Rapid Detection of the *Mycobacterium tuberculosis* Complex by Use of Quenching Probe PCR (geneCube)

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Early detection of tuberculosis (TB) is essential for infection control. The geneCube (Toyobo) is a novel fully automated gene analyzer that can amplify target DNAs within 60 min. In this study, we evaluated the ability of the geneCube to directly detect *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium avium* complex (MAC) in clinical specimens. The results were then compared with those obtained using conventional culture, microscopy, and the Cobas Amplicor assay (Roche). We examined a total of 516 frozen samples from 69 patients who showed culture-positive infection (73 samples; 39 MTBC, 32 MAC, and 2 mixed infections) and from 354 patients who were culture negative (443 samples). Assays using the geneCube had a sensitivity of 85.4% and a specificity of 99.8% for detection of MTBC and a sensitivity of 85.3% and a specificity of 99.8% for detection of MAC. These results are similar to those obtained using the Amplicor system but were obtained much more rapidly (1 h with the geneCube versus 5.5 h with the Amplicor system). The geneCube thus enables a significant shortening of the assay time with no loss of sensitivity or specificity.

Tuberculosis (TB) is a major public health problem worldwide. According to a recent World Health Organization (WHO) report, 1.1 million people around the world died of TB in 2010 (29). In Japan, the number of TB patients has been declining yearly; nonetheless, 24,170 new cases were reported in 2009 (28). The prevalence of TB is higher in Japan than in Western countries, and it remains a serious public health problem, even now. Reducing the incidence of TB will require optimal control of infection and early diagnosis. However, members of the *Mycobacterium tuberculosis* complex (MTBC) grow slower than other bacteria and so require a long incubation time for diagnosis in culture. To overcome that situation, more rapid diagnosis with genetic testing using PCR is now widespread in hospital laboratories (1–3, 6–15, 17–21, 24, 26, 31).

The Cobas Amplicor MTB (Roche Diagnostics, Basel, Switzerland) PCR assay for direct detection of MTBC is popular in many countries, and numerous studies have been conducted to evaluate the system (2, 6–9, 11–13, 19–21, 24). This assay was approved by the FDA for testing of respiratory samples but requires about 6 h and cannot be applied to screening of nonrespiratory samples due to the presence of a PCR inhibitor in those tissues. Recently, the geneCube system (Toyobo, Osaka, Japan), a fully automated gene analyzer, was developed as a new detection method that can detect and distinguish between DNA from MTBC and *Mycobacterium avium* complex (MAC) within 60 min. This assay technique is based on real-time PCR technology targeting the *dnaJ* genes of MTBC and MAC and uses quenching probes (Qprobes) to confirm the presence of MTBC or MAC (25, 27, 30). In the present study, we compared the ability of the geneCube system to directly detect MTBC and MAC with that of the Cobas Amplicor system, using the standard culture method as the reference.

MATERIALS AND METHODS

Clinical specimens and patients. We used a total of 516 samples from 423 patients in this study. The samples were sent to the laboratory at the University of Fukui Hospital for genetic testing and MTBC culture be-

tween January 2008 and March 2012. The collection of clinical samples for this study was approved by the Review Board Committee of University of Fukui Hospital. Of the 516 samples, 73 were culture positive (39 TB, 32 MAC, and 2 mixed infections) and 443 were culture negative. Prior to assay, the samples were frozen at -80°C . There were 344 pulmonary samples (273 sputum, 24 bronchial aspirate, 34 bronchial washing, and 13 bronchial brushing liquid samples) and 172 nonpulmonary samples (35 pleural fluid, 23 abscess fluid, 25 feces, 23 urine, 19 joint fluid, 15 cerebrospinal fluid, 10 tissue, 4 gastric secretion, and 18 other samples).

Pretreatment of samples. Frozen sediments were thawed and divided into two equal portions, one for the geneCube assay and the other for the Cobas Amplicor assay. After mixing 1 to 15 ml of the specimen sample with twice as much *N*-acetyl-L-cysteine (NALC)-NaOH (final concentration, 2%), the samples were vortexed for 20 s and then left at room temperature for 15 min. Thereafter, the mixture was adjusted to 50 ml by addition of cold sterile 67 mM phosphate buffer (pH 6.8) and then centrifuged for 20 min at $3,000 \times g$ (4°C). After discarding the supernatant, the pellet was mixed with 1 ml of the phosphate buffer for detection using each method.

Staining and culture. Smears were prepared and stained using the method of Ziehl-Neelsen. Acid-fast bacilli (AFB) were counted in more than 300 microscope fields at $\times 1,000$ magnification. The results were graded by experimenters blinded to the results of the other tests as follows: $-$, no bacteria found in 300 fields; $+$, >1 and <9 AFB in 100 fields; $2+$, ≥ 10 AFB in 100 fields. Medium containing 3% solid potassium dihydrogen phosphate (3% Ogawa medium; Nissui, Tokyo, Japan) or one BacT/Alert MP culture bottle (bioMérieux, Marcy l'Etoile, France) was used for the culture of AFB. Samples were maintained at 37°C for up to 8 weeks to confirm the presence of AFB. Specimens were observed once each week.

Received 25 June 2012 Returned for modification 1 August 2012

Accepted 27 August 2012

Published ahead of print 29 August 2012

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doi:10.1128/JCM.01654-12

Cobas Amplicor assay. Prepared samples were examined using the Cobas Amplicor system as instructed by the manufacturer. One hundred microliters of prepared sample was added to 500 μ l of wash solution, and the mixture was then centrifuged for 10 min at $13,000 \times g$. After discarding the supernatant, a lysis solution was added to the pellet and the tubes were incubated for 45 min at 60°C. After the incubation, 100 μ l of neutralization solution was added. Fifty-microliter aliquots of the resultant mixture was then added to 50 μ l of master mix plus internal controls, and PCR amplifications were carried out; that is, the amplification tubes were closed, and the amplification ring was transferred to the Cobas Amplicor system. Provided with the kit were one positive control and one negative control for each vessel ring, which were included in each run. The Cobas Amplicor automates the PCR amplification and detection procedure. To discriminate between positive and negative findings, the results were calculated using the system software with a cutoff set to an optical density (OD) at 650 nm of 0.35. We performed all procedures on-site at the University of Fukui Hospital laboratory, and those performing the assays were blinded to the culture results.

geneCube assay. geneCube is a device that can be used for automated gene analysis, including nucleic acid extraction, amplification, and detection. This device can handle a maximum of eight samples at a time and analyze up to four items at the same time. After installing the reagents and consumables, nucleic acid extraction requires approximately 15 min, and amplification and detection can be completed in 22 min.

DNA extraction was performed using the geneCube Prep module. Each decontaminated and concentrated clinical sample was added to 300 μ l of lysis reagent, and the mixture was incubated at 80°C for 10 min. Following centrifugation ($2,300 \times g$ for 2 min), 500 μ l of the supernatant was transferred to the geneCube Prep column. To collect the DNA, silica monolith was allowed to set in a syringe, after which the sample supernatant was aspirated and discharged 10 times using the silica-filled syringe. The DNA in the sample was bound to the silica monolith under the high-guanidine conditions, and after washing the silica twice with 60% ethanol, the DNA was eluted using elution buffer.

PCR was carried out using nucleic acid solution, internal control mix, KOD polymerase (16, 23) mix, and primer-probe mix. By measuring the positive and negative controls as samples, we were able to verify the accuracy of the results. After the reagents were mixed automatically in the geneCube system, they were added to a single plastic capillary by centrifugation. The capillary was then transported automatically to a thermal cycler. The PCR protocol entailed denaturation at 94°C for 30 s, followed by 50 cycles of denaturation at 97°C for 1 s, annealing at 58°C for 3 s, and extension at 63°C for 5 s. The PCR products were then automatically subjected to melting temperature (T_m) analysis, which consisted of denaturation at 98°C for 30 s, annealing at 39°C for 30 s, and then heating to 75°C at a rate of 0.09°C/s. The amplified target DNA was hybridized with a fluorescently labeled oligonucleotide (a Qprobe) (27). Upon binding to the target DNA, the Qprobe fluorescence was quenched by the guanine bases in the target. However, the fluorescence reappeared as the Qprobe dissociated from the melting target. By detecting this change in fluorescence intensity, we were able to distinguish *Mycobacterium* species. This melting curve analysis for T_m measurement was performed using a program provided with the geneCube, and the results were shown on geneCube's display. We performed all procedures on-site at the University of Fukui Hospital laboratory, and those performing the assays were blinded to the culture results. In addition, the turnaround and hands-on/hands-off times of the geneCube and Cobas Amplicor systems were compared using 5 samples (average per day in our laboratory).

Sequence of AFB. The 16S rRNA gene was amplified using universal PCR primers (22), after which the amplified gene product was purified using a QIAamp PCR purification kit (Qiagen, CA) and directly sequenced using a BigDye Terminator cycle sequencing kit (Life Technologies, CA) on an ABI 3130 sequencer (Biosystems, CA).

Statistical analysis. The results of the nucleic acid amplification technique (NAAT) were compared with those of culture for MTBC and MAC.

Because the analysis was done using known AFB-positive and -negative specimens, all of the specimens were considered to be true positives (Tps) or true negatives (Tns). The NAAT results were classified as Tp, false negative (Fn), Tn, or false positive (Fp). The sensitivity and specificity were calculated as [number of Tps/(number of Tps + number of Fns)] $\times$ 100 and [number of Tns/(number of Tns + number of Fps)] $\times$ 100, respectively.

RESULTS

Specificity of geneCube reagent. Genetic testing for both MTBC and MAC using the geneCube system was performed using the same 516 clinical samples. The 39 MTBC-positive samples were identified as MAC negative. Similarly, the 32 MAC-positive samples were identified as MTBC negative. In addition, two samples that showed mixed MTBC and MAC infections in culture were identified by the geneCube system as positive for both *M. tuberculosis* and MAC. Using the geneCube system, therefore, we were able to specifically identify MTBC and MAC infections.

Results obtained with each detection method. Frozen sediments were thawed and divided into two equal portions, one for the geneCube assay and the other for the Cobas Amplicor assay. In this study, the results obtained from 12 samples (sputum, 1; pericardial fluid, 1; feces, 10) assayed using the Cobas Amplicor were invalid due to the presence of a PCR inhibitor. Using the geneCube assay, in contrast, valid results were obtained from all 12 of these samples, which is indicative of the greater amplification performance of the geneCube. We excluded those 12 samples from the study.

Table 1 summarizes the results for detection of MTBC using the Cobas Amplicor and geneCube. Forty-one samples were classified as TB positive on the basis of detection of *M. tuberculosis* in culture. Of those, 19 (46.3%) were smear positive and 22 (53.7%) were smear negative. On the basis of the negativity of the cultures for *M. tuberculosis*, 431 of 472 samples were classified as TB negative. Overall, 35 of 472 samples yielded positive PCR results with the Amplicor and 437 yielded negative results. By comparison, 36 of 472 samples yielded positive PCR results with the geneCube and 436 yielded negative results. Of the 41 TB-positive culture samples, 35 gave positive results with the geneCube, for an overall sensitivity of 85.4% (Cobas Amplicor, 82.9%). The sensitivity for smear-positive specimens ($n = 19$) was 100% (Cobas Amplicor, 94.7%), and it was 72.7% for smear-negative specimens ($n = 22$) (Cobas Amplicor, 72.7%).

Similar sensitivities were calculated when analyzing respiratory TB-positive specimens ($n = 29$). With the geneCube, the sensitivities for smear-positive and smear-negative samples were 100% and 73.3%, respectively. Among nonrespiratory samples ($n = 12$), all smear-positive TB samples ($n = 5$; 3 abscess fluid, 1 urine, 1 joint fluid) and 5 of the 7 smear-negative TB samples (all abscess fluid) were identified as TB positive using the geneCube, giving sensitivities of 100% and 71.4%, respectively. Among the 431 culture-negative samples, one sample each was identified as TB positive using Cobas Amplicor and geneCube. Notably, this amplification-positive but culture-negative sample had been obtained from a TB patient during anti-TB chemotherapy.

Table 2 shows the results for the detection of MAC using the two systems. The 465 samples tested included 34 culture-positive samples. Using geneCube or Cobas Amplicor, MAC was detected in the same 29 samples (sensitivity, 85.3%). Positivity for MAC

TABLE 1 Performance of Cobas Amplicor and geneCube assays based on *M. tuberculosis* culture results

		No. of specimens													
		Culture-positive specimens ^a						Culture-negative specimens ^b							
		PCR positive		PCR negative		PCR positive		PCR negative		PCR positive		PCR negative			
		Overall specimens	Respiratory specimens	Overall specimens	Respiratory specimens	Overall specimens	Respiratory specimens	Overall specimens	Respiratory specimens	Overall specimens	Respiratory specimens	Overall specimens	Respiratory specimens	Sensitivity (%)	
Specimen	Assay	34	23	7	6	1	1	1	1	430	281	82.9 (75.1–84.9) ^d	79.3 (68.8–82.1)	99.8 (99.0–100)	96.6 (98.6–99.9)
		35	25	6	4	1	1	1	1	430	281	85.4 (77.6–87.4)	86.2 (76.1–89.0)	99.8 (99.0–100)	96.6 (98.6–99.9)
Smear positive	Cobas	18	13	1	1 ^c	0	0	0	0	0		94.7 (94.7–94.7)	92.9 (92.9–92.9)		
	geneCube	19	14	0	0	0	0	0	0	0		100.0 (100–100)	100.0 (100–100)		
Smear negative	Cobas	16	10	6	5	1	1	1	1	430	281	72.7 (59.4–76.4)	66.7 (48.9–72.1)	99.8 (99.1–100)	99.6 (98.7–99.9)
	geneCube	16	11	6	4	1	1	1	1	430	281	72.7 (59.4–76.4)	73.3 (55.7–78.8)	99.8 (99.1–100)	99.6 (98.7–99.9)

^a Data are for 41 overall specimens and 29 respiratory specimens.

^b Data are for 431 overall specimens and 282 respiratory specimens.

^c This sample (sample 311) was positive for *M. tuberculosis* and MAC in culture, and the Cobas result was as follows: *M. tuberculosis* negative (Cobas OD, 0.12), MAC positive (OD, >4.00).

^d Data in parentheses are 95% confidence intervals.

infection (PCR positive but culture negative) was obtained with one patient receiving anti-TB chemotherapy.

Agreement between results from geneCube and Cobas. The correlation between the results obtained with the Amplicor and geneCube are summarized in Table 3. For MTBC, the match rate was 98.9% (467/472). Among the 5 mismatched cases, the 2 samples that were Cobas positive and geneCube negative were sample 671 (100 CFU/ml; sputum sample; Cobas OD, 0.89) and sample 338 (>1,000 CFU/ml; pus sample; Cobas OD, 1.33). These samples were positive for MTBC in culture (sequencing was not carried out). Samples 127, 195, and 311 were Cobas negative and geneCube positive. With these samples, both sequencing and culture were positive for MTBC. The overall agreement between Cobas and geneCube was 98.9%.

Characteristics of geneCube and Cobas Amplicor assays. Compared to the Cobas used routinely in our laboratory, the geneCube real-time PCR system was easier to use because the work-flow design is automated from DNA extraction to detection of TB. As the manual operations entail only incubation and centrifugation of the clinical samples, the total hands-on time is less than 1 min/sample (Table 4). Moreover, the time required to obtain a result is markedly shorter with the geneCube system (~1 h) than the Cobas system (~5.5 h). Finally, the cost of the geneCube system was also lower than that of the Cobas system.

DISCUSSION

In 2009, the Centers for Disease Control and Prevention recommended using at least one molecular technique when testing for MTBC in a patient (4). One of the most widely used molecular techniques for detection of MTBC is Cobas Amplicor. In the present study, we observed that in terms of both sensitivity and specificity, the performance of the geneCube system for detection of MTBC or MAC was comparable to that of the Cobas Amplicor. The geneCube *M. tuberculosis* assay offers an overall sensitivity of 85.4% and a specificity of 99.8% when using culture results as a standard reference. The overall sensitivity of 85.4% is similar to that obtained with other NAATs used to test respiratory and non-respiratory specimens (range, 58.3% to 96.6%; average, 82.2%) (1–3, 6–15, 17–21, 24, 26, 31). In contrast to several other studies, which found that the sensitivities of various NAATs were reduced when used with nonrespiratory specimens, the sensitivity of the geneCube *M. tuberculosis* assay with nonrespiratory samples (83.3%, 10/12) was comparable to that with respiratory samples (86.2%, 25/29). The sensitivity of the geneCube *M. tuberculosis* assay with smear-negative specimens was also higher (72.7%) than that seen with most other NAATs (range, 44.8% to 79.5%; average, 61.2%) (1, 2, 6–8, 10, 13–15, 19, 20, 24, 26, 31). Similarly, in the case of MAC, both Cobas Amplicor and geneCube identified the same MAC-positive samples.

We obtained a total of 11 false negatives with the geneCube *M. tuberculosis* and MAC assays (Tables 1 and 2). Ten of 11 specimens produced positive cultures only after incubation for 19 days, which is indicative of the low AFB counts (<500 CFU/ml) in these samples. When molecular amplification assays yield negative results with culture-positive specimens, it is normally presumed that the reason is a low AFB load, the presence of polymerase inhibitors, and/or an unequal distribution of AFB in the test specimens.

The Clinical and Laboratory Standards Institute recommends that when inoculating liquid culture medium, at least one selective

TABLE 3 Agreement between results from geneCube and Cobas Amplicor assays

Cobas result	No. of specimens with the following geneCube result:	
	Positive	Negative
MTB (<i>n</i> = 472)		
Positive	33	2 ^a
Negative	3 ^b	434
MAC (<i>n</i> = 465)		
Positive	30	0
Negative	0	435

^a The 2 samples that were Cobas positive and geneCube negative were 671 (100 CFU/ml; sputum sample; Cobas OD, 0.89) and 338 (>1,000 CFU/ml; pus sample; Cobas OD, 1.33). These samples were positive for MTBC in culture (sequencing was not carried out).

^b Samples 127, 195, and 311 were Cobas negative and geneCube positive. With these samples, both the sequencing and cultures were positive for MTBC.

solid medium be inoculated at the same time (5). It is therefore widely accepted that two types of media (one liquid, one solid) should be used for each mycobacterial culture. However, we used either egg-based solid medium (3% Ogawa) or Middlebrook broth (BacT/Alert MP bottle) for each culture, not both. Consequently, our culture results are not “gold standards” with which to confirm true positive and negative. In that regard, we had two samples that were PCR positive and culture negative, and we cannot be certain whether they were false or true positive. This is a limitation of the present study.

The principle concern with the Cobas Amplicor system is the incidence of invalid specimens. In the present study, there were 12 invalid samples (sputum, 1; pericardial fluid, 1; feces, 10) that could not be analyzed using Cobas Amplicor. Reischl et al. (20) reported that 78.2% of feces samples (54/69) contained PCR inhibitors. For that reason, it is noteworthy that there were no invalid results with the geneCube assay. This likely reflects the fact that the geneCube assay uses *KOD* polymerase, which has a higher synthesis rate (number of bases per second) and a higher fidelity than *Taq* polymerase (16, 23). In addition, the sensitivity and accuracy of Qprobe PCR will depend on the primer design, and several sets of PCR primers have been designed for the geneCube assay, enabling one to select the primer set with the best amplification efficiency. In that regard, Takewaki et al. (25) and Yamada-Noda et al. (30) compared the nucleotide sequences of the *dnaJ* genes among *Mycobacterium* species and introduced a new phylogenetic analysis that provided a phenotypic classification that was in good agreement with the conventional phenotypic classification of slowly growing species. Moreover, PCR analysis of *dnaJ* has

TABLE 4 Main characteristics of geneCube and Cobas Amplicor assays

Characteristic	geneCube	Cobas
Work-flow design	Batch mode	Batch mode
Sample preparation	Incubation and centrifugation of a sample (13 min/sample)	Wash and centrifugation of a sample (13 min/sample)
DNA extraction	Aspirated and discharged using a silica-filled syringe (automatic)	Incubation with a lysis solution (45 min)
Hands-on time (min/sample)	~1	~5
Hands-off time	~50 min	~4 h
Time to results (h)	~1	~5.5
Cost	~\$25	~\$40

TABLE 2 Performance of the Cobas Amplicor and geneCube based on MAC culture results

Specimen	Assay	No. of specimens		MAC culture-positive specimens ^a				MAC culture-negative specimens ^b				Sensitivity (%)		Specificity (%)	
		PCR positive		PCR negative		PCR positive		PCR negative		Overall		Overall		Overall	
		Overall	Respiratory	Overall	Respiratory	Overall	Respiratory	Overall	Respiratory	Overall	Respiratory	Overall	Respiratory	Overall	Respiratory
		specimens	specimens	specimens	specimens	specimens	specimens	specimens	specimens	specimens	specimens	specimens	specimens	specimens	specimens
Overall	Cobas	29	27	5	5	5	1	1	1	430	281	85.3 (76.3–87.7) ^c	84.4 (74.9–86.9)	99.8 (99.1–100)	99.6 (98.6–99.9)
	geneCube	29	27	5	5	5	1	1	1	430	281	85.3 (76.3–87.7)	84.4 (74.9–86.9)	99.8 (99.1–100)	99.6 (98.6–99.9)
Smear positive	Cobas	15	15	0	0	0	0	0	0	0	0	100.0 (100–100)	100.0 (100–100)	99.8 (99.1–100)	99.6 (98.7–99.9)
	geneCube	15	15	0	0	0	0	0	0	0	0	100.0 (100–100)	100.0 (100–100)	99.8 (99.1–100)	99.6 (98.7–99.9)
Smear negative	Cobas	14	12	5	5	5	1	1	1	430	281	73.7 (58.8–78.0)	70.6 (54.4–75.4)	99.8 (99.1–100)	99.6 (98.7–99.9)
	geneCube	14	12	5	5	5	1	1	1	430	281	73.7 (58.8–78.0)	70.6 (54.4–75.4)	99.8 (99.1–100)	99.6 (98.7–99.9)

^a Data are for 34 overall specimens and 32 respiratory specimens.

^b Data are for 431 overall specimens and 282 respiratory specimens.

^c Data in parentheses are 95% confidence intervals.

proven effective for detection and identification of *Mycobacterium* at the species level.

In 2010, WHO endorsed a new rapid test (Xpert MTB/RIF) for detection of TB and rifampin resistance (29). This test will revolutionize TB care and control by providing accurate diagnoses for numerous patients within about 2 h, in comparison to the up to 3 months for current tests to yield results. The geneCube assay cannot detect rifampin resistance, which is a weakness of the present assay that should be improved in the future.

In summary, the geneCube assay is a simple method that is well adapted for routine use in clinical laboratories and has proven to be suitable for detection of both MTBC and MAC in respiratory specimens and in panels of respiratory and nonrespiratory smear-positive specimens. The sensitivity of the geneCube system was found to be comparable to that of the Cobas Amplicor assay, and its specificity was excellent. Notably, less than 1 h was required to obtain results once the specimens were treated with NALC-NaOH. It thus appears that this novel method would be suitable for routine use and rapid diagnosis of TB.

ACKNOWLEDGMENTS

We thank Yoshihiro Soya and Keiichi Shiuchi of Toyobo Corporation for their excellent technical advice on the geneCube.

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