clinical status) for samples from all sites, the overall sensitivity was 84% and the overall specificity was 97%. Specificity results for each of the sites were greater than 96%, yielding a positive predictive value (PPV) and a negative predictive value (NPV) of 82 and 97%, respectively, for a specimen population that was approximately 15% positive (following resolution of discrepant results).

Results for smear-positive and smear-negative categories were reviewed separately and are presented in Fig. 2B and C. Sixty-five of 117 (55.5%) specimens from patients with confirmed cases of tuberculosis were scored as smear positive; 62 of the 65 smear-positive specimens (95.3%) were positive when tested with the Galileo system. Fifty-two samples from patients with confirmed cases of tuberculosis were smear negative; 36 of these 52 samples (69.2%) were positive with the Galileo system.

Six of 42 samples (14.3%) from which *Mycobacterium* spp. other than *M. tuberculosis* (MOTT) were cultured were positive by the Galileo assay, compared to the 2.4% false-positive rate observed for samples yielding no *Mycobacterium* spp. Comparison of these two categories by chi-square analysis yielded a significant value (chi-square value, 18.07 with one degree of freedom; value for >99.9% significance, 10.8); however, the expected value for the number of positive results in the MOTT category is less than five, implying that more samples containing MOTT should be tested to confirm this difference. Given the earlier documentation of the specificity of the assay for *M. tuberculosis* complex organisms, at least some of these infections may represent unreported mixed infections rather than actual false-positive results.

DISCUSSION

Previously, inclusivity testing of all five *M. tuberculosis* complex members showed that *M. bovis* and *M. tuberculosis* strains were detected by hybridization and Q-Beta replicase amplification, as predicted by sequence data (43). When multiple samples of 10-fold dilutions of nine *M. tuberculosis* strains were tested, the lower limit of detection was determined to be <10 to 300 CFU.

In this study, a single *M. microti* sample produced a RT similar to those for the other members of the complex tested; however, the slope value was high, and by the established software algorithm, the result was therefore scored as a test failure. The dye binding slope value for this sample was 165, only 5 units (3.1%) above the cutoff limit of 160. Explanations for an aberrant slope value include amplification of contaminant (nonreporter probe) replicable RNA, a rare instance of legitimate amplification of reporter probe outside the previously assigned slope characteristic boundaries, or an inappro-

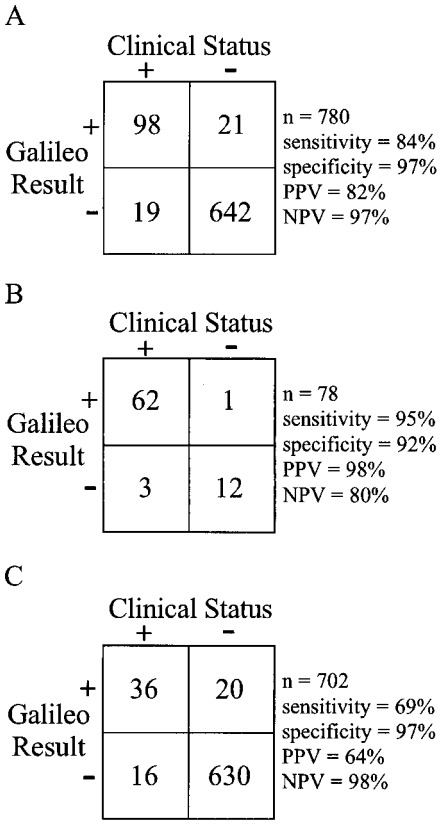

FIG. 2. Galileo assay result for *M. tuberculosis* versus clinical status after resolution of discrepant results. (A) Overall results for all samples; (B) results for smear-positive samples; (C) results for smear-negative samples; *n*, number of samples tested.

priate combination of amplification reagents. The limit of 160 was chosen statistically to conservatively exclude infrequent responses resulting from contaminant replicating RNA species which produce higher slopes, i.e., generally >200 (9). However, the choice of 160 as a limit resulted in an occasional failure to detect otherwise positive specimens. Previously, we have shown that total RNA from *M. microti* was readily detected by a manual Q-Beta replicase amplification assay (38, 39). While we would have considered this particular sample to have a true-positive result on the basis of the RT, the coincidental occurrence of a high slope highlights the potential need for review of slope value limit criteria and/or refinement of analytical software. *M. africanum* was not tested in this study by using the Galileo instrument because of a procedural error. However, when previously tested in the manual format, *M. africanum* gave positive results similar to those obtained with other *M. tuberculosis* complex organisms (39). These results are consistent with the observation that the target regions of both *M. africanum* and *M. microti* rRNA to which the capture probe binds differ from the *M. tuberculosis* target sequence by only one nucleotide (43).

Exclusivity studies with samples from dense cultures of mycobacterial species other than *M. tuberculosis* complex indicated that high levels of rRNA target from these organisms were negative in the assay. Only 18 of 61 such organisms (29%) produced RTs in the Galileo assay within the 25-min read period, and these were above the cutoff value (i.e., 13.5 to 20.8 min; RT data not shown). RTs for approximately equivalent masses of cells of the *M. tuberculosis* complex were 4.21 to 5.13 min, supporting the specificity of the assay.

We tested *M. celatum* in five separate assays, because this and a few other MOTT organisms have the potential to yield false-positive results when tested in the Gen-Probe MTD assay (20). Samples of *M. celatum* were consistently negative when tested in the Galileo assay.

The comparison of unresolved Galileo assay results with culture results indicated 79% sensitivity and 93% specificity. Twenty-seven of the 48 apparently false-positive samples were resolved to the positive category on the basis of a review of microbiological and clinical histories. One site providing approximately 20% of the total study samples accounted for 74% of the resolved discrepant results. All 20 samples at this site with false-positive results were resolved and reclassified as true positive for *M. tuberculosis*, increasing the prevalence of samples from patients with confirmed cases of tuberculosis at this site from 15 to 25%. Most of the false-negative samples were smear negative; however, the results for none of these samples were resolved, presumably because target levels were below the lower detection limit of the assay. False-negative results for single sputum samples are well known across diagnostic methods; hence, classical protocols mandate testing of multiple samples to establish a reliable result for a given point in time for a given patient. Data from repeat testing of specimens giving discordant results have not been reported here. Additional studies will be required to verify the optimal number of specimens to be tested by amplified hybridization methods to ensure results with predictable accuracy. As suggested previously (40), the present study demonstrates somewhat better overall assay performance with fresh specimens than that observed previously when testing specimens which had been stored for variable and lengthy periods.

Following resolution of the results in our study, of 117 samples from patients with confirmed tuberculosis, 98 were positive by amplification, yielding a sensitivity of 83.8%. Six hundred forty-two of 663 *M. tuberculosis*-negative samples were also negative by the Galileo assay, for a specificity of 96.8%. Given an overall disease prevalence of 15% in this study population, the PPV and NPV were 82 and 97%, respectively.

Smear results were reviewed for all samples. Of the 117 samples from patients with a diagnosis of tuberculosis, 65 were smear positive and 52 were smear negative. Sixty-two of the 65 smear-positive samples (95.4%) were detected by the Galileo assay. A total of 69.2% (36 of 52) of the smear-negative, culture-positive samples were detected by the assay.

Culture has been considered the gold standard reference method in mycobacteriology; however, we were able to resolve the results for 27 samples from patients with histories supporting or proving pulmonary tuberculosis which were positive when tested by Q-Beta replicase amplification and negative by culture. By these criteria the resultant average sensitivity of culture at the three sites in this study was only 98 of 117 (84%). This is consistent with observations in the literature documenting the risk of loss of viability during classical decontamination of sputum (27). Such a situation might well yield noncultivable organisms while allowing nucleic acid targets (RNA or DNA) that are detectable by amplification to remain. Additionally, one might also expect that some samples from patients on antimycobacterial therapy would be culture negative while remaining smear and Galileo assay positive.

There was a difference between the frequency of false-positive results for samples containing MOTT compared to that for the samples which grew no *Mycobacterium* spp. (14.3% for MOTT-positive samples versus 2.4% for MOTT-negative samples). The chi-square value obtained when these populations are compared suggests that they are different at greater than the 99.9% confidence level. However, in the analysis the expected value for the MOTT category was less than five samples, which weakens the significance of the test. The five MOTT-containing specimens which were Galileo assay positive (initially reported as *M. tuberculosis* negative) consisted of three samples containing *M. avium-M. intracellulare*, two samples containing *M. kansasii*, and one sample yielding an unidentified MOTT organism. However, of the non-*M. tuberculosis* complex organisms listed in Table 1, none were positive in the assay when tested at high target levels. Previously, we have shown that negative results were obtained when 10^7 CFU of *M. avium* was tested in the automated assay (40). Additionally, 23S rRNA sequence information (43) indicates that *M. kansasii*, *M. celatum*, *M. xenopi*, *M. gastri*, and *M. gordonae* have very similar sequences in the target region and none of these were positive by the Galileo assay with a high level of target input. During analysis of discrepant results, we found that one sample reported as MOTT came from a patient with clinical tuberculosis. In an earlier preclinical study (40) we found that samples smear positive for MOTT were negative by the Galileo assay. However, it is possible for pulmonary specimens from certain patient groups to be reported on preliminary culture as containing MOTT and to also harbor low levels of *M. tuberculosis* which may go undetected by conventional methods.

There is a growing literature on the two commercially available amplified probe-based products for the direct detection of *M. tuberculosis*; the reader is directed to a more extensive list of references included in several of the recent reports on PCR (Amplicor) and the Amplified *M. tuberculosis* Test (examples of which include references 4, 5, 8, 10, 11, 14, 15, 19, 22, 25, 31, 36, and 41). The sensitivities and specificities of these products have been reported to be similar, i.e., ranging from approximately 67 to 94% sensitivity and from 94 to 100% specificity. As has recently been noted, very few studies have provided a direct comparison of these two products (22, 52). Evaluation of the published studies is therefore difficult given the wide range of protocols, reference methods, and study populations (19).

When testing smear-positive specimens, both the Gen-Probe MTD and the Roche Amplicor assays perform well. However, the performance with smear-negative specimens is much more variable. A recent study with the Amplicor assay found a sensitivity of only 40% among smear-negative specimens (5). Even after considerable testing and improvements, the overall performance is sufficiently constrained that FDA has limited initial claims for both of the commercially available products and has indicated that the assay should be used with smear-positive specimens only (3). Use of these products still remains somewhat tedious, and these products can be subject to contamination and sample inhibition issues and provide results after 6 to 8 h. Additionally, two other amplification products for the detection of *M. tuberculosis* are in earlier stages of commercial development, i.e., nucleic acid sequence-based amplification (50) and strand displacement amplification (16). In general, any of the probe-based methods can readily be designed to provide high specificity, particularly with model systems. Differentiation and acceptance of these products will rest primarily on the sensitivity determined in a number of clinical settings, validated predictive values, and importantly, overall ease of use (including sample processing).

The present study represents the first clinical trial of Q-Beta replicase amplification and the Galileo platform. However, the initial results, including the ability to detect 69.2% of smear-negative, *M. tuberculosis*-positive specimens, are very encouraging and may be superior to the results obtained with commercially available products. In resolving discrepant results, we relied on a review of pertinent clinical information, and we have not included data from retesting of samples or additional testing of specially collected samples. In other studies in which amplification methods were used, such retesting has resulted in improved performance (52). Given the variability of sputum samples from the same patient and the practical difficulty of splitting specimens for direct comparisons, it is possible that such retesting would have yielded improved overall sensitivity of the Q-Beta replicase assay.

While the closed-pack disposable design of the Galileo instrument offers many advantages with regard to controlling amplicon or specimen cross-contamination and, to a smaller degree, control of biohazards, it would also be desirable to simplify the assay, perhaps making it amenable to use with the instrumentation commonly found in diagnostic laboratories. Recent investigations have focused on splitting the midivariant RNA detector probe into two pieces which can replicate only when ligated following hybridization with specific target. Using this "smart probe" approach, 10^2 target molecules of *Chlamydia* or human immunodeficiency virus RNA have been detected in model systems by using only one round of RTC (42, 47). These initial results with a simplified assay are very encouraging and should stimulate further feasibility studies on adapting Q-Beta replicase amplification to existing instruments.

Rapid identification of *M. tuberculosis* following culture by automated hybridization has been reported previously (29). However, here we have reported results obtained with what we believe to be the first fully automated hybridization diagnostic system for testing clinical specimens directly. Concerted efforts at enhancing diagnostic procedures have been undertaken in most large U.S. mycobacteriology laboratories (6). These and improved, vigorous public health practices have resulted in encouraging moderation of the overall occurrence of tuberculosis in the United States (17). However, the potential threat of infections caused by multiple-drug-resistant strains persists, and infection rates among the most susceptible human populations remain staggering throughout the world (18). We believe that as they are validated, simplified, and become commercially available, new diagnostic approaches, such as the one that we have described here, will find wide utility for the more effective control of tuberculosis and many other important infectious diseases.

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