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Clinical usefulness of the nested polymerase chain reaction in the diagnosis of extrapulmonary tuberculosis

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Abstract

Objective. To evaluate the effectiveness of nested polymerase chain reaction (PCR) for diagnosis of extrapulmonary tuberculosis (ETB), as well as the impact of PCR results on clinical management. Materials and Methods. We conducted a study of nested PCR tests in 45 patients and a review of patient hospital files, calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Results. PCR was positive in 51% of cases; PCR sensitivity for diagnosing TB was 86%, specificity was 79%, PPV was 76%, and NPV was 88%. When solely analyzing urine samples, sensitivity and NPV increased to 100%. PCR exerted an influence on management in 27% of patients. Conclusions. PCR for rapid diagnosis of extrapulmonary TB has an adequate effect, which improves when performed on urine. The results of PCR exerted an acceptable impact on the clinical management of these patients.

Key words: tuberculosis; extrapulmonary tuberculosis; polymerase chain reaction; nested polymerase chain reaction

Resumen

Objetivo. Evaluar la eficacia de la reacción en cadena de la polimerasa (PCR) anidada para el diagnóstico de tuberculosis extrapulmonar, así como el impacto de sus resultados en el manejo clínico. Material y métodos. Se realizó PCR anidada en 45 pacientes y se llevó a cabo la revisión de expedientes. Se calculó sensibilidad, especificidad, valor predictivo positivo (VPP) y valor predictivo negativo (VPN). Resultados. La PCR fue positiva en 51% de los casos, la sensibilidad fue de 86%, la especificidad de 79%, el VPP de 76% y el VPN de 88%. Al analizar solamente las muestras de orina, la sensibilidad y VPN se incrementaron a 100%. La PCR influyó en el manejo de 27% de los pacientes. Conclusiones. La PCR para el diagnóstico rápido de TB extrapulmonar tiene una eficacia adecuada, la cual mejora cuando se realiza en orina. El resultado de la PCR tuvo un impacto aceptable en el manejo clínico de estos pacientes.

Palabras clave: tuberculosis; tuberculosis extrapulmonar; reacción en cadena de la polimerasa; reacción en cadena de la polimerasa anidada

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The diagnostic approach to tuberculosis (TB) has changed little since the era of Robert Koch, whose rapid diagnostic test for TB was bacilloscopy, which is insensitive and unspecific. Species identification with the traditional Löwenstein-Jensen culture, considered the bacteriologic gold standard, requires 4 to 12 weeks for development. Liquid culture media developed by Middlebrook (7H9 and -12) for radiometric and colorimetric systems allow for the development of Mycobacterium tuberculosis (MTB) within a 2 to 3-week period, detecting small inocula such as those of 200–300 colony-forming units per ml.

Other TB diagnostic methods include blood cultures for extrapulmonary TB (ETB) and serologic tests by Enzyme-linked ImmunoSorbent Assay (ELISA, which do not present sufficient sensitivity and specificity to be useful and determination of adenosine deaminase. The development of DNA amplification techniques such as polymerase chain reaction (PCR) has focused the attention of investigators toward this direction. The concept of specific genetic-material amplification at detectable levels would be very attractive. The use of PCR in TB diagnosis is defined in a limited fashion; studies to date include small samples, different initiators, different diagnostic criteria, and distinct or poorly explained gold standards as well as clinical criteria, rendering comparison among these studies difficult. PCR use in the identification of MTB has exhibited excellent results, with a sensitivity that can range from 65 to 100% and a specificity of 98%, in addition to the reduction of waiting days to initiate treatment (from 3 to 5 days, approximately); thus, some institutions have implemented its use as a diagnostic resource. Among the disadvantages found of PCR are its high cost ($125 US dollars [USD]) and the need for adequate infrastructure, as well as requiring qualified personnel to perform this technique. However, no other method offers the population a rapid, sensitive and relatively accessible result.

With regard to clinical usefulness, articles have been published on PCR in pulmonary TB (PTB), correlating clinical, culture and PCR data. One of the largest series was the evaluation of 844 respiratory tract samples from 421 patients over a period of six months. When carrying out a comparison with the culture, a sensitivity of 93.6% and a specificity of 97.8% were found, with a positive predictive value (PPV) of 95.5% and a negative predictive value (NPV) of 70%. The usefulness of PCR has been discussed in articles and editorials, with resulting controversy concerning its application. This is due to the fact that although PCR has a high sensitivity, the presence of contamination or the inability to detect infection with live bacilli has limited its use, and that it cannot be substituted for the culture.

ETB represents an even greater diagnostic problem than PTB because it presents with less frequency and occurs with little liberation of bacilli, as well as the fact that it is localized in sites that are difficult to access. This combination of situations gives rise to a difficulty in bacteriologic confirmation, thus implying more invasive procedures for sample obtention. Due to the relatively low number of cases, diagnosis of ETB is initially omitted and clinical signs remain undetected by the majority of clinics.

Concerning tuberculous meningitis (TBM), this disease develops with greater frequency when a meningeal calcification or a sub-cortical focus (Rich focus) discharges its contents into the subarachnoidal space. It is known that TBM is often accompanied by miliary TB, but the interrelationship between the development of Rich focus and miliary TB continues to be controversial. Conventional bacteriologic methods rarely detect MTB in cerebrospinal fluid (CSF) and are, thus, of limited use in TBM diagnosis; TBM’s suggestive clinical characteristics are frequently supported by indirect evidence, such as CSF examination and computed tomography (CT) of the head.

Genitourinary TB (GUTB) continues to be a serious diagnostic problem for the microbiological laboratory, because the World Health Organization (WHO) has proscribed performing bacilloscopy in urine due to its low specificity, since acid fast bacilli (AFB) are environmental saprophytes. In addition, in developing countries such as Mexico the use of the culture is unfortunately not accessible at all clinical care levels.

Due to the previously mentioned situations, the objective of the present study was to evaluate the clinical usefulness and optimization of PCR for rapid molecular diagnosis of extrapulmonary tuberculosis.

Materials and Methods

The study was approved by the Ethics Committee at the Infectology Hospital at “La Raza” Medical Center and all participants gave written informed consent. We performed the nested PCR test on extrapulmonary samples from 45 patients from January 2001 to August 2002. We then carried out a retrospective study based on data obtained from the Clinical Microbiology Laboratory and a review of patient files. These patients were seen at the Mexican Institute of Social Security’s (IMSS) La Raza Medical Center in Mexico City, a tertiary-level hospital that generally cares for patients with chronic illnesses. We reviewed clinical annotations on patients from 10 days after the request date for the PCR to analyze its impact on patient management. Similarly, we reviewed later clinical annotations to decide whether the final
diagnosis of the case was or was not TB, considering the treating physician’s clinic as the gold standard, including treatment response. In this respect, we determined the usefulness of PCR with regard to confirmation of the clinical suspicion, diagnostic support, treatment restoration in the case of a positive test, and compatibility with the remainder of laboratory and medical office examinations. All suspected ETB patients were considered as confirmed cases (positive culture or AFB smear), highly probable cases (meeting all the clinical criteria and with all supporting evidences being positive, but having no bacterial isolation) and cases without TB.

We studied 25 samples from the cerebrospinal fluid (CSF) of patients with tuberculosis of the central nervous system (CNS) and 20 samples of urine from patients with GUTB.

Polymerase chain reaction (PCR): the mycobacterial strain utilized as a positive control was M. tuberculosis H37Rv. The DNA was isolated with guanidine isothiocyanate and phenol utilizing 500 µl of the TRIZol reagent (Gibco BRL) according to the procedure described by Chomczynski. The specimens were also processed in the same way. The DNA was resuspended in 50 µl of distilled water after precipitation with ethanol at 75%. This solution was heated at 55 ºC for 20 min. We determined its absorbance relationship at 260/280 nm and took 5 µl for amplification by PCR of genes coding for the 32-kDa protein, the MTP40 species-specific protein and the IS6110 sequence insertion. The initiator sequences employed for amplifying the species-specific gene were PT1 (5’CCG CAA CGC GCC GTC GGT GG) and PT2 (5’CCC CCC ACG GCA CCG CCG GG), with a resulting fragment of 396 bp. For IS6110 insertion-element amplification, the specific MercatoBenzoThiazolet (MTB)-complex initiators were IS5 (5’CCG AGA CGG TGC GTA AGT GG) and IS6 (5’GAT GGA CCG GCA CTT GC), with a 984-bp amplification fragment. The specific initiators for amplifying the gene coding for the antigen α of 32-kDa present in all described mycobacteria (genus-specific) were MT1 (5’TTC CTG ACC AGC GAG CTG CCG) and MT2 (5’CCC CAG TAC TCC CAG CTT GC), with a 506-bp amplification fragment. 

All reactions were taken to a final volume of 50 µl containing 100 ng of purified DNA from the reference strain as well as from each clinical specimen, reaction buffer 1X, 2.5 U of Taq polymerase, 0.2 mM of each triphosphate deoxynucleoside, and 20 pM of each of the three pairs of initiators. The reaction was carried out in a thermocycler (Biometra). Cycles included initial denaturation at 94 ºC for 5 min, followed by 35 repeated denaturation cycles at 94 ºC for 1 min, annealing at 71 ºC for 2 min, and extension at 72 ºC for 3 min. Subsequently, a final extension was carried out at 72 ºC for 10 min.

To increase amplification sensitivity, we performed nested PCR, amplifying an internal segment of the specific gene designed by Del Portillo; in the second PCR corresponded to nucleosides 44–65 (PT3, 5’-CAC CAC GTT AGG GAT GCA CTG C-3’) and 244–265 (PT4, 5’-CTG ATG GTCC ACC ACG TTC G-3’), which amplified the 223-bp internal region. For this second step, we took 5 µl of the multiplex PCR product and transferred this into 45 ml of the pre-mixed solution containing the PCR regents at the same previously described concentration. Amplification was repeated for 30 cycles with the same time and temperature parameters as described previously, except for an annealing at 75 ºC for 2 min, an extension at 72 ºC for 2 min, and a final extension at 72 ºC for 7 min. After the 1/10 amplification of the PCR reaction mixture, this was analyzed by electrophoresis in agarose at 1.5% containing 0.5 µg/ml of ethidium bromide and was visualized with an ultraviolet (UV) transilluminator.

Analysis: we calculated sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) according to Galen & Gambino, as compared with the treating physician’s clinic, including treatment response and the prevalence of TB in the studied population.

Results

With regard to patients with a presumptive diagnosis of ETB, TB of the central nervous system (CNS) was present in 25 (49%), specifying cerebral affection in five patients (9.8%), medullar in three (6%), meningal in nine (18), unspecified in eight (17%), GUTB in 20 (39%) patients, renal-level in 19 (37%), and gall bladder in one (2%) patient (Table I).

Of the previously mentioned cases, ETB diagnosis was confirmed in 25 (56%) patients by their clinical picture, including the treatment and culture, acid fast bacilli and medical office studies. Among PCR results, 23 (51%) positive tests were reported. The clinic considered that the PCR result supported the final diagnosis (whether positive or negative) in 41 (91%) patients. PCR sensitivity for diagnosing ETB was 86%, specificity was 79%, PPV was 76%, and NPV, 88% (Table II).

Confirmatory studies were requested in 44 (98%) patients; positive bacilloscopy and culture were found in sixteen (36%) patients and a positive radiologic study finding was observed in 30 (67%) patients. PCR result-based treatment was initiated in twelve (27%) patients (Table III).

To evaluate clinical usefulness, we documented the PCR result and the treating physician’s final decision...
PCR for diagnosis of tuberculosis

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Para iniciar el tratamiento, así como el resultado obtenido caso por caso. De los 25 pacientes con diagnóstico presuntivo de TB en CNS, solo 14 fueron finalmente considerados positivos para TB; de estos, nueve (68%) presentaron resultados positivos con PCR y el tratamiento se inició basado en estos resultados en cuatro (29%) pacientes. No obstante, en cinco (38%) casos con alta sospecha clínica, el tratamiento se inició a pesar de resultados negativos por PCR. Finalmente, TB diagnosis was discarded in 11 patients, nine (82%) of whom had negative results by PCR, with treatment modification based on these results in five (45%). One (9%) false positive result was reported in which the patient had a diagnosis of HIV and Hodgkin lymphoma (Table IV).

Regarding PCR test in urine, we analyzed 20 cases; of these, nine were finally considered as positive for TB and all presented positive results by PCR, with three

### Table I
**Affected site localization in patients with presumptive diagnosis of tuberculosis. Investigation Unit in Immunology and Infectology, La Raza Medical Center, Mexico. 2001-2002**

<table>
<thead>
<tr>
<th>Localization</th>
<th>No. of patients (n) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS</td>
<td>25 (100)</td>
</tr>
<tr>
<td>Meningeal</td>
<td>9 (36)</td>
</tr>
<tr>
<td>Cerebral</td>
<td>5 (20)</td>
</tr>
<tr>
<td>Medullar</td>
<td>3 (12)</td>
</tr>
<tr>
<td>NS*</td>
<td>8 (32)</td>
</tr>
<tr>
<td>GUTB</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Renal</td>
<td>19 (95)</td>
</tr>
<tr>
<td>Bladder</td>
<td>1 (5)</td>
</tr>
</tbody>
</table>

* NS: not specified; CNS: Central nervous system; GUTB: Genitourinary tuberculosis

### Table II
**Polymerase chain reaction in patients with extrapulmonary tuberculosis. Investigation Unit in Immunology and Infectology, La Raza Medical Center, Mexico. 2001-2002**

<table>
<thead>
<tr>
<th></th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive PCR</td>
<td>23 (51)</td>
</tr>
<tr>
<td>Support for final diagnosis (positive or negative)</td>
<td>41 (91)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>(86)</td>
</tr>
<tr>
<td>Specificity</td>
<td>(79)</td>
</tr>
<tr>
<td>PPV</td>
<td>(76)</td>
</tr>
<tr>
<td>NPV</td>
<td>(88)</td>
</tr>
</tbody>
</table>

n: number of cases

PCR: polymerase chain reaction

TBE: extrapulmonary tuberculosis

PPV: positive predictive value

NPV: negative predictive value

### Table III
**Complementary studies requested. Investigation Unit in Immunology and Infectology, La Raza Medical Center. Mexico, 2001-2002**

<table>
<thead>
<tr>
<th></th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmatory studies</td>
<td>44 (98)</td>
</tr>
<tr>
<td>Bacilloscopy and positive cultures</td>
<td>16 (36)</td>
</tr>
<tr>
<td>Radiology</td>
<td>30 (67)</td>
</tr>
<tr>
<td>PCR-associated treatment initiation</td>
<td>12 (27)</td>
</tr>
</tbody>
</table>

PCR: polymerase chain reaction

### Table IV
**Polymerase chain reaction results for tuberculosis of the central nervous system and genitourinary tuberculosis. Investigation Unit in Immunology and Infectology, La Raza Medical Center. Mexico, 2001-2002**

<table>
<thead>
<tr>
<th></th>
<th>TB in CNS GUTB</th>
</tr>
</thead>
<tbody>
<tr>
<td>With TB and positive PCR</td>
<td>9/14 (68)</td>
</tr>
<tr>
<td>PCR-associated treatment initiation</td>
<td>4 (29)</td>
</tr>
<tr>
<td>Without TB and negative PCR</td>
<td>9/11 (82)</td>
</tr>
<tr>
<td>Modification of treatment</td>
<td>5 (45)</td>
</tr>
</tbody>
</table>

Sensitivity | (64) | (100)
Specificity | (82) | (82)
PPV | (82) | (82)
NPV | (64) | (100)

TB: tuberculosis
CNS: central nervous system
GUTB: genitourinary system
PPV: positive predictive value
NPV: negative predictive value
(33%) initiating treatment based on this result. In cases considered negative, initial treatment was modified based on the PCR result in six (54%) patients. Two false positives occurred due to presenting negative cultures and these patients were found to be asymptomatic (Table IV).

Six patients presented a positive culture in CSF and only two in urine. The patients’ evolution with presumptive diagnosis but without bacteriologic confirmation was clinically toward tuberculous disease.

For renal TB, abdominal radiographs, intravenous urography, retrograde pyelography and computed tomography (CT) demonstrated various patterns of calcifications, including amorphous, granular and lobar patterns.

Discussion

The clinical presentation of ETB is frequently atypical; tissue samples for confirmation of the diagnosis can be difficult to obtain on some occasions, and conventional diagnostic methods possess low sensitivity. The availability of CT, magnetic resonance (MR), and endoscopy aid greatly in anatomic localization.

Our study coincided with the literature, finding only 36% of cases of ETB with a positive culture. This information is useful retrospectively because several weeks are necessary for the culture to develop. In the present study, PCR exhibited greater sensitivity than microscopy and the culture, and could facilitate therapeutic decisions for patients with a clinical suspicion of ETB, as was found by other authors.

For diagnosis of GUTB, although a high index for clinical suspicion is necessary, PCR can be useful for cases in which bacteriologic and clinical diagnoses of TB are not conclusive. We found PCR to be a very rapid diagnostic method for GUTB. It was sensitive, specific, and prevented waiting to initiate treatment because the sensitivity, specificity, PPV, and NPV for ETB were 76, 84, 82, and 78%, respectively; on analyzing only urine samples sensitivity and NPV rose to 100%.

In our study, nested PCR dramatically increased sensitivity and specificity of DNA amplification over the conventional single-step PCR, in that initial multiplex PCR did not detect any sample as positive. The fact that amplification by nested PCR improves sensitivity and specificity renders it necessary for rapid diagnosis of ETB in the clinical laboratory.

In Mexico, there are very few studies that have detected the usefulness of PCR in ETB. Our data are in agreement with some, while a discrepancy exists with another.

Rapid molecular diagnostic tests for PTB influence decisions concerning the initiation of antituberculosis therapy (antiTB) depending on the clinical suspicion. This includes patients for whom bacilloscopy is positive but clinical suspicion is intermediate or low, and patients for whom bacilloscopy is negative but clinical suspicion of PTB is high or intermediate.

Nevertheless, in the area in which clinical data are most scarce—molecular diagnosis in non-respiratory samples such as sterile body fluids (especially urine, CSF, and pleural fluids), and gastric and tissue aspirates including formalin-fixed samples—participation is required by multiple institutions to carry out an optimal clinical study design to evaluate PCR in extrapulmonary samples. Protocols should be developed to standardize sample processing with a sufficient number of protocols at each site to obtain an adequate quantity of positive results and to be able to validate these.

In conclusion, even though this is a preliminary study, our results permitted us to claim the usefulness of this fast and reliable diagnostic procedure as an important tool against TB when used together with the clinical data available. Rapid molecular diagnosis of ETB in our environment possesses adequate efficiency, which improves when it is carried out in urine. The PCR result exerted an acceptable impact on the clinical management of these patients. At present we are evaluating a larger number of clinical samples, comparing these with cultures such as the gold standard.

References
