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EXPERIMENTAL / LABORATORY STUDIES

National Tuberculosis Reference Laboratory Experience in Multi Center Quality Control Programs for Molecular Diagnostics of Tuberculosis*

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Abstract: Nucleic acid amplification methods to detect Mycobacterium tuberculosis complex (MTBC) in clinical specimens are increasingly used to diagnose tuberculosis.

A number of nucleic acids amplification assays have been developed to detect MTBC DNA in clinical material. These assays differ in their requirements for sample volume and sample preparation, methods of amplification, and methods of detection. There are advantages and disadvantages of all assays; thus, there is probably no assay which is best suited to all situations. Quality control programs for molecular diagnostics (QCMD) based on nucleic acid amplification have not been widely implemented in clinical laboratories and remain limited to a few tests. Development of specific QCMD trials based on methodologic proficiency testing and directed to the evaluation of analytical aspects common to the majority of PCR –based tests may be valuable. The purpose of this study was to evaluate the specifity and sensitivity of molecular amplification methods for MTBC detection and our laboratory performance. Proficiency panel specimens were obtained from QCMD 2002 TB Proficiency Panel for the Assessment of Mycobacterium tuberculosis Nucleic Acid Detection Methodologies Program, Scotland, UK. The proficiency panel consisted of 12 (8 sputum and 4 diluted samples) samples containing a range of concentrations of cultured Mycobacterium bovis BCG in either pooled sputum or presented as a decontaminated and washed cell pellet . Negative samples were also included in the panel. In the present study, all samples sent in the framework of QCMD 2000, were evaluated using three different diagnostic methods, namely COBAS Amplicor MTB, Gen Probe MTD, Taq Man RT-PCR / Gene Amp 7700 sequence detection system. All steps of the tests used during the study were made in accordance with standard protocols and instructions of the manufacturer. According to these results, COBAS Amplicor MTB test yielded consistent results in 10 (83.3%), Gen Probe MTB test in 8 (66.66%) ve RT-PCR test in 6 (50%). Our success rates were 100 % (8/8), in sputum samples and 50 % (2/4) in diluted samples. Multicenter quality control programs are quite illuminating for the determination of laboratory efficacy and the performance of the tests. It is our suggestion that nucleic acid amplification methods employed for rapid diagnosis of M. tuberculosis should be subjected to internal and external controls and made routine accordingly.

Key Words: Tuberculosis, Quality Control, Molecular Diagnosis, PCR

Introduction

Recently, in addition to the conventional methods, molecular methods have been used in the diagnosis and monitorisation of mycobacterial infections, due to their rapidity and wide reference ranges. Many commercial and in house methods are being used in routine laboratories. However, the specificity and sensitivity of tests vary largely according to the methods used in laboratories. These studies have a high cost and require experienced and trained personnel as there is no quality standard for each test, in order to determine the reliability of tests and sensitivity and specificity of methods especially those used for clinical diagnosis (1-7). Therefore, each laboratory carrying out routine tests should perform external control using multi-center quality control programs in addition to internal quality control, which is of great

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significance for evaluating reliability and efficacy. The purpose of this study was to evaluate the specificity and sensitivity of molecular amplification methods for MTBC detection and our laboratory performance.

Materials and Methods

Study Groups: Proficiency panel specimens were obtained from QCMD 2002 TB Proficiency Panel for the Assessment of *Mycobacterium tuberculosis* Nucleic Acid Detection Methodologies Program (Scotland, UK). The proficiency panel consisted of 12 samples containing a range of concentrations of cultured *Mycobacterium bovis BCG* in either pooled sputum or presented as a decontaminated and washed cell pellet . Negative samples were also included in the panel.

Specimen Processing: Samples 1 to 8 required decontamination and liquefaction of the whole 250 ml sputum sample to be performed in the microtube containing the sample. Samples 9 to 12 were to be considered as pellets from clinical samples after decontamination and washing, and to be treated accordingly. Eight sputum samples (250 ml) were decontaminated according to Dio-Safeprocess " decontamination and concentration kit (Diomed, Inc., Istanbul, Turkey). In summary, each specimen was collected containing NALC and glass beads in a sterile 50-ml conical polypropylene screw-cap centrifuge tube and added to a volume of NaOH (final concentration, 3%) solution equal to the volume of the specimen. The tubes were agitated on a vortex mixer, and kept for 15 min at room temperature (20 to 25 ^{0}C). Each mixture was diluted to the 50-ml level with sterile 0.067M phosphate buffer (pH : 6.8). After centrifugation at 4,000Xg for 10 min, the supernatant was removed. Each pellet was resuspended in 1.0 ml sterile 0.067 M phosphate buffer (pH : 6.8).

Gen Probe MTD (AMTD): The Gen-Probe MTD assay (Gen-Probe, Inc., San Diago, California) was performed according to the manufacturer's instructions.

Each run included positive and negative amplification controls. 450 µl of specimen was placed in a tube containing lysing solution, vortexed, and sonicated for 15 min.

A 25-µl volume of lysate was transferred into an amplification tube containing amplification reagent. Tubes were incubated for 15 min at 95°C in a water bath. Tubes were transferred to a 42°C water bath and left to cool for 5 min. Enzyme reagent (25 µl) was added to each tube and mixed, and the mixture was incubated for 30 min at 42°C. Hybridization reagent (100 µl) was added to each tube, vortexed, and incubated for 15 min at 60°C in a water bath. Tubes were removed, 300 µl of selection reagent was added, and the mixture was vortexed and incubated for 15 min at 60°C in a water bath. Tubes were cooled and placed in a luminometer to determine the number of relative light units (RLU) produced by the reaction. The cut off value was set by the manufacturer : samples with values of ≥ 30,000 RLU were considered positive, and samples with values of < 30,000 RLU were considered negative.

COBAS Amplicor MTB: The COBAS Amplicor MTB (Roche Diagnostics, Inc., Branchburg, USA) was performed according to the manufacturer's instructions. In brief, 100 ml of sediment was washed in 500 ml of Specimen Wash Solution. After lysis at 60 ^{0} C for 45 min in 100 ml of Specimen Lysis Reagent, the samples were neutralized by addition of 100 ml of Specimen Neutralization Reagent. Fifty microliters from each of the prepared specimens, negative controls and positive controls were transferred to tubes containing the master mix and loaded into the COBAS Amplicor MTB apparatus for the automated amplification and detection process. Amplification results of the *M.tuberculosis* – specific probe (MTB) and of the internal control (MCC) were recorded in optical density (OD) units (cut off, 350 OD).

Taq Man Real Time PCR (RT-PCR) protocol: RT-PCR assay (Perkin Elmer/ Applied Biosystems, Foster City, CA, USA) was performed according to the manufacturer's instructions. The test was conducted in two stages, namely DNA extraction and amplification. In the DNA extraction procedure, the sonication method recommended by the manufacturer was used.

1- DNA extraction: A 300 ml sample was centrifuged at 5000 rpm. After the supernatant was removed, 300 ml distilled water was added. This procedure was repeated three times. After irrigation, the pellet was suspended with 300ml distilled water. The suspension was transferred to lysing tubes. The tubes were kept first in a sonicator, then in 95 ^{0}C heat block for 20 minutes. Samples were transferred to microcentrifuge tubes and centrifuged at 5000 rpm for 3 minutes. The supernatant

was transferred to a clean tube and used as template DNA.

2-Amplification: In the amplification procedure, TBCP1 (5'-GATCTCGTCCAGC GCCGCTTCG-3') ve TCBP2 (5'-ACCGACGCCTACGCTCGCAGG-3') primers, which amplify 163 bp fragment of IS6110 gene location at 1355 bp length in *Mycobacterium tuberculosis* genome was used. The amplified product was detected by using specific fluorescent probes (FAM – GCTACCCACAGCCGGTTAGGTGCTGGTG-TAMRA.) For each sample, 5 ml of template DNA was incorporated into 45 ml PCR containing the amplification Tuberculosis Real Time PCR mix. Amplification procedure was carried out in the ABI PRSIM 7700 SDS (Applied Biosystems / Gene Amp 7700) system. The optimized TaqMan RT-PCR protocol included an initial step at 50 ^{0}C 2 min, then 95 ^{0}C 10 min, followed by a touchdown PCR protocol using the following conditions: 95 ^{0}C for 15 s, 61.5 ^{0}C for 1 min for 40 cycles. The threshold cycle (Ct) value is the cycle at which there is a significant increase in fluorescence, and this value is associated with an exponential growth of PCR product during the log-linear phase. Test results were calculated according to the Ct value which was compared with positive and negative controls. A Ct value of 10-20 was considered high positive, 25-30 medium, 30-35 low positive and 35 or higher negative.

Results

In the present study, 8 sputum and 4 diluted samples sent in the framework of QCMD 2002, were evaluated using three different diagnostic methods, namely the COBAS Amplicor MTB, Gen Probe MTD, Taq Man RT-PCR / Gene Amp 7700 sequence detection system. All steps of the tests used during the study were made in accordance with standard protocols and instructions of the manufacturer. Results were interpreted quantitatively in COBAS Amplicor MTB and Gen Probe MTD tests according to cut off values, and in the RT-PCR test according to amplification curve and Ct values.

When all results were evaluated comparatively, in samples 1, 2, 4 , 5 , 11 and 12, the same results were obtained in all three tests. In other samples, results were different in different tests. Results are outlined in Table 1.

Table 1. The cut off value and study results of panel samples.

		Study Results					
		COBAS AMPLICOR MTB		Gen Probe MTD		TaqMan RT-PCR 7700	
Number of sample	Type of sample	Cut-off (OD)	Results([a])	Cut-off (RLU)	Results([b])	Ct Value	Results([c])
1	Sputum	3.764	+	699903	+	27.52	+
2	Sputum	0.725	+	578187	+	31.66	+
3	Sputum	0.432	+	2837823	+	40.00	-
4	Sputum	0.029	-	3660	-	40.00	-
5	Sputum	3.764	+	2319978	+	33.84	+
6	Sputum	1.046	+	2925148	+	40.00	-
7	Sputum	0.024	-	3987	-	33.91	+
8	Sputum	4.000	+	4256	-	30.22	+
9	Dilution	0.455	+	5306	-	40.00	-
10	Dilution	2.833	+	4088	-	40.00	-
11	Dilution	0.015	-	4277	-	40.00	-
12	Dilution	0.049	-	4703	-	40.00	-

([a]): The interpretive criteria for the COBAS AMLICOR MTB assay reading were as follows: OD ≥ 0.350, positive; OD of < 0.350, negative; OD of (*.***) ≥ 4.000, positive as manufacturer's ecommendation(OD:Optic Density).

([b]): The cutoff value was set by the manufacturer : samples with values of ≥ 30,000 RLU were considered positive, and samples with values of <30,000 RLU were considered negative(RLU: Relative Light Unit).

([c]): The threshold cycle (Ct): 10- 20 high positive,25-30 medium , 30-35 low positive and 35 or higher negative.

The characteristics of the samples prepared using different dilutions of *Mycobacterium bovis* BCG for study in the QCMD 2002 quality control program and our test results are illustrated in Table 2.

According to these results, of the 12 samples evaluated in the study, the COBAS Amplicor MTB test yielded consistent results in 10 (83.3%) , Gen Probe MTB test in 8 (66.66%) ve RT-PCR test in 6 (50%) .

Discussion

Diagnostic techniques based on amplification have the potential to increase the sensitivity for detecting mycobacteria as well as to dramatically reduce the time usually necessary to detect and identify these organisms in clinical specimens. Often quite demanding as far as technical equipment and operation skills are concerned, many of the protocols for detecting MTBC do not, fit easily into a clinical laboratory's work flow. In addition to the amplification technique, including lysing methods, target nucleic acids, primers and the procedures used to detect amplified products are used. The reported senstivities and specifities are difficult to compare. Therefore, quality control in routine tests is the most important step. It has become possible to determine the reliability and laboratory performance of the tests by means of multi-centered quality control programs.

In the present study, 12 standardized samples were evaluated in the framework of the QCMD *Mycobacterium tuberculosis* Proficiency Programme. At this stage, each laboratory used its own methods. Results of all participating laboratories were evaluated by the QCMD 2002 committee and a report was prepared. Of these methods, the most suitable ones in our laboratory were COBAS Amplicor MTB, Gen Probe MTD and In-House Real Time PCR.

According to reference results reported in the QCMD-2002 program, our success rates were 100 % (8/8), in sputum samples and 50 % (2/4) in diluted samples. DNA copy numbers in the suspensions of samples 9 and 12, that yielded incompatible results, were reported to be 10 and 100 copies / sample, respectively. The number of DNA copies in the main suspensions of samples 9 and 12, which yielded inconsistent results, were reported to be 10 and 100 copies/ sample respectively. In order to have the necessary volume required for three different tests, sputum samples were diluted after decontamination procedure and dilution samples were directly diluted at the ratio of 1/100, to obtain a final volume of 1.0 ml It was thought that copies in these samples may not be distributed homeogenously and adequately and that therefore, there may be inconsistencies between three different tests and reference results. Cut-off value of sample 9, which included 10 copies/ 10ml sample in the

Table 2. The test results of QCMD 2002 quality control program and our study results.

					Present study Results			
Number of sample	Type of sample	Volume	CFU/ Sample	Number of DNA copies	COBAS Amplicor MTB	Gen Probe MTD	TaqMan RT-PCR 7700	Expected Results
1	Sputum	250ml	6500	6500	+	+	+	+
2	Sputum	250ml	650	650	+	+	+	+
3	Sputum	250ml	650	650	+	+	-	+
4	Sputum	250ml	0	0	-	-	-	-
5	Sputum	250ml	2.4	10.000	+	+	+	+
6	Sputum	250ml	6500	6500	+	+	-	+
7	Sputum	250ml	0	0	-	-	+	-
8	Sputum	250ml	60	250000	+	-	+	+
9	Dilution	10ml	0.002	10	-	-	-	+
10	Dilution	10ml	0.24	1000	+	-	-	+
11	Dilution	10ml	0	0	-	-	-	-
12	Dilution	10ml	0.024	100	-	-	-	+

main suspension, was 0.455 in COBAS Amplicor MTB test. As this value was close to negativity (OD of < 0.350), it was considered a doubtful positive and studied again. As negative results were obtained in the repeated study, this sample test result were reported to be negative. In all participating labratories, this sample was defined correctly at the rate of 23% and incorrectly at the rate of 74% and doubtful at the rate of 2%.(+/-). Likewise, the cut off value of sputum sample number 3 was negative, but since the sample examined was sputum and FDA approved especially respiratory tract sample, it was regarded to be a true positive.

In the framework of the program, diagnostic performance of each test used in all laboratories was evaluated separately according to the number of DNA copies in each sample (Table 3). In the table, it is shown that the 4 methods used most frequently by the participating laboratories are COBAS Amplicor MTB, GenProbe MTD ve Real Time PCR. Of these methods, the test that displayed the best performance both with sputum and diluted samples was observed to be COBAS Amplicor MTB. Our success with these tests was as follows: COBAS Amplicor MTB test 83.3, % Gen Probe MTB test 66.6% ve RT-PCR test 50.0 %.

According to the studies and the data of the manufacturer, the rate of obtaining M. tuberculosis DNA for each test was as follows (ml): COBAS Amplicor MTB 10 copies, Gen Probe MTD 1copies , RT-PCR 10 copies. According to the principles of the QCMD-2002 program, participating laboratories are not informed in advance about the amount of bacteria and the number of DNA copies in the samples. In addition, the study protocol sent to us together with sample panel does not include clear information regarding maximum dilution of the samples and maximum number of biomolecular tests that can be studied with this sample. As the aim of the present study was the external control of all three methods, original diluted samples (i.e, samples 9-12) were diluted at the rate of 1/100 to reach 1ml volume which would be sufficient for all three tests. Therefore, we consider that the test performance in these samples that contained few number of DNA copies (especially 9 and 12) may be influenced not only by the method and laboratory application but also by the quantity of samples studied. Therefore, it would be more suitable to focus mostly on samples of sputum. In 8 sputum samples, COBAS Amplicor MTB test had 100%, Gen Probe MTD test 87.5% and RT-PCR test 62.5 %success rates. The cause

Table 3. Number of correct results per panel member and type of assay.

		Percentage Correct									
			Commercial					In-House			
Sample Number	Contents DNA copies per sample	All Tests n=82(%)	Roche Cobas n=27(%)	Roche Manual n= 5(%)	BD Probe TEC n=4(%)	Gene Probe TMA n=16(%)	Com'cial Other* n=2(%)	Real-Time PCR n=10(%)	Nested/ Semi-nested PCR n=6(%)	PCR + Gel n=5(%)	PCR +Hyb** n=7(%)
TB02-04	0	79(96)	26(96)	5(100)	4(100)	15(94)	2(100)	10(100)	6(100)	4(80)	7(100)
TB02-0	0	76(93)	25(93)	5(100)	4(100)	15(94)	1(50)	9(90)	6(100)	4(80)	7(100)
TB02-02	650	64(78)	22(81)	5(100)	3(75)	8(50)	2(100)	7(70)	5(83)	5(100)	7(100)
TB02-03	650	47(57)	10(37)	0(0)	3(75)	10(63)	2(100)	6(60)	4(67)	5(100)	7(100)
TB02-01	6500	79(96)	25(93)	5(100)	4(100)	16(100)	2(100)	9(90)	6(100)	5(100)	7(100)
TB02-06	6500	77(94)	23(85)	5(100)	4(100)	16(100)	2(100)	9(90)	6(100)	5(100)	7(100)
TB02-05	10000	80(98)	27(100)	5(100)	4(100)	14(88)	2(100)	10(100)	6(100)	5(100)	7(100)
TB02-08	250000	76(93)	27(100)	5(100)	4(100)	11(69)	2(100)	9(90)	6(100)	5(100)	7(100)
TB02-11	0	78(95)	26(96)	5(100)	4(100)	15(94)	2(100)	10(100)	6(100)	3(60)	7(100)
TB02-09	10	19(23)	8(30)	1(20)	0(0)	2(13)	1(50)	1(10)	3(50)	1(20)	2(29)
TB02-12	100	47(57)	23(85)	4(80)	2(50)	2(13)	2(100)	3(30)	4(67)	2(40)	4(57)
TB02-10	1000	60(73)	26(96)	5(100)	3(75)	2(13)	2(100)	8(80)	5(83)	3(60)	6(86)

of the negative results obtained in 8 no. sputum sample, although there are 250.000 DNA copies, could not be explained . In In – House RT- PCR tests, the amplification step is standardized by the producer, but there is no standard method for the extraction step. Methods used in this step are selected according to the manufacturer or laboratories.

In In -house PCR tests, although the chance of diagnosis increases with the quality of probes and primers, the most important stage influencing the success of the test is the extraction of DNA with high purification. In the present study, in accordance with the recommendations of the manufacturer, the sonication extraction method was used and 62.5% success was obtained in sputum samples, as there is no detailed information on extraction and amplification methods of laboratories achieving high success rates with the same test . It is our belief that our low level of performance may be especially related to extraction.

Nucleic acid amplification methods are used all over the world for rapid diagnosis of MTBC. However, there is no standard method that can be used in all laboratories. Specificity and sensitivity of tests vary according to laboratory procedure and performance of the tests. Multicenter quality control programs are quite illuminating for the determination of laboratory efficacy and the performance of the tests.

We conclude that nucleic acid amplification methods to be employed for rapid diagnosis of *Mycobacterium tuberculosis complex* should be subjected to internal and external controls and made routine accordingly.

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References

1. Noordhoek GT, Kolk AHJ, Bjune G. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. J Clin Microbiol 32 : 277-284, 1994.
2. Noordhoek GT, Embden JV, Kolk AHJ. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among 30 laboratories. J Clin Microbiol 34 :2522-2525, 1996.
3. Crawford JT. New technologies in the diagnosis of tuberculosis. Semin Respir Infect 9 : 62-70, 1994.
4. Centers for Disease Control and Prevention. Update: nucleic acid amplification tests for tuberculosis. Morb Mortal Wkly Rep 49:593-594, 2000.
5. Rosenstraus M, Wang Z, Chang SY et al. An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. J Clin Microbiol 36: 191-197, 1998.
6. Bennedsen J, Thomsen V, Pfyffer GE et al. Utility of PCR in diagnosing pulmonary tuberculosis. J. Clin. Microbiol 34:1407-1411, 1996.
7. Scarparo C, P. Piccoli A, Rigon G et al. Comparison of enhanced *Mycobacterium tuberculosis* Amplified Direct Test with COBAS AMPLICOR *Mycobacterium tuberculosis* assay for direct detection of *Mycobacterium tuberculosis* complex in respiratory and extrapulmonary specimens. J. Clin. Microbiol. 38:1559-1562, 2000.
8. Noordhoek GT, Mulder S, Wallace P et al. Multicentre quality control study for detection of Mycobacterium tuberculosis in clinical samples by nucleic amplification methods. Clin Microbiol Infect 10 : 295-301,2004.