Clinical utility of the polymerase chain reaction in the diagnosis of extrapulmonary tuberculosis

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Clinical utility of the polymerase chain reaction in the diagnosis of extrapulmonary tuberculosis. A.H.J. Kolk, L.F.F. Kox, J. van Leeuwen, S. Kuijper, H.M. Jansen. ©ERS Journals Ltd 1998.

ABSTRACT: This study examines the diagnostic utility of the polymerase chain reaction (PCR) in 156 patients (five human immunodeficiency virus (HIV) seropositive) suspected of extrapulmonary tuberculosis.

The results of PCR in 226 samples from 11 different sites were compared with the results of microscopy and culture.

Positive culture results were predicted in 86% of samples by PCR but in only 31% by microscopy. Specificity of PCR was 92%. In cases with culture-proven tuberculosis, PCR identified all 11 microscopy positive cases and 19 of 24 (79%) of the microscopy-negative cases. In four patients, PCR excluded the diagnosis of tuberculosis in microscopy-positive samples, which were later shown to contain mycobacteria other than *Mycobacterium tuberculosis* or laboratory contaminants. In 20 patients (microscopy, PCR and culture negative) a trial of antituberculous drugs was given, but patients showed no improvement and treatment was stopped. In 17 patients, all culture negative (in nine PCR was positive, three of whom also had positive microscopy) the diagnosis was probable tuberculosis based on clinical findings and response to treatment.

This polymerase chain reaction has a much higher sensitivity than microscopy and can facilitate therapeutic decisions for those with suspected extrapulmonary tuberculosis.

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Microscopy with the auramine or Ziehl-Neelsen (ZN) stain is frequently negative in cases of extrapulmonary tuberculosis (TB). This means that treatment may be started and continued until the culture results confirm or refute the diagnosis at least 3 weeks later. The polymerase chain reaction (PCR), which permits direct identification of the *Mycobacterium tuberculosis* complex [1, 2] is more sensitive than microscopy, has a much lower detection limit and gives results within 24 h of receipt of the sample. The few studies which have evaluated PCR for the diagnosis of extrapulmonary TB have included a high percentage of human immunodeficiency virus (HIV)-seropositive patients. The sensitivity of PCR in these studies ranges 42–93% [3–6]. Previously, we and others showed that PCR can be used for the detection of pulmonary TB cases in routine laboratories [7–9]. Using our improved PCR technique [2], we evaluated the contribution of PCR to the diagnosis of TB in patients suspected of having extrapulmonary TB. Patients with suspected tuberculous meningitis were excluded from this analysis and are the subject of a separate publication [10].

Materials and methods

Patients

Two hundred and twenty six specimens were obtained from 156 patients residing in the Netherlands who were

suspected of having TB on the basis of symptoms, signs and radiological findings as judged by the treating clinician at the time of presentation. None of the patients had a recent (≤5 yrs) diagnosis of mycobacterial disease. The specimens were taken before antituberculous treatment was started.

Comparisons between conventional techniques (microscopy and culture) and PCR were made on samples from a single specimen. The specimens were obtained from various tertiary hospitals in the Netherlands and were sent by regular mail at ambient temperature to the Royal Tropical Institute. They arrived within 48 h and were processed on the same day and stored at 4°C. The specimens included pleural fluid (n=55), tissue biopsies (n=52), blood (n=56), pus from wound (n=32), bone marrow (n=6), ascites (n=2), synovial fluid (n=5), pericardial fluid (n=3), urine (n=13) and faeces (n=2).

Treatment of specimens

On receipt at the local laboratory, the specimen was split and a portion was sent to us for testing with PCR. The remaining portion was handled as detailed below. Protocols for specimen processing were standardized [11].

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Microscopy

Auramine and ZN staining on each specimen were performed according to the method described by SMITHWICK [12] and GROOTHUIS and YATES [11].

Culture

Specimens were cultured on two different solid media: Löwenstein-Jensen and Coletsos or Ogawa and on liquid medium: Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), or the Septi-Chek AFB system (Becton Dickinson, Microbiology Systems, Cockeysville, MD, USA). The specimens were incubated at 37°C for 2 months and examined weekly for growth. The isolated mycobacteria were identified by biochemical means and by the Accuprobe system (GenProbe, San Diego, CA, USA).

Polymerase chain reaction

Large volume specimens (*e.g.* pleural fluid, ascites and urine) were concentrated by centrifugation at 4,000×g before deoxyribonucleic acid (DNA) isolation. DNA was isolated as described previously [2]. The PCR was based on the IS6110 insertion element; Pt18 (5'-GAA CCG TGA GGG CAT CGA GG-3') and INS2 (5'-GCG TAG GCG TCG GTG ACA AA-3') were used as primers and 5' biotinylated Pt3 (5'-GAA CGG CTG ATG ACC AAA CT-3') as probe [1, 2]. To detect inhibition, the modified target *Mycobacterium smegmatis* 1008 DNA [9, 13], or *M. tuberculosis* DNA was added to a portion of the sample [1, 2]. All samples were tested in duplicate. A sample was judged as having inhibition when 50 fg of *M. smegmatis* 1008 DNA (corresponding to 10 bacteria or 10 IS6110 copies) or 10 fg of *M. tuberculosis* DNA (corresponding to two bacteria or 16 IS6110 copies) could not be detected by agarose gel electrophoresis. In such cases another DNA extraction method was used [2].

PCR was performed without knowledge of the clinical status of the patient or the result of microscopy. PCR results were reported before the culture results became available. Clinicians were told the PCR was experimental and was undergoing evaluation and that a negative PCR did not exclude TB.

Diagnosis

A clinical case definition of TB, named final diagnosis, was used as a reference to determine the utility of all diagnostic tests. Clinicians participating in this study followed the standard definitions of diagnosis recommended by the Royal Dutch Organisation against Tuberculosis [14]. The final diagnosis of TB was based on the following features: clinical findings; radiological findings including chest radiograph and computed tomography scan of the diseased site (when appropriate); auramine and ZN staining; culture; skin test results; histological findings (*e.g.* presence of giant cells or necrosis in caseating granulomata); and the response to antituberculous treatment.

Patients fulfilling the criteria of categories 1–3 below were considered to have a final diagnosis of TB (the results of the PCR test are not included in the criteria for diagnosis):

- 1) Confirmed TB, when *M. tuberculosis* was grown from at least one specimen.
- 2) Probable TB, when the patient had suggestive clinical findings, responded to antituberculous treatment and had one or more of the following: a strong skin test or a skin test conversion; changes in the chest radiograph compatible with the diagnosis; or microscopy positive for mycobacteria or histology showing a caseating granuloma.
- 3) Possible TB, when the patient had only clinical findings and responded to treatment. This means that if the patient improved on antituberculous treatment, even if all microbiological tests for TB were negative, the final diagnosis was still TB.

Patients not belonging to any of these categories were considered not to have TB.

Results

The majority of the specimens sent to our laboratory for PCR for *M. tuberculosis* were taken from patients in whom difficulties in diagnosis were encountered by experienced clinicians or microbiologists in tertiary referral centres. Around one third of the patients were born and had resided in the past outside the Netherlands. Approximately half of these patients had a final diagnosis of TB. About 40% of the patients were hospitalized. Importantly, only five of the 156 patients were HIV seropositive, of whom four had confirmed TB.

Comparison of results of PCR with those of microscopy and culture

The results of PCR and microscopy were available within 24 h. PCR was positive in the specimens from 40 patients and microscopy in the specimens from 18 of the 156 patients with suspected extrapulmonary TB (table 1). The sensitivity of the PCR in patients with a positive culture but negative microscopy was 79% (19 of 24 patients).

Table 2 shows the results in 226 specimens of PCR and microscopy compared with the gold standard for microbiological diagnosis, culture. From these results it is clear that PCR is more often positive than microscopy or culture of the same specimen. Consequently, PCR has a lower specificity than microscopy in comparison with culture. A comparison of the results of the three diagnostic methods in patients, rather than specimens, is given in table 3. The sensitivity of the PCR for patients suspected of extrapulmonary TB was 86%, which is dramatically higher than the 31% for microscopy.

Comparison of the results of PCR, microscopy and culture with the final diagnosis

The final diagnosis was TB when the patient fulfilled the criteria for confirmed, probable or possible TB. For ease of analysis we have grouped patients according to the

Table 1. — Result of polymerase chain reaction (PCR), microscopy and culture in 156 patients compared with final diagnosis

Patient group code	Patients n	Microscopy*	Culture* <i>Mycobacterium tuberculosis</i>	Antituberculous drugs	Response to antituberculous treatment	Final diagnosis [§]
PCR-positive patients						
1	11	+	+	Yes	Yes	Confirmed TB
2	19	-	+	Yes	Yes	Confirmed TB
3	3	+ [‡]	-	Yes	Yes	Probable TB
4	6	-	-	Yes	Yes	Probable TB
5	1	-	-	No	NA	Not TB
Total	40					
PCR-negative patients						
6	3	+	- [†]	No	NA	Not TB
7	1	+ [‡]	-	Yes	No	Not TB
8	20 [§]	-	-	Yes	No	Not TB
9	79	-	-	No	NA	Not TB
10	5 [#]	-	+	Yes	Yes	Confirmed TB
11	3	-	-	Yes	Yes	Probable TB
12	5	-	-	Yes	Yes	Possible TB
Total	116					

*: PCR, culture (of *Mycobacterium tuberculosis*) or microscopy were considered positive when at least one specimen was positive; [§]: the final diagnosis of tuberculosis (TB) was based on culture, microscopy, histological findings, clinical and radiological findings and the response to antituberculous treatment, and was considered positive when the patient belonged to the confirmed, probable or possible TB group; [‡]: only a few mycobacteria were seen; [†]: mycobacteria other than *M. tuberculosis* were seen; [§]: two patients were human immunodeficiency virus (HIV) seropositive; [#]: three patients were HIV seropositive. +: positive; -: negative; NA: not applicable.

Table 2. — Results of polymerase chain reaction (PCR), microscopy and culture in 226 specimens from 156 patients

	Culture* <i>Mycobacterium tuberculosis</i>		Sensitivity+	Specificity+
	Positive	Negative		
PCR				
Positive	30	10	86	95
Negative	5	181		
Microscopy				
Positive	11	8	31	96
Negative	24	183		

*: culture was positive when *Mycobacterium tuberculosis* was isolated; ⁺: sensitivity and specificity were calculated with culture as gold standard.

results of PCR, microscopy, culture and final diagnosis, as shown in table 1. In the 11 patients in group 1 (table 1) PCR confirmed that the mycobacteria seen by microscopy belonged to the *M. tuberculosis* complex. In group 2, PCR predicted the positive culture results in 19 patients with negative microscopy specimens. Nine of these patients were suspected of having TB and had a positive radiograph or skin test, in the 10 other patients treatment was started on clinical grounds alone. In group 3, microscopy revealed only a few mycobacteria, the positive PCR supported the clinical evidence although culture was negative. PCR was the only positive laboratory test in the six patients in group 4. These patients all responded to treatment, started on the basis of clinical findings and a suspicious radiograph or positive skin test.

The 74 yr old female in group 5 had cancer; two biopsies were PCR-positive but no antituberculous treatment was given. This patient may have had endogenous reactivation of a previous TB infection due to a failure of the immune system.

In four patients microscopy, was positive, but PCR was negative (table 1; patient groups 6 and 7). Later, mycobacteria not belonging to the *M. tuberculosis* complex were

grown from the three patients in group 6. These three patients did not receive therapy because the clinicians decided to wait for the culture results which ruled out TB. The negative PCR results may have influenced their decision. The patient in group 7 was treated for TB but showed no improvement and treatment was stopped; the radiograph was suggestive and there were only a few acid-fast bacteria in the pleural fluid.

The 20 patients in group 8 had a trial of treatment with antituberculous drugs, but showed no improvement and treatment was stopped. In addition to clinical evidence, 10 had supportive radiological evidence and five had a positive skin test. The final diagnosis was not TB. Six patients in this group died. The 79 patients in group 9 did not receive treatment because the clinicians felt there was not enough evidence to initiate therapy. Culture remained negative in all these patients. Treatment was started before culture results were known in four patients in group 10

Table 3. — Results of polymerase chain reaction (PCR), microscopy and culture in 156* patients

	Culture+ <i>Mycobacterium tuberculosis</i>		Sen. [†]	Spec. [‡]	PPV	NPV
	Positive	Negative				
PCR						
Positive	30	10	86	92	75	96
Negative	5	111				
Microscopy						
Positive	11	7	31	94	61	83
Negative	24	114				

*: multiple specimens were obtained from some patients, and a patient was considered positive if one specimen was positive in the designated test; ⁺: culture results were counted as positive when *M. tuberculosis* was isolated; [†]: sensitivity (Sen.), specificity (Spec.), positive and negative predictive values of PCR and microscopy were calculated using culture results as the gold standard. PPV: positive predictive value; NPV: negative predictive value.

Table 4. — Results of polymerase chain reaction (PCR) and microscopy in samples from sites that were culture positive for *Mycobacterium tuberculosis**

Sample origin	Samples n	Patients n	Positive samples n		
			Culture	PCR	Microscopy
Pus	14	14	14	14	6
Biopsy	11	11	11	10	2
Pleural fluid	9	9	9	5	3
Pericardial fluid	1	1	1	1	-†

*: detailed results of culture microscopy and PCR for each of the 226 samples are available from the authors; †: negative in microscopy.

the basis of clinical findings and a positive skin test or radiograph. The five patients in this group had a positive culture with a few colonies after long incubation of specimens (>7 weeks) from extrapulmonary sites (four with pleural fluid and one with a skin biopsy). The one patient with a culture-positive skin biopsy was treated with anti-tuberculous medication after the results of culture were known. The PCR was negative in all of these specimens from these patients. These are the only five patients in whom PCR failed to predict the positive culture results.

The three patients of group 11 were negative in PCR, microscopy and culture. The diagnosis was probable TB, since all responded to treatment; in two patients the skin test was positive (>10 mm) and in one the radiograph was suspicious. The five patients of group 12 suspected of extrapulmonary TB were negative in PCR, microscopy and culture, but responded to treatment which was started on clinical grounds alone.

We have analysed the results in samples of different origin, restricting the analysis to samples from sites that were culture positive (table 4). These results show that PCR can give rapid, accurate diagnosis in samples of pus and in tissue biopsies, but is poorer in pleural fluid. The diagnostic yield of culture and PCR was low in blood, urine and bone marrow; only one of 56 blood samples, one of 13 urine samples and none of six bone marrow samples were PCR positive and all were culture negative.

Discussion

This PCR has been evaluated for the diagnosis of extrapulmonary TB in 156 patients, 151 of whom were HIV seronegative and nonimmunosuppressed. Most published studies have reported only on sputum specimens [8, 15–17] and have included a high proportion of HIV or acquired immune deficiency syndrome (AIDS) patients [5]. We have shown that PCR could make a major contribution to the initial management of the patient when only symptoms, signs and microscopy are available.

In microscopy-positive cases PCR can confirm or exclude the presence of *M. tuberculosis* within 24 h. For the microscopy-negative cases, PCR is much faster than culture. In the culture-proven cases, 31% were identified by microscopy whereas 86% were diagnosed by PCR.

Taking culture as the gold standard for the diagnosis, our results with PCR on specimens from many different

sites are comparable or better than the published results in which sputum or extrapulmonary specimens have been tested [5, 6, 8, 15–17]. The choice of PCR kit is not important, but the attention to detail with which the whole method is conducted is of fundamental importance [18].

Problems can arise when specimens contain very few mycobacteria. Due to sampling error, one sample may contain *M. tuberculosis* whereas the other sample does not. This may be the explanation for the discrepancies in patient group 10 (table 1). In one case only a fragment of a skin biopsy could be tested in PCR (which was negative) whereas a larger fragment could be used for culture (which was positive). In four other patients, all with a positive radiograph, pleural fluid was negative in PCR, but culture was positive. PCR can give rapid, accurate results in samples from pus and tissue biopsies, but it is inferior to culture in samples of pleural fluid. The low sensitivity of PCR in pleural fluid suggests that more work is needed in this area to improve the assay. In the meantime, when a rapid diagnosis is required, other tissue (e.g. pleural biopsy) should be obtained for testing.

There is controversy about the results of PCR in blood from patients with TB. Our experience of PCR in blood specimens in 191 patients (54% HIV seropositive) suspected of extrapulmonary TB, has also shown a low rate of detection [19]. AGUADO *et al.* [20] also reported a low detection rate of PCR in blood specimens in sputum-smear positive patients with *M. tuberculosis*. To date, only CONDOS *et al.* [21] have found that PCR in blood is useful for the diagnosis of tuberculosis.

In this study, the results of the PCR could have influenced patient management decisions, since the clinician was told the results when they became available. However the clinician was also told that the test was being evaluated in a research setting. The different decisions made by clinicians faced with negative microscopy and PCR results are illustrated in groups 8, 9, 11 and 12 (table 1). In the absence of any positive identification of *M. tuberculosis* it is unclear whether antituberculous therapy contributed to the improvement in the treated patients or whether they were overtreated. These observations show that laboratory results are necessary, but not sufficient, for the decision to treat or not to treat as tuberculosis.

The results of this study demonstrate the role of the polymerase chain reaction for *Mycobacterium tuberculosis* complex in the diagnosis of extrapulmonary tuberculosis. In human immunodeficiency virus-seronegative patients, polymerase chain reaction may be unnecessary when positive microscopy accords with the clinical and epidemiological findings suggesting tuberculosis. Isolation of the mycobacterial strain by culture is important for drug susceptibility testing. Polymerase chain reaction should be performed when microscopy is positive, but there is a chance that infection is due to mycobacteria other than *Mycobacterium tuberculosis*, e.g. in the immunosuppressed or human immunodeficiency virus-seropositive patient. Such opportunistic mycobacteria can be identified directly in clinical material with a polymerase chain reaction based on the deoxyribonucleic acid encoding the 16S ribosomal ribonucleic acid [22, 23]. The polymerase chain reaction should be performed in all microscopy-negative specimens from patients suspected of tuberculosis, to obtain a rapid diagnosis. If both microscopy and polymerase

chain reaction are negative in the suspected case, repeated testing is required.

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