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Multicenter Evaluation of Two Commercial Amplification Kits (Amplicor, Roche and LCx, Abbott) for Direct Detection of *Mycobacterium tuberculosis* in Pulmonary and Extrapulmonary Specimens

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Direct detection of Mycobacterium tuberculosis *was performed in parallel with the Amplicor* M. tuberculosis *test (Roche Diagnostic System, USA) and the LCx* M. tuberculosis *(Abbott Diagnostic Division, USA) on 697 samples, collected from 481 patients, in three different Italian laboratories. Though both systems are licensed only for pulmonary specimens, 113 extrapulmonary specimens (represented mainly by pleural fluids, cerebrospinal fluids and urines) were included in the study. Amplification results were compared with acid-fast microscopy, culture, and identification of isolates. Final clinical diagnosis was used to resolve discrepant results.* M. tuberculosis *was detected in 105 specimens by both assays, whereas 561 were agreeing negatives; 21 and 6 of the remaining true-positive samples scored positive with LCx only and with Amplicor only, respectively. There were three false-positives with LCx and one false-positive with Amplicor. The diagnostic sensitivity of both methods was significantly better when only respiratory specimens were considered (78% versus 59% in nonrespiratory samples with Amplicor, and 88% versus 65% with LCx). Our data reveal a significantly better sensitivity of the LCx (*p = 0.026*) and a slight better specificity of the Amplicor assay. It is noteworthy that 16 of the 21 Amplicor-negative specimens in which LCx detected* M. tuberculosis *were culture negative, thus suggesting that the higher diagnostic sensitivity of the latter assay is attributable to its better analytical sensitivity. However, the majority of such samples originated from patients under antimicrobial treatment, which makes uncertain the clinical significance of such increased sensitivity. Considering true-positive for LCx and true-negative for Amplicor, the 16 culture-negative/LCx-positive/Amplicor-negative specimens resulted true-positives after the resolution of discrepancies, the final overall sensitivity and specificity values of the LCx assay were not significantly different from the ones of the Amplicor assay.*

INTRODUCTION

Tuberculosis is re-emerging as a major problem, not only in the third world, but also in developed countries, where numerous community outbreaks have been reported (Raviglione et al. 1995). Furthermore the presence of multiple drug resistance in several

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strains of *Mycobacterium tuberculosis* (Perronne 1993) can greatly worsen the outcome of the disease, with an increase of fatality ratios. A prompt diagnosis of new cases is a prerequisite for the prevention of secondary cases (Tenover et al. 1993).

The availability of commercial amplification kits made easier the introduction of molecular diagnostic procedures in mycobacteriology laboratories. Sensitivity and specificity of such commercial assays have been thoroughly assessed, but little has been done to compare them directly. LCx *M. tuberculosis* (Abbott Diagnostic Division, Abbott Park, Ill, USA) is the most recent commercial amplification kit, and we carried out a multicenter assessment of its performance, in direct comparison with the well tested (Beavis et al. 1995; Bergmann and Woods 1996; Carpentier et al. 1995; Cartuyvels et al. 1996; D'Amato et al. 1995, 1996; Devallois et al. 1996; Huang et al. 1996; Ichiyama et al. 1996; Moore and Curry 1995; Piersimoni et al. 1997; Schirm et al. 1995; Soini et al. 1996; Wobeser et al. 1996) Amplicor *M. tuberculosis* assay (Roche Diagnostic System, Branchburg, NJ, USA).

MATERIALS AND METHODS

Clinical Specimens

The comparison was based on 697 clinical specimens from 481 patients, and it was performed in three different laboratories (Table 1). Whereas laboratory B included into the comparison 303 consecutively received samples, laboratories A and C limited the analysis to about 50% of specimens processed during the experimentation period, which lasted 3 months. A volume of the sample lower than 5 mL and the provenance from divisions known for their low prevalence of *M. tuberculosis* isolations were the main criteria adopted for the exclusion.

Respiratory specimens, for which both amplification methods to be tested are licensed, represented the majority of our study sample (83.8%), but we also included 113 specimens from nonrespiratory body sites (Table 2). All samples from nonsterile sites were processed according to the NALC-NaOH digestion-decontamination method presenting a final NaOH concentration of 1% (Nolte and Metchock 1995). On centrifuged sediments, resuspended to a final volume of 2 mL in distilled water, fluorescent acid-fast microscopy, culture, and both the amplification assays were performed. Culture was performed by inoculating 0.2 mL in a couple of Lowenstein-Jensen slants, which were incubated at 37°C for 8 weeks, and 0.5 mL in a radiometric vial (Bactec, Becton Dickinson, Towson, MD, USA), incubated at the same temperature up to 6 weeks. Mycobacteria isolated were identified by means of conventional tests (Nolte and Metchock 1995), high-performance liquid chromatography (Tortoli and Bartoloni 1996) and/or DNA-probes (Stockman 1992).

TABLE 2 Specimens Tested

Specimen	Samples	Patients
Sputum	389	219
Bronchial aspirate	192	161
Pleural fluid	29	28
Cerebrospinal fluid	28	25
Urine	23	16
Biopsy specimen	15	12
Pus	13	13
Gastric aspirate	3	2
Ascites fluid	3	3
Synovial fluid	2	2
Total	697	481

Amplicor *M. tuberculosis* Assay

Amplicor test was performed according to the manufacturer's instructions as reported before (Beavis et al. 1995). In short, 100 μL of each pre-processed sample were washed with 500 μl of Wash Solution and the pellet was resuspended with 100 μL of Sputum Lysis Reagent and incubated for 45 min at 60°C. Inactivated tube was added with 100 μL of Sputum Neutralization Reagent and 50 μL were transferred to an amplification tube containing 50 μL of previously prepared Master Mix (by adding to the amplification reagents the contamination-preventing system uracil-*N*-glycosylase, AmpErase). Each series of samples was performed in parallel with a positive and three negative controls. The amplification protocol consisted of 2 min at 50°C, two cycles of 98°C for 20 s, 62°C for 20 s, and 72°C for 45 s, and 35 cycles of 94°C for 20 s, 62°C for 20 s and 72°C for 45 s, with a

TABLE 1 Specimens Processed by Participating Centers

Center	Specimens (no.)	Pulmonary (no.)	Extra-pulmonary (no.)	Culture positive for *M. tuberculosis* (%)	Amplicor positive (%)	LCx positive (%)
A	315	231	84	20.00	20.00	23.17
B	303	291	12	6.60	8.58	10.89
C	79	62	17	34.18	28.11	29.11
Total	697	584	113	15.78	16.06	18.50

final maintenance at 72°C in a thermal cycler. Amplification was followed by denaturation of double-stranded amplicons with 100 μL of Denaturation Solution. Microwell plates coated with a DNA probe specific for *M. tuberculosis* complex were used for the capture of amplification products and final detection was achieved by incubating the washed microwells with 100 μL of Avidin-Horseradish Peroxidase Conjugate and by adding, after further washing, 100 μL of the Substrate reagent. The optical density was measured at A_{450} and samples with absorbency equal to or greater than 0.35 were considered positive.

LCx *M. tuberculosis*

Manufacturer's recommendations were followed for LCx test (Ausina et al. 1997). Briefly, 500 μL of each pre-processed sample were transferred to a ready to use screw-cap microcentrifuge tube containing Respiratory Specimen Buffer and glass beads; the tube was subsequently centrifuged and the pellet was washed with 1 mL of Resuspension Buffer and suspended with 500 μL of the same buffer. The suspension, after inactivation for 20 min at 95°C was lysed by sonication for 10 min and 100 μL of supernatant were transferred to a tube containing the amplification mixture. Amplification was performed for 37 cycles in a thermal cycler as follows: 94°C for 1 s, 64°C for 1 s and 69°C for 40 s with a 25°C holding at the end of the last cycle. For each series of tests (including no more than 20 samples) provided negative control and calibrator were prepared in duplicate and subjected to the same amplification procedure as the samples. Amplified tubes were transferred unopened to the carousel of LCx analyzer, which directly detects the amplification products by a microparticle enzyme immunoassay reporting the results as fluorescence rates that are compared to the calibrator rate; results greater than 30% of calibrators average are considered positive.

Resolution of Discrepancies

A resolution of conflicting results was made according to the final judgement of the clinician in charge after revision of clinical, radiological, and immunological (PPD reactivity) data.

Statistical Analysis

The differences between positivity rates were compared using the χ^2 method or Fisher's exact test, when appropriate, whereas differences between means were analyzed by Student's *t* test.

RESULTS

One hundred thirty-five specimens grew mycobacteria in culture, 110 of which were identified as *M. tuberculosis;* of the latter, 84 came from microscopy-positive samples, 26 from negative ones. The 25 non-tuberculous strains included eight *Mycobacterium avium,* eight *Mycobacterium xenopi,* five *Mycobacterium kansasii,* three *Mycobacterium gordonae,* and one *Mycobacterium celatum.*

The two amplification methods yielded very close values of sensitivity and specificity in comparison with culture (Table 3).

An agreement between the two amplification procedures was obtained for 105 positive and 561 neg-

TABLE 3 Initial Comparison of Amplicor and LCx Assays, with Culture, for Detection of *M. tuberculosis*

Specimen	No. of specimens for which *M. tuberculosis* culture results were:				Sensitivity		Specificity
	Positive and amplification results were:		Negative and amplification results were:				
	Positive	Negative	Positive	Negative	%	p[a]	%
All (Amplicor)	90	20	22	565	81.82	n.s.	96.25
All (LCx)	89	21	40	547	80.91		93.19
Smear positive (Amplicor)	81	3	13	23	96.43	n.s.	63.89
Smear positive (LCx)	77	7	16	20	91.67		55.56
Smear negative (Amplicor)	9	17	9	542	34.62	n.s.	98.37
Smear negative (LCx)	12	14	24	527	46.15		95.64
Respiratory (Amplicor)	80	14	22	468	85.11	n.s.	95.51
Respiratory (LCx)	79	15	37	453	84.04		92.45
Nonrespiratory (Amplicor)	10	6	0	97	62.50	n.s.	100.00
Nonrespiratory (LCx)	10	6	3	94	62.50		96.91

[a] n.s., not significant.

ative samples (Table 4). Conflicting results were found in 31 cases, 24 positives with LCx alone and 7 with Amplicor alone. Three of the positive results obtained by LCx alone were on samples from patients not presenting any sign or symptom of tuberculous disease and were thus considered as false positives. The only false-positive result obtained with Amplicor concerned a specimen which subsequently grew *M. celatum.* The remaining 27 discrepancies (21 LCx positives, 6 Amplicor positives) were scored as true positive: 11 were culture-confirmed, 5 were resolved on clinical grounds and 11 were obtained from patients with a previous cultural positivity.

After resolution of discrepancies LCx turned out to be significantly more sensitive than Amplicor (Table 5) both on sample considered as a whole and, once the samples were disagreed, on ones smear-negative and respiratory. As expected, the diagnostic sensitivity of either method was clearly higher on smear-positive than on smear-negative specimens (Table 5). The diagnostic sensitivity on nonrespiratory specimens, although significantly lower (p = 0.008) than that on respiratory ones, remained near 60% with either amplification method.

LCx, differently from Amplicor, detected several culture-negative samples originating from patients who had previously grown *M. tuberculosis.* The time elapsed from the last positive culture ranged between 35 and 110 days.

The overall specificity was 99.82% for Amplicor and 99.45% for LCx (p = n.s.). Samples scoring a false-negative amplification result were 11 by LCx, 23 by Amplicor, and 8 by both assays. Not always, these false-negatives implied a misdiagnosis, in fact, among patients who yielded more than one sample, the LCx test detected tuberculosis in five of seven cases (71%); the Amplicor in 9 of 17 (53%; p = n.s.).

DISCUSSION

Usually the evaluations of amplification systems resort to final resolution, on the basis of clinical diagnosis of tuberculosis, only for cases characterized by results disagreeing with the culture. In this study a further source of discrepancies is represented by the parallel use of two biomolecular methods that revealed a number of disagreements between the amplification results. While, limiting the use of resolution to discrepancies among amplification and culture, Amplicor and LCx seem characterized by practically overlapping values of sensitivity and specificity, once adopted discrepancies resolution following the latter criterion, a significantly better sensitivity of LCx emerges. The importance of this difference (Table 4), which does indeed reflect a better sensitivity of LCx, is however hardly valuable; in fact, from a clinical point of view, the significance of detection of *M. tuberculosis* DNA in specimens from patients under antimycobacterial treatment, like many in our study, remains still undetermined.

Both the inoculum size (100 versus 500 μL) and the possible presence of inhibitors could explain the lower sensibility of the Amplicor assay, in fact, although neither Amplicor nor LCx implement inhibitors detection, the latter assay includes a step for their removal.

The number of false positive results, three with LCx and one with Amplicor, suggests a better specificity, although statistically nonsignificant, for the latter. The only explanation for the three false-positives obtained with LCx seems to be an accidental contamination during the handling of the samples: a carryover in the detection phase can be excluded by the position of tubes in the LCx carousel, because none of the three followed a positive specimen.

The sample which scored positive with Amplicor and negative with LCx yielded *M. celatum* in culture. Cross reactivity of *M. tuberculosis* and *M. celatum* Type 1 has been already reported for the DNA probes AccuProbe *M. tuberculosis* (Gen-Probe, San Diego, Calif. USA) and has been imputed to the close similarity of genetic sequences of the two species within 16S rRNA region (Butler et al. 1994). Interestingly both Amplicor and AccuProbe aim to a stretch within this gene, whereas the target chosen for LCx is the gene encoding for the protein antigen b.

The sensitivity and specificity of the Amplicor assay have been estimated extensively, with rather

TABLE 4 Direct Comparison of Amplicor and LCx Assays

Culture result	Amplicor positive LCx positive	Amplicor negative LCx negative	Amplicor positive LCx negative	Amplicor negative LCx positive
Positive	84	15	6	5
Negative	21	546	1[a]	19[b]
Total	105	561	7	24

[a] False positive.
[b] Including three false-positives.

TABLE 5 Assessment of Amplicor and LCx Assays, after Resolution of Discrepancies, in Comparison with the Clinical Diagnosis of Tuberculosis

Specimen	No. of specimens for which clinical diagnosis of tuberculosis was:				Sensitivity		Specificity (%)
	Positive and amplification results were:		Negative and amplification results were:				
	Positive	Negative	Positive	Negative	(%)	p[a]	
All (Amplicor)	111	36	1	549	75.51	0.026	99.82
All (LCx)	126	21	3	547	85.71		99.45
Smear positive (Amplicor)	94	6	0	20	94.00	n.s.	100.00
Smear positive (LCx)	93	7	0	20	93.00		100.00
Smear negative (Amplicor)	17	30	1	529	36.17	0.002	99.81
Smear negative (LCx)	33	14	3	527	70.21		99.43
Respiratory (Amplicor)	101	29	1	453	77.69	0.020	99.78
Respiratory (LCx)	115	15	1	453	88.46		99.78
Nonrespiratory (Amplicor)	10	7	0	96	58.82	n.s.	100.00
Nonrespiratory (LCx)	11	6	2	94	64.71		97.92

[a] n.s., not significant.

variable results, ranging from 61.82 (D'Amato et al. 1995) to 95.60 (Beavis et al. 1995) for sensitivity and from 80.33 (Soini et al. 1996) to 99.33 (Bergmann and Woods 1996) for specificity, before resolution of discrepancies. The composition of the study samples may be a major responsible of such a variability, higher estimates being obtained in studies with a high prevalence of positive specimens. Our estimates, which are based on the pooled results form centers with varied prevalence of positive specimens, rightly fall in the middle of the range of published ones. Only six evaluations of LCx assay have been reported so far. Though the purpose of the first study (Yuen et al. 1997), in which the LCx assay was compared with the automated version of the Amplicor test implementing the Cobas Amplicor (Roche) instrumentation, was very close to ours, the results were quite different from ours and from the ones of the remaining five reports (Ausina et al. 1997; Gamboa et al. 1898; Lindbråthen et al. 1997; Moore and Curry 1998; Tortoli et al. 1997). In such comparison both methods showed in fact very low values of sensitivity, particularly so on smear-negative specimens; furthermore in that study, differently from ours, Amplicor performed better than LCx in terms of sensitivity. In our opinion the use of whole samples for microscopy and cultures, and of diluted samples for the amplifications, might have lowered the level of sensitivity. The discrepancy in results concerning direct comparison of the two methods remains unexplained, all the more since as at present no data are available that allow to hypothesize an improvement of Amplicor performance related to the introduction of Cobas automation.

Concerning the possible use of Amplicor and of LCx for the detection of *M. tuberculosis* in nonrespiratory specimens, we believe that the lower diagnostic sensitivity is more likely to be related to a lower microbial density than to the source. Extrapulmonary specimens are in fact in most cases paucibacillar as confirmed in the present study by the prevalence of microscopy positives (11%) clearly below that of samples originating from the lung (18%). In this view the limitation of amplification techniques to pulmonary specimens only does not seem justifiable, provided that there is the awareness, for extra pulmonary likewise for all weakly positive specimens, of possible false negative results.

Albeit both methods are licensed for a qualitative evaluation, they report amplification results in a quantitative manner as a numeric score that is compared to a cutoff value. However, only in LCx system the sample to cutoff ratio seemed to be related to the mycobacterial burden of the sample. LCx signal, differently from the one of Amplicor, was in fact significantly higher in smear-positive (5.06 ± 1.49) than in smear-negative samples (3.13 ± 1.48; $p < 0.01$), and, among culture-negative specimens, on samples obtained from patients with a diagnosis of tuberculosis in comparison to the others. Besides, LCx-positives scored significantly higher when Amplicor too was positive than when it was negative ($p < 0.01$).

Both tests can be completed within 6 h, and both require a labor intensive processing of the samples before the amplification step. They differ however in the identification step, which is fully automated in the LCx assay, while it requires a further handling with the Amplicor. Also, Amplicor uses eight-well stripes, which restricts the number of samples which can be batch processed; on the contrary, with LCx the batch run is only limited by the carousel capacity (24 places). In both methods the inclusion of four con-

trols per session must also be considered when assessing the working efficiency and cost.

On the basis of our experience we thus conclude that the novel LCx assay performs at least as well as the thoroughly validated Amplicor assay, the increased sensitivity being slightly counterbalanced by a small loss of specificity. The automation of LCx certainly represents an important extra bonus when the comparison, as in this study, involves the manual version of Amplicor. In the presence of balanced performances the cost effectiveness may become the decisive factor; unfortunately the great variability of prices in various European countries does not allow to reach unambiguous conclusions.

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