Original Article

Mycobacterium tuberculosis complex detected by modified fluorescent in situ hybridization in lymph nodes of clinical samples

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Abstract

Introduction: Lymph node tuberculosis (TB) is the leading cause of extrapulmonary tuberculosis and is the most frequently identified type in Aguascalientes, Mexico. Conventional diagnosis has serious limitations for rapid detection of extrapulmonary tuberculosis in clinical samples. Here PCR and modified FISH have been tested as complementary diagnosis methods for extrapulmonary tuberculosis.

Methodology: The specific insertion sequence IS6110 for *Mycobacterium tuberculosis* complex was used to perform PCR and build DNA and PNA FISH probes (20bp). PCR and modified DNA and PNA FISH assays were performed to evaluate 41 lymph node paraffin-embedded tissue samples, in comparison with the histopathology diagnosis, which was considered the gold standard (22 positive and 19 negative).

Results: In comparison with histopathology diagnosis PCR showed 62.5 % sensitivity and 77.8 % specificity (χ^2 = 4.583 p< 0.05). Modified DNA FISH showed 71.4% sensitivity and 84.6% specificity (χ^2 = 11.21 p< 0.05). PNA FISH showed 66.7% sensitivity and 60.0% specificity (χ^2 = 2.93 p> 0.05). Ziehl Neelsen stain was positive in only four cases of 22 lymph node samples positive to histopathology. In contrast, PCR and modified DNA FISH were positive in 20 cases of the same group. The negative cases were coincident in all tests.

Conclusions: PCR and DNA FISH showed a significant increase in the number of cases detected and also showed higher sensitivity and specificity compared with data reported by traditional methodology. In developing countries, these techniques could help to complement the early diagnosis and timely treatment of extrapulmonary tuberculosis.

Key words: fluorescent *in-situ* hybridization; FISH, tuberculosis; *Mycobacterium tuberculosis* complex; PCR; insertion sequence *6110*; peptide nucleic acid; PNA

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Introduction

Tuberculosis (TB) is an infectious disease caused by species of the *Mycobacterium tuberculosis* complex (MTC) [1,2,3,4]. About 70% of tuberculosis is pulmonary and the rest is extrapulmonary, but this data can vary from country to country [5,6]. Lymph node tuberculosis (LNTB) is the leading cause of extrapulmonary tuberculosis [6,7]. Aguascalientes, México, has a high incidence of extrapulmonary TB (48 %) detected by histopathology and the most frequently identified form is LNTB [8].

LNTB diagnosis depends on the clinical history, symptoms, clinical findings, and purified protein derivative (PPD) reaction of the patient. This diagnosis depends mainly on the bacilli culture obtained after fine needle aspiration biopsy or excisional biopsy, with the microscopic demonstration of the acid-fast bacilli or Ziehl Neelsen stain (ZN) [9,10,11,12,13]. While these methods could not distinguish between non-tuberculous mycobacterium (NTM) and MTC, presumptive differentiation between MTC and NTM can be made by growth characteristics (rough and cream colonies) and by microscopic observation of cording formation on ZN stain of a positive culture. However, this is not a definitive differentiation because some NTM also produce cording. Conclusive identification of MTC can be obtained using conventional tests, such as the niacin test, nitrate reduction or catalase production, but these procedures are time-consuming [14].

Histopathological analysis by hematoxylin-eosin staining (HE) is used for the diagnosis of mycobacterial infection on formalin-fixed, paraffin-embedded tissue samples according to the morphology of the lesions. The demonstration of

mycobacteria on formalin-fixed, paraffin-embedded tissue samples by ZN staining shows low sensitivity because the formalin fixation as well as the low bacilli concentration impairs the microbiological yield [9,15,16]. Even when lymph node biopsies are taken, they are fixed in formalin for histopathological analysis and the culture in most cases is not made [17]. Another complication at present is that TB is difficult to differentiate from other diseases and may produce misdiagnoses that can lead to fatal complications [7]. In Mexico, LNTB diagnosis is a critical problem due to the difficulty in culturing *Mycobacterium tuberculosis* (MTB) [16].

Currently, almost half of the TB cases diagnosed in the Latin American and Caribbean region are bacteriologically confirmed. In most of these, however, the confirmation is made by microscopic smear examination. This rapid, low-cost and quite specific technique allows the detection of highly infectious pulmonary cases that are sources of infection in the community so they may be treated. However, it is well-known that mycobacterial species cannot be differentiated by acid-fast bacilli (AFB) staining, and information on *M. bovis* cases remains limited. Even in the United States, where culture is routinely used in the diagnosis of TB, many laboratories do not identify positive cultures beyond the level of the MTC [18].

New methodologies, such as PCR and *in situ* hybridization (ISH) [19] that are faster and more sensitive and specific than current TB diagnosis procedures are necessary. Several commercial products based on PCR have been used and approved for molecular mycobacteria identification, but these tests are not extensively used in Mexico because they need special infrastructure and specialized technicians to perform them, and they also cost more than traditional techniques [20,21,22,23].

PCR and molecular techniques cost more in comparison to AFB and culture methods; however, they are ideal for rapid localization of single-copy genes or small DNA segments. Therefore, even if they are used in fixed samples, these characteristics make them a cost-effective tool in the fight against TB [24].

In addition to high sensitivity and specificity, ISH allows the visualization of bacilli in clinical samples [25,26]; ISH also allows the direct observation of bacterial distribution and morphology in the infected tissue [27,28,29]. ISH is improved with the use of fluorescence (FISH) for both DNA or RNA probes [27,30,31,32,33,34,35,36,37]. However, conventional oligonucleotides (100 to 500 bp) used for DNA ISH or FISH have penetration problems because of the nature of the mycobacterial cellular surface, which shows low permeability for these molecules [11,27,28,38]. To solve this problem, some studies have used peptide nucleic acid (PNA) instead of conventional DNA probes because PNA are hydrophobic and therefore more permeable for bacterial cell walls and plasmatic membranes. These PNA probes have been used to detect mycobacteria in environmental biofilms, sputum, bacterial cultures, experimental infected animals, and in clinical samples such as skin and lung tissues. In comparison with conventional DNA probes used for ISH or FISH, PNA probes are also much smaller (20bp) [2,11,22,34,39,40,41].

Since cell permeability decreases with the molecular weight of the probes, we used DNA probes with a low molecular weight (20pb) for FISH assays instead of the usual probes (100 a 500 pb) in our study. The objective was to significantly increase the permeability of the designed DNA probes and therefore also to increase the sensitivity of the tests, but without significantly reducing specificity. The specific insertion sequence IS6110 for *Mycobacterium tuberculosis* complex was used to perform PCR and to build the DNA and PNA FISH probes (20bp) for MTC diagnosis in lymph node biopsies. The results of PCR and modified DNA and PNA FISH assays were compared with a histopathologically established TB diagnosis, which is considered the gold standard here.

Methodology

Ethics statement

The study protocol was approved by the local human ethics committee at the Miguel Hidalgo Centenarian Hospital (MHCH) in Aguascalientes, México.

Clinical specimens

Clinical specimens histopathologically characterized by the pathology service at MHCH were used. A total of 41 biopsies of lymph nodes embedded in paraffin were collected between January 2007 and December 2008. From them 22 showed histopathological examination positive (HEP) to mycobacterial infection and 19 showed histopathological examination negative (HEN) to mycobacterial infection. Only four samples of the HEP group were positive by Ziehl-Neelsen (ZN) staining and the rest were negative. None of the

samples was confirmed by bacilli culture in Löwenstein-Jensen medium. All patients in this study were negative to human immunodeficiency virus (Figure 1).

Mycobacterium reference strains

The mycobacterium reference strains *M. tuberculosis* H37Rv (American Type Culture Collection, USA) and *M. bovis* BCG ATCC 35734 were used as controls to validate PCR and FISH procedures. All samples were tested twice.

DNA isolation and PCR

The tissue sections were placed in an Eppendorf tube in 5% Chelex-100 resin, 0.3% Tween 20 and 0.03% Triton X-100 (Sigma-Aldrich, Toluca, Edo. De México, México), heated at 94°C for 30 minutes, and the supernatant was used in PCR assays [42].

The IS*6110* PCR assay was performed as described previously [43,44]. Briefly, the primers used were (5´ CCT GCG AGC GTA GGC GTC GG3´ and 5´CTC GTC CAG CGC TTC GG 3´) to amplify a 123-bp fragment. A PCR kit was used (Invitrogen, Carlsbad, CA, USA). The reactions were subjected to 10 minutes at 94°C, followed by 35 cycles of 30 seconds each at 94°C, 30 seconds at 65°C and 30 seconds at 72°C, with a final extension at 72°C for 5 minutes. The PCR products were visualized under UV light after agarose gel (2.5%) electrophoresis with ethidium bromide staining.

Oligonucleotide probes

To improve the cell penetration capability of the probes, low molecular weight specific probes (20bp) were built for MTB detection by FISH. The sequence reported by Eisenach *et al.* [43] for MTB detection by PCR was used [11,22,34,39,40,41]. This sequence was used to make both the DNA (Alpha DNA Montreal, Canada) and PNA probes (Bio-Synthesis Inc, Lewisville, TX, United States) [43,44]. DNA and PNA probes were labeled with 6-carboxyfluorescein in the N terminus (5´).

FISH

Serial 5 µm thick sections were cut and deparaffinized in xylene (100% 5 minutes), followed by 5-minute stepwise immersions in ethanol in decreasing concentrations, ending in distilled water. Then pretreatment was made in two steps: in the first step, the sections were immersed three times in PBS, (5 minutes each); in the second step, the sections were immersed for 15 minutes in a solution of proteinase K (10 µg/mL) in 50 mL of Tris buffer at 37°C and then for 10 minutes in a jar containing citric acid (1mM) at 96°C. Slides were refreshed for approximately 30 minutes and air dried [19].

FISH assay was performed as described previously [34,39]. Slides with tissue sections were preheated to hybridization temperature. Aliquots (30 µL) containing the following hybridization mixture were applied to each slide: containing 10% (wt/vol) dextran sulfate (Merck, Darm-stadt, Germany), 10 mM NaCl (J.T. Baker, Xalostoc, Edo. de México, México), 30 % (vol/vol) formamide (Pharmacia Biotech AB, Uppsala, Sweden), 0.1% (wt/vol) sodium pyrophosphate (Merck, Darmstadt, Germany), 0.2% (wt/vol) polyvinylpyrrolidone (Sigma Chemical Co, St Louis, MO, USA), 0.2% (wt/vol) Ficoll (Fluka Chemie AG, Basel, Switzerland), 5 mM EDTA [disodium ethylenediaminetetra-acetate dihydrate] (USB, Cleveland, Ohio, USA), 0.1% (vol/vol) Triton X-100 (USB, OH, USA), 50 mM Tris-HCl (Invitrogen, Carlsbad, California, USA) (pH 7.5) and a fluorescent probe with a final concentration of 1 µM. The slides were covered with cover glass and then placed in a preheated moisture chamber in the dark at 60°C for 60 minutes. After brief immersion in bi-distilled water, slides were washed in preheated washing buffer (5 mM Tris, 15 mM NaCl, 0.1% [vol/vol] Triton X-100 [pH 10]) at hybridization temperature for 30 minutes. Following a brief immersion in bi-distilled water, slides were air-dried and mounted with one drop of prolong Gold (Invitrogen).

Microscopy

Images were taken with a Zeiss Axioscope 40 microscope and captured using the Image Pro Plus System (Cybernetics, Bethesda, MD, USA). Images were also taken with a confocal microscope (LEICA DMI 4000 B) and analyzed with LAF software (Leica, Germany).

Statistical analysis

The results of individual tests IS6110 PCR, IS6110 DNA FISH and IS6110 PNA FISH were compared to the histopathological examination that was considered the gold standard. Chi square (χ^2) test was performed to test significance. A confidence interval of 95% was considered, and a p value less than 0.05 was considered statistically significant. Tests were evaluated by sensitivity, specificity, positive predictive value, negative predictive value,

Figure 1. Study flow diagram and results of PCR and FISH analyses for 41 ganglionar samples

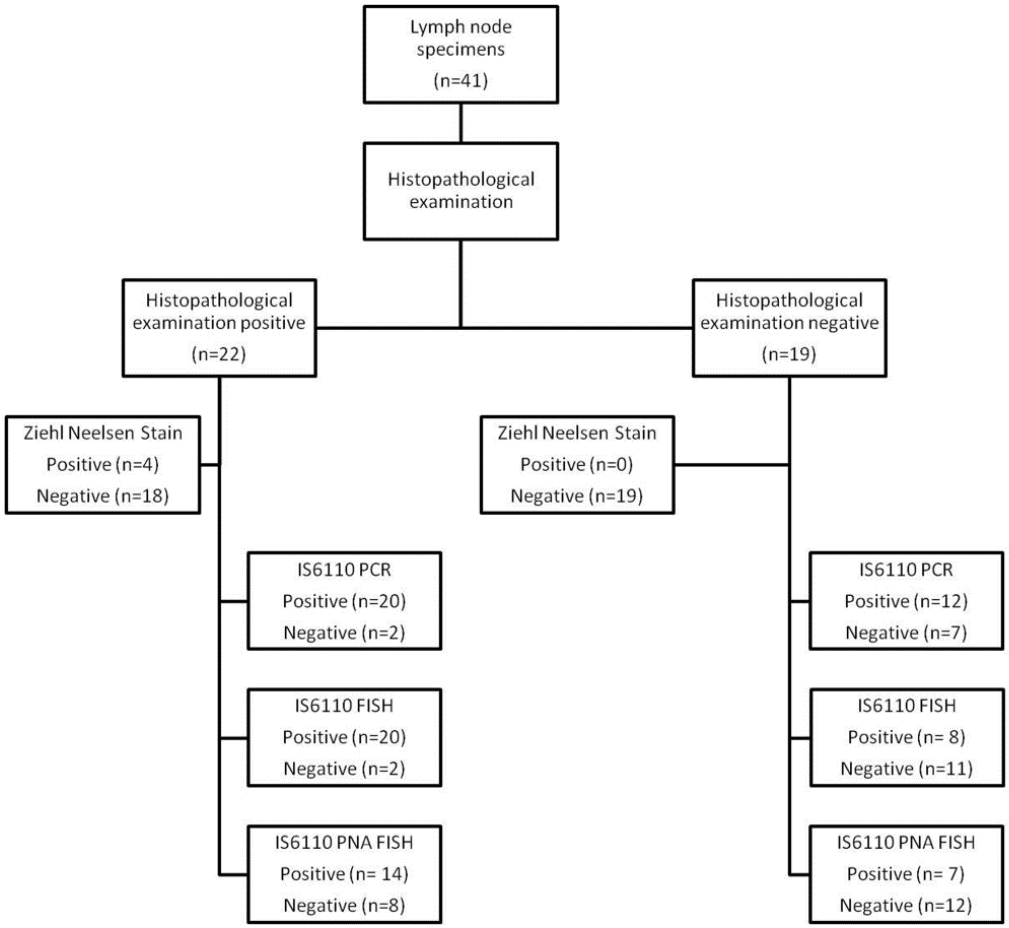
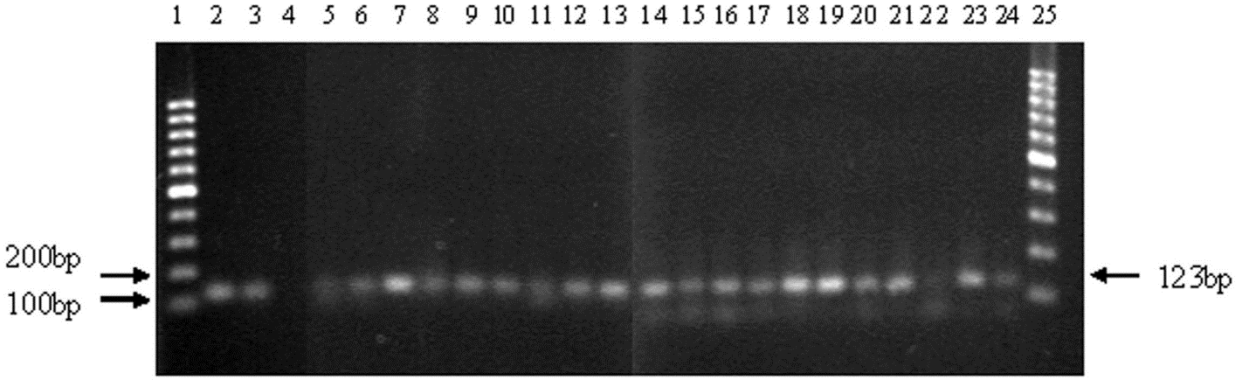
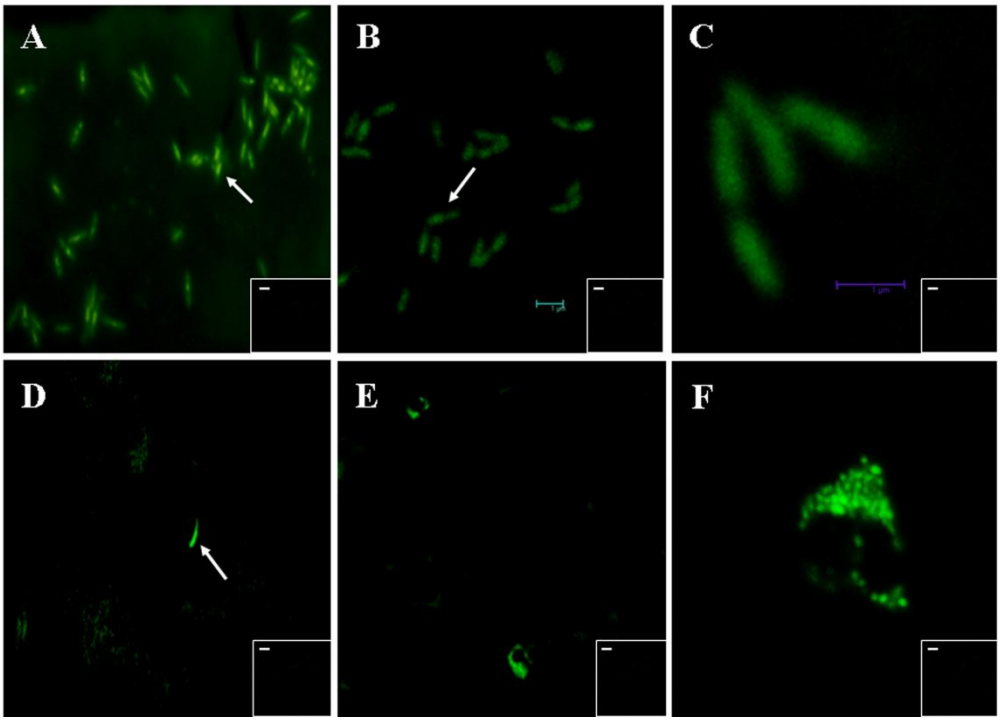


Figure 2. PCR products of the sequence IS6110 were analyzed on 2.0% agarose gel electrophoresis followed by ethidium bromide stain



Lanes 1 and 25, 100-bp ladder; 2, *M. bovis* BCG ATCC 35734; 3, *M. tuberculosis* H37Rv; 4, negative control; 5-24, lymph node clinical samples, positive with 123-bp amplified product.

Figure 3. Visualization by fluorescence in situ hybridization of *M. bovis* BCG ATCC 35734



(A & B) and *M. tuberculosis* H37Rv (C) cultures, hybridized with IS6110-FITC (green). (A) Images were taken with microscope Zeiss Axioscope 40 and captured using the Image Pro Plus System (Cybernetics). Original magnification ×1000 (B & C) micrographs were taken with a confocal microscope LEICA DMI 4000 B and analyzed with the software LAF (Leica, Germany). Original magnification ×630. Bar = 1 micrometer; (D, E & F) Lymph node sections (biopsies from patients positive to PCR IS6110) hybridized with IS6110-FITC (green) to detect *Mycobacterium tuberculosis* complex. Micrographs were taken with a confocal microscope LEICA DMI 4000 B and analyzed with the software LAF (Leica, Germany). Original magnification ×630. (-) Negative control. Arrows indicate a positive *Mycobacterium tuberculosis* complex bacillus (green).

and accuracy. Positive predictive value was considered the proportion of subjects with positive test results who were correctly diagnosed. Negative predictive value was considered the proportion of subjects with a negative test result who were correctly diagnosed. Accuracy indicated the efficiency of the test [45]. The Kappa coefficient was used for the evaluation of an agreement between two or more observations. We also used the Kappa test as performed by Viera [46] to verify our results.

The IS6110 PCR, IS6110 DNA FISH and IS6110 PNA FISH results were classified as true positives (Tp), true negatives (Tn), false positives (Fp), and false negatives (Fn). Sensitivity was calculated as [Tp/(Tp+Fn)]*100 and specificity was calculated as [Tn/(Tn + Fp)] *100. Positive predictive value (PPV) was calculated as Tp obtained/total number of positives; negative predictive value (NPV) was calculated as Tn obtained/total number of negatives; and Accuracy was calculated as [(Tp + Tn)/n] * 100, where n = 41[45].

Results

The IS6110 PCR

PCR IS6110 was positive in all tests of mycobacterium strains used as positive controls, specifically *M. tuberculosis* H37Rv (American Type Culture Collection, USA,) and *M. bovis* BCG ATCC 35734. On the histopathological examination positive (HEP), PCR IS6110 was positive in 20 samples and negative in two samples. On the histopathological examination negative (HEN) group, PCR was positive in 12 samples and negative in seven samples (Figure 1). PCR was negative in one lymph node sample with histopathological diagnosis of Hodgkin lymphoma by ZN and negative culture (negative control) (Figure 2).

Fluorescent in-situ *hybridization (FISH)*

As a first test of specificity, IS6110 DNA and PNA oligonucleotide fluorescent probes were applied to pure cultures, including *M. tuberculosis* H37Rv (American Type Culture Collection, USA,) and *M. bovis* BCG ATCC 35734, which were used as positive inner controls. A lymph node sample with histopathological diagnosis of Hodgkin lymphoma

Table 1. Results of PCR and FISH analyses for 41 specimens

Parameter [a]	Lymph node specimens (n = 41)		
	IS6110 PCR	IS6110 FISH	IS6110 PNA FISH
Sensitivity (%)	62.5, (CI, 54.4; 66.9)	71.4, (CI 61.3; 76.4)	66.7, (CI 51.8; 79.3)
Specificity (%)	77.8, (CI, 48.9; 93.4)	84.6, (CI, 62.9; 95.4)	60.7, (CI, 44.4; 73.3)
PPV (%)	90.9, (CI, 79.1; 97.3)	90.9, (CI, 78.1; 97.3)	63.6, (CI, 49.4; 75.7)
NPV (%)	36.8, (CI, 23.2; 44.2)	57.9, (CI, 43; 65.3)	63.2, (CI, 46.7; 77.1)
Accuracy (%)	65.9, (CI, 53.2; 72.7)	75.6, (CI, 61.8; 82.5)	63.4, (CI, 48.2; 76.4)
χ^2	4.583*	11.21*	2.93

[a]PPV, positive predictive Value; NPV, negative predictive value; χ^2, chi square test of significance, * (P< 0.05).CI, Confidence Interval 95%. HEP, histopathological examination positive; HEN, histopathological examination negative.

was used as a negative control, which was ZN and culture negative. Hybridization of positive controls showed intense and abundant fluorescent signals (Figure 3), with an aggregate pattern similar to the ZN staining pattern. The negative controls showed no fluorescent signals when they were probed with both the IS6110 DNA and PNA probes.

FISH IS6110 with DNA probes in the HEP group showed a positive result in 20 samples and a negative result in two samples, in perfect coincidence with PCR positive and negative results. In the HEN group, samples were positive in eight samples and negative in 11 samples. FISH IS6110 with PNA probes in the HEP group showed a positive result in 14 samples, and a negative in eight samples. When the HEN group was tested (19 samples), seven samples tested positive for FISH IS6110-PNA and 12 tested negative (Figure 1). Statistical results are shown in table 1.

The concordance percentage for IS6110 PCR and histopathological results was 65.8%. In comparison, the concordance percentage for IS6110 FISH and histopathological data was 75.6%. The concordance percentage for IS6110 PNAFISH was 63.4%. The Kappa value for inter-observer variability in estimating the IS6110 PCR, IS6110 DNAFISH and IS6110 PNAFISH was 1 according to the two trials for each test, because the results were the same in all three tests.

Discussion

LNTB is the type of tuberculosis most frequently identified in Aguascalientes, Mexico [17]. Extrapulmonary tuberculosis is difficult to differentiate from other diseases and is often misdiagnosed [7]. Various methods for diagnosis used in different countries could be one reason for the high levels of LNTB found. Fukushima and collaborators in Japan [47] propose that tuberculosis should be suspected in any patient with respiratory symptoms. Sputum tests for acid-fast bacteria should be performed at least three times initially. If findings on chest X-ray films are equivocal, high-resolution computed tomography should be performed to confirm details of shadows and to detect minimal pulmonary shadows or cavitary lesions. Physicians from all specialties should be repeatedly informed about the risk of tuberculosis and should include tuberculosis in the differential diagnosis in patients suspected to have pulmonary diseases. However, this type of management control is not frequently practiced in all parts of Mexico.

An essential element in the management of LNTB is the availability of a rapid, sensitive, specific and reliable diagnosis. The laboratory diagnosis is largely based on ZN staining, followed by direct microscopy identification and culture for mycobacterium. Direct microscopic examination by ZN staining has very low sensitivity and often is less specific than molecular tests [10,15,19,48]. The isolation of mycobacterium is not only time-consuming but also has low sensitivity in LNTB samples, due to the low concentration of bacilli [2,45,48]. Since demonstration of ZN in histopathological specimens and the bacilli recovery by culture are both quite difficult, the diagnosis of LNTB is often based on the presence of granulomatous inflammation with or without caseation [45].

In recent years, PCR methods have increasingly been developed for the most important species of mycobacteria. Some tests are already commercially available in Mexico but are not used as a complementary diagnosis tool [45,48,49]. Because there are multiple copies of the IS6110 insertion sequence in the MTB, it is an attractive target for PCR amplification, and has been tested in intestinal TB and Crohn's disease [50]. Our PCR data showed similar sensitivity and specificity compared with the

results reported by these authors. PCR based on IS6110 amplification showed to be a rapid, sensitive and specific test reliable for routine use for the diagnosis of TB in different clinical specimens [21,44,51,52].

The use of other molecular methods for the detection of mycobacteria in tissue samples, such as ISH or FISH, have been used with similar results to those of PCR [22,28,34]. ISH allows a faster diagnosis than culture methods and also detects fewer bacilli than current PCR methods. Furthermore, unlike PCR, ISH provides detailed, tissue-based morphological information [22] including expression of several MTB genes in tuberculous granulomas that could be implicated in the pathogenesis of bacteria [27].

In this study IS*6110* oligonucleotides DNA and PNA of 20 bp labeled with 6-carboxyfluorescein were built, following the same sequence as those used in the PCR test described by Lefmann *et al*. [34] and Naser *et al*. [35]. These oligonucleotides showed high specificity and sensitivity [44,45,48,49]. Our results showed higher sensitivity and specificity in the detection of MTC in comparison to ZN staining, PCR, and modified FISH. However, while these oligonucleotides can distinguish between NTM and MTC, they cannot distinguish between *Mycobacterium tuberculosis* and BCG, because BCG is part of MTC [47].

In our study we found that 90.9% of the samples were positive by PCR; furthermore, when using modified DNA FISH, 63.63 % were positive with PNA probes. FISH IS6110 using DNA probes showed better results compared to PNA probes. All three tests showed highly consistent results between both positive and negative samples.

False negative results were probably due to too few bacilli contained in the sample. MTB might also not be distributed evenly in the tissue, and occasionally loss of bacilli can occur during DNA extraction [50].

We observed the following different patterns of MTC aggregates: free bacilli, bacilli around granulomatous lesions, and intracellular bacilli. Lymphadenitis was the most common manifestation of infection due to non-tuberculous mycobacteria in otherwise healthy children [52]. There are few reports n Mexico on NTM strains and those that are available are generally associated with HIV [53]. As described, DNA or PNA IS*6110* oligonucleotides discriminate between NTM and MTC [1,50]. NTM was not included as a control in this work; therefore, more studies incorporating this data should be conducted.

Although PCR is used to confirm TB diagnosis in Mexico [54], it is not common in all hospitals throughout the country because of the lack of necessary infrastructure. The initial cost for equipment and technician training is high. However, once the initial expenses have been covered, PCR and DNA FISH testing are not costly. Becausethey provide resultsrapidly, the use of PCR and modified DNA FISH technology could help to complement the early diagnosis of LNTB and could have particular relevance in developing countries such as Mexico, which have high incidence of extra pulmonary tuberculosis.

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