

## EFFECTS OF TISSUE HANDLING AND PROCESSING STEPS ON PCR FOR DETECTION OF *Mycobacterium tuberculosis* IN FORMALIN-FIXED PARAFFIN-EMBEDDED SAMPLES

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### SUMMARY

Development and standardization of reliable methods for detection of *Mycobacterium tuberculosis* in clinical samples is an important goal in laboratories throughout the world. In this work, lung and spleen fragments from a patient who died with the diagnosis of miliary tuberculosis were used to evaluate the influence of the type of fixative as well as the fixation and paraffin inclusion protocols on PCR performance in paraffin embedded specimens. Tissue fragments were fixed for four h to 48 h, using either 10% non-buffered or 10% buffered formalin, and embedded in pure paraffin or paraffin mixed with bee wax. Specimens were submitted to PCR for amplification of the human beta-actin gene and separately for amplification of the insertion sequence IS6110, specific from the *M. tuberculosis* complex. Amplification of the beta-actin gene was positive in all samples. No amplicons were generated by PCR-IS6110 when lung tissue fragments were fixed using 10% non-buffered formalin and were embedded in paraffin containing bee wax. In conclusion, combined inhibitory factors interfere in the detection of *M. tuberculosis* in stored material. It is important to control these inhibitory factors in order to implement molecular diagnosis in pathology laboratories.

**KEYWORDS:** Tuberculosis; PCR; Diagnosis; Paraffin-embedded tissue.

### INTRODUCTION

Brazil occupies the sixteenth position in the number of tuberculosis cases per year among the 22 countries concentrating 80% of tuberculosis cases in the world<sup>30</sup>.

Precise and fast diagnosis of tuberculosis is of utmost importance nowadays and standardization of reliable methods for detection of *Mycobacterium tuberculosis* in clinical samples is a major goal, not only in high-burden countries but also throughout the world.

Detection of the tuberculosis bacillus in clinical and pathology laboratories is usually performed by microscopic observation after Ziehl-Neelsen (ZN) staining. This is a rapid and affordable test, but sensitivity is low compared to molecular techniques and it does not allow the identification of the *Mycobacterium* species present in the specimen<sup>6,11</sup>.

Polymerase Chain Reaction (PCR) is highly specific and sensitive compared to ZN and culture<sup>4,6,9,26,29</sup>. It is also rapid, cheap and can be performed in most clinical and pathology laboratories<sup>22</sup>.

Amplification of the insertion sequence IS6110 has been used for many years for diagnosis of tuberculosis<sup>5,20,21,27</sup>. This element is present in genomes of members of the *M. tuberculosis* complex. Copy numbers

vary from 0 to 25, integrated in different genome regions<sup>5,26,29</sup>. Its presence in multiple copies is the main advantage for its use, which increases amplification sensitivity. *M. tuberculosis* lacking this insertion sequence would not be detected by PCR-IS6110 but this event is rare, and has not been described in Brazil. Also the absence of this insertion element in other species of mycobacteria impairs its use for diagnosis of diseases caused by non-tuberculous mycobacteria.

Several authors have proposed the use of diagnostic molecular methods using formalin-fixed and paraffin-embedded tissue (FFPE)<sup>9,13,27</sup>. This material can be stored and represents an important source for both prospective and retrospective studies. PCR can detect DNA in small amounts of biological material and for this reason also it is useful for diagnosis<sup>5,6</sup>.

Nevertheless, there are difficulties in obtaining good results using PCR in FFPE tissue<sup>16</sup>. PCR efficiency in FFPE samples may be influenced by several factors, which affect tissue integrity<sup>7,22</sup>, such as the fixation protocol, the age of the paraffin block, and the presence of endogenous or exogenous inhibitors of the reaction<sup>1,7</sup>.

Optimization of rapid diagnostic tests is an important laboratory goal, especially in developing areas with limited resources. In this work we have compared different protocols for tissue fixation and paraffin

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embedding in a controlled experiment for a PCR diagnostic testing for tuberculosis.

## MATERIAL AND METHODS

**Samples with controlled fixation and paraffin embedding:** Fresh tissue specimens of spleen and lung from an AIDS patient, who died from miliary tuberculosis, strongly positive for acid-fast bacilli, were subjected to different protocols of tissue fixation and paraffin embedding. As negative control, a non-infected block from spleen was tested. Fixation was carried out for 4h, 6h, 12h, 24h, or 48h, at room temperature, using formalin phosphate-buffered at pH 7.0 (formaldehyde 37%, monosodic phosphate 47 mM, disodic phosphate 28 mM) or 10% formalin in distilled water. Embedding was performed using paraffin (Retrowax-Parafinas Nordeste) either pure or mixed with bee wax, 2:1. Table 1 shows the distribution of the different protocols.

**Tissue processing for PCR:** From each paraffin-embedded specimen, 3 to 10 µm-thick sections were cut, using a new blade for each

sample. Paraffin was extracted with four 30 min incubation with xylol (MERCK, Germany) followed by four washes with 100% ethanol. Tissue was digested with proteinase K 600 µg/mL (final concentration) in TE (Tris-HCl 1 mM, EDTA 0.5 mM pH 8) and Tween 20 0.5% for 16 h at 37 °C. Samples were subjected to heat-shock in liquid nitrogen, for one min, and incubated for 10 min at 95 °C. DNA was extracted with phenol-chloroform-isoamyl alcohol 25:24:1 and precipitated with 10% sodium acetate 3M pH 4.8 and 2.5 volumes of absolute ethanol (MERCK). The precipitate was resuspended in TE plus RNase and kept at 4 °C. DNA concentration was determined in spectrophotometer at 260 nm wave length, and purity was evaluated by analysis of readings at 260 nm and 280 nm (GeneQuant, Amersham Biosciences do Brasil Ltda).

**Amplification of human beta-actin gene:** A 135 bp fragment of the human beta-actin gene was amplified using 0.5 µM of primers β<sub>5</sub>: AGCGGGAAATCGTGCGTG and β<sub>R</sub>: GGTGATGACCTGGCCGTC in reactions containing 50 mM KCl, 10 mM Tris-HCl, pH 8.0, 1.5 mM MgCl<sub>2</sub>, 100 µM dNTPs, and 1 U of Taq polymerase (Invitrogen). DNA (0.2 µg) was amplified with one cycle at 96 °C for five min, 40 cycles at

**Table 1**

Results of PCR for amplification of the human beta-actin gene and IS6110 in lung and spleen samples subjected to different fixation and embedding protocols

fxT (h)	Tpf	Incl	tissue				β-actin
			spleen		lung		
			IS6110	H	IS6110	H	
4	F10%	Pw	+	+	-	+	+
4	F10%	Pp	+	+	+	+	+
4	BF	Pw	+	+	-	+	+
4	BF	Pp	+	+	-	-	+
6	F10%	Pw	-	+	-	+	+
6	F10%	Pp	+	+	+	+	+
6	BF	Pw	-	+	+	+	+
6	BF	Pp	+	+	+	+	+
12	F10%	Pw	+	+	-	-	+
12	F10%	Pp	-	-	+	+	+
12	BF	Pw	-	+	-	+	+
12	BF	Pp	+	+	+	+	+
24	F10%	Pw	+	+	-	-	+
24	F10%	Pp	-	+	+	+	+
24	BF	Pw	+	+	-	+	+
24	BF	Pp	+	+	+	+	+
48	F10%	Pw	-	-	-	-	+
48	F10%	Pp	-	+	+	+	+
48	BF	Pw	+	+	+	+	+
48	BF	Pp	-	+	+	+	+

fxT = fixation time (hours), Tpf = type of fixative, Incl = paraffin inclusion, F10% = 10% non-buffered formalin, BF = buffered formalin, Pw = paraffin with bee wax, Pp = pure paraffin, H = hybridization with probe complementary to IS6110.

96 °C for two min, 60 °C for two min and 72 °C for two min and a final extension cycle at 72 °C for seven min.

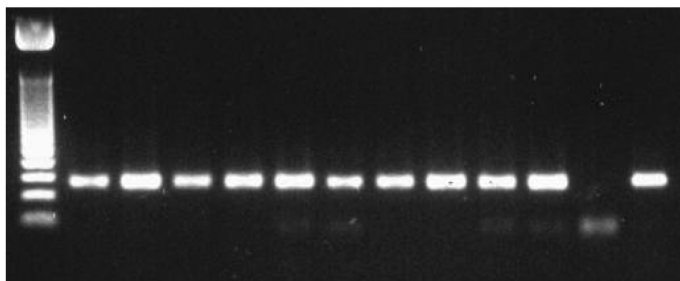
**Amplification of insertion sequence IS6110:** A 123 bp fragment from the insertion sequence IS6110 was amplified using 0.5 µM of the primers IS1: CCTGCGAGCGTAGGCCGTCGG and IS2: CTCGTCCAGCGCCGCTTCGG<sup>5</sup> in reactions containing 50 mM KCl, 10 mM Tris-HCl, pH 8.0, 1.5 mM MgCl<sub>2</sub>, 100 µM dNTPs, and 1 U of Taq Polymerase. Amplification cycles were one cycle at 96 °C for five min, 40 cycles at 96 °C for one min, 65 °C for one min and 72 °C for one min and a final extension cycle at 72 °C for seven min.

**Detection of amplification products:** PCR products were visualized in 2% agarose gels in buffer TBE (Tris-borate 45 mM, EDTA 1 mM) under UV light, after electrophoresis at 100 V/min and ethidium bromide staining.

**Southern blot and hybridization:** Amplification products in agarose gels were blotted onto nylon membranes (Hybond N-plus, Amersham Biosciences do Brasil Ltda) using Vacuum Blotter apparatus (BioRad, Hercules, CA, USA). Membranes were incubated in NaOH 0.4N for two min and in SSC 0.1X / SDS 0.1% at 65 °C for 15 min (SSC 1X is 150 mM NaCl, 15 mM sodium citrate pH 7.0). DNA was probed, in different experiments, with products of amplification of beta-actin and IS6110. Probes were covalently labeled with peroxidase by glutaraldehyde and detected according to the procedure of the ECL Direct System (Amersham Biosciences do Brasil Ltda). Hybridization was visualized after exposure of the membranes to X-ray film (X-OMAT Kodak, Rochester, NY, USA).

**RESULTS**

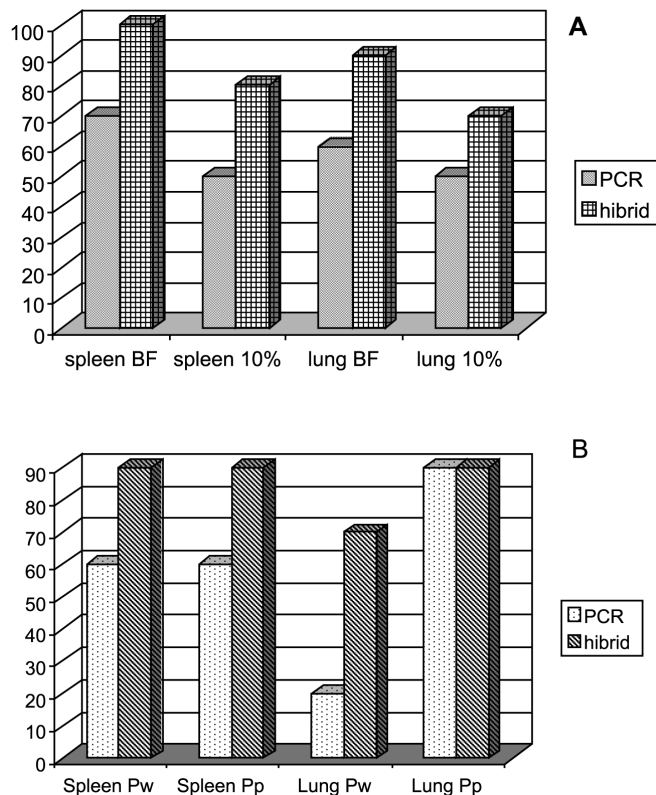
No consistent differences in the quantity of isolated DNA were observed between the samples. Table 1 shows the different specimen preparation protocols and results obtained with PCR reactions and hybridization. PCR of the human beta-actin gene generated amplicons with all 40 tested samples, confirming the presence of amplifiable DNA in all samples (Fig. 1). The different fixation times used (4h, 6h, 12h, 24h and 48h) did not influence the amplification results.



**Fig. 1** - Agarose (2%) gel electrophoresis of amplified beta-actin fragments from 10 lung samples. 1: 4 h / buffered formalin, 2: 4 h / 10% non-buffered formalin, 3: 6 h / buffered formalin, 4: 6 h / 10% non-buffered formalin, 5: 12 h / buffered formalin, 6: 12h / 10% non-buffered formalin, 7: 24 h / buffered formalin, 8: 24 h / 10% non-buffered formalin, 9: 48 h / buffered formalin, 10: 48h / 10% non-buffered formalin. All samples were embedded in paraffin with bee wax. N: negative control, P: positive control (purified DNA from *M. tuberculosis* H37Rv).

**Fixation protocols:** IS6110 amplicons were visualized in agarose gels with 60% of the lung specimens fixed in buffered formalin and were detected in 90% of the same specimens after hybridization of the amplified fragments. When lung specimens were fixed in 10% non-buffered formalin, positivity of PCR decreased to 50% by gel visualization and to 70% after hybridization.

IS6110 amplicons from 70% of the spleen specimens fixed with buffered formalin were visualized in gel. After hybridization, all specimens gave positive results. Amplicons were visualized in agarose gels with 50% of the 10% non-buffered formalin fixation specimens and were detected in 80% of these specimens after hybridization (Fig. 2A).



**Fig. 2** - **A:** Positivity of PCR-IS6110 (PCR) and hybridization (hibrid) in lung and spleen specimens fixed with buffered formalin (BF) and 10% non-buffered formalin (10%). **B:** Positivity of PCR-IS6110 (PCR) and hybridization (hibrid) in lung and spleen specimens embedded in paraffin with bee wax (Pw) and pure paraffin (Pp).

**Embedding protocols:** Lung samples embedded in paraffin with bee wax generated amplicons in 20% of the analyzed samples, by gel visualization; this percentage increased to 70% after hybridization. Only one out of 10 samples embedded in pure paraffin was negative in both agarose gel visualization and hybridization (Fig. 2B and Table 1).

No differences were observed when spleen samples embedded in pure paraffin or paraffin with bee wax were compared. Amplicons were visualized in agarose gels with 60% of the samples and after hybridization with 90% of the samples (Fig. 2B). In one sample from each group, amplicons not visualized in gels were also negative after hybridization (Table 1).

## DISCUSSION

The FFPE procedure is adequate for tissue morphology conservation but may negatively affect nucleic acid preservation and consequently also molecular diagnostic procedures. Analyzable DNA fragments usually have less than 300 bp to 500 bp, probably due to strand fragmentation to which RNA is much more susceptible<sup>24,28</sup>. In order to evaluate factors contributing to the low efficiency of PCR in FFPE samples, an experiment using two different tissue types (lung and spleen) and different time of fixation, fixatives, and paraffin embedding protocols was performed. A single protocol for DNA extraction was used, based on that proposed by WRIGHT & MANOS<sup>31</sup>, but including additional steps of DNA extraction with phenol-chloroform-isoamyl alcohol and ethanol precipitation.

Amplification of the human beta-actin gene, used as control of DNA extraction, was obtained with all samples. The high efficiency of this test might be related to the primers used, which amplify a 135 bp fragment instead of the 404 bp fragment described by RICHTER *et al.*<sup>19</sup>. The size of the amplicon is a decisive factor for the amplification success<sup>8</sup>, because DNA in FFPE tissues is often degraded by fixation and embedding procedures<sup>12,23,28</sup>.

DNA concentration may also influence PCR results. DIAZ *et al.*<sup>4</sup> used 0.5 mg to 2 mg of DNA in the tests for the detection of *Mycobacterium tuberculosis* DNA in tissue and obtained 100% positive result in all biopsy samples, which were also culture proven cases of tuberculosis. MARCHETTI *et al.*<sup>18</sup> tested different DNA concentrations (1 µg, 3 µg and 5 µg) in PCR for amplification of the IS6110 insertion sequence. Best results in terms of sensitivity were obtained with 1 µg and 3 µg and the highest number of false-negative results was obtained with DNA at a concentration of 5 µg, probably as a consequence of the presence of inhibitors<sup>1,8</sup>. In this work, we have used 0.2 µg of DNA per reaction and this concentration was sufficient to generate beta-actin gene amplicons with all samples.

RISH *et al.*<sup>20</sup> suggested that DNA fragmentation in FFPE tissues might be influenced by the fixative and fixation time. In our study, amplification results were not clearly influenced by the different fixation times tested (4h, 6h, 12h, 24h and 48h).

Despite the few numbers of samples fixed for 48h, apparently there was less interference of the use of non-buffered formalin in the molecular results in the samples fixed longer than 24h.

In a future project, it would be interesting to test, in a large series, archival samples, fixed in non-buffered formalin and stored for long period of time.

Similar results were obtained by GREER *et al.*<sup>7</sup>, who evaluated different fixation times (1h, 4h and 24h) and by JOHANSEN *et al.*<sup>16</sup> who obtained excellent results using fixation times between 16h and 18h. In our experiment, the samples were collected and fixed in less than 24h, which may have contributed to the high positivity in the human beta-actin gene amplification. INOUE *et al.*<sup>14</sup> concluded that tissues have to be fixed immediately for one day to remain viable for amplification by PCR of fragments up to 200bp.

Regarding the fixative, buffered formalin presented better results

than 10% non-buffered formalin both with lung and spleen samples, with positivity of 50% and 50%, respectively. For the samples fixed in buffered formalin, the positivity was 60% and 70% for lung and spleen, respectively, which suggested that 10% non-buffered formalin may be inadequate for samples that will be submitted to molecular biology testing.

Our results paralleled those obtained by MARCHETTI *et al.*<sup>18</sup> using FFPE tissues fixed with buffered formalin. Nevertheless, the results differ from those obtained by JOHANSEN *et al.*<sup>16</sup>, who described good results with the use of 10% non-buffered formalin. It is possible that the quality of the water used for formalin dilution or other unknown factors might have contributed to these discrepant results. AN *et al.*<sup>1</sup> described the presence of PCR inhibitors in frozen samples, indicating that they can be endogenous and not induced by fixation or other steps of specimen preparation.

Bee wax is commonly added to paraffin to facilitate the sectioning of FFPE samples. The presence of bee wax did not influence PCR for human beta-actin gene amplification but influenced negatively the amplification of the *M. tuberculosis* IS6110 fragment. This was observed in lung tissue, in which amplicons were detected only in 20% of the samples embedded in paraffin with bee wax against 90% of the samples embedded in pure paraffin. On the contrary, similar results were obtained with spleen specimens embedded in paraffin with bee wax and pure paraffin.

Hybridization can be used to increase sensitivity of detection of amplification products, especially in paucibacillary samples<sup>3,15,25</sup>. In the present study, IS6110 amplification products from five lung and six spleen samples not visualized in gels were detected after hybridization experiments. However, hybridization failed to detect amplicons obtained with four lung and two spleen samples (Table 1). The present results confirmed overall increase in sensitivity after hybridization; however some authors did not observe any significant increase in sensitivity after hybridization of PCR products<sup>5,17</sup>.

False negative results of IS6110 PCR might have resulted from a low efficiency of the mycobacterial cell wall lysis or uneven distribution of bacilli in the samples<sup>1,16</sup>. In addition, for the testing, only part of biopsy samples were analysed. As the distribution and quantity of bacilli vary from field to field, this might be an additional explanation for false negative results.

False negative results of molecular testing using FFPE tissue have also been described when the fixation protocol is not known<sup>10</sup>, after inadequate fixation<sup>2</sup>, and as a result of excessive dilution of DNA<sup>18</sup>. To increase the efficiency of cell wall lysis of the organisms, the increase of the temperature as well as the concentration of the proteinase K might be two helpful technical additional procedures. HEINMOLLER *et al.*<sup>10</sup> did not demonstrate the presence of mycobacteria by PCR in FFPE lymph node samples, but amplicons were generated when fresh samples were used.

Considering data presented in Table 1, the number of spleen and lung test samples generating IS6110 amplicons visualized in agarose gels was comparable (12 with spleen and 11 with lung samples). On the other hand, after hybridization, amplicons were detected with 18 spleen samples but only with 16 lung samples. Perhaps the histological organization of the lungs, showing sparse cellularity with respect to organ

volume, may contribute for the production of insufficient amounts of available mycobacterial DNA<sup>1</sup>.

When the factors that produced negative effect in amplification detection were combined, i.e. specimens fixed with 10% non-buffered formalin, embedded in paraffin with bee wax, and obtained from lung, positivity of PCR-IS6110 was null. This result indicates that these combined factors profoundly affect the detection of *M. tuberculosis* in stored paraffin blocks. In conclusion, an effort for implementation of non-inhibiting fixation and embedding protocols has to be made in pathology laboratories in order to put into practice efficient molecular techniques for tuberculosis diagnosis.

## RESUMO

### Efeitos das etapas de tratamento e processamento do tecido na PCR para detecção de *Mycobacterium tuberculosis* em amostras fixadas em formalina e incluídas em parafina

O desenvolvimento e a padronização de métodos confiáveis para a detecção de *Mycobacterium tuberculosis* em amostras clínicas é um objetivo importante nos laboratórios de todo o mundo. Neste trabalho, fragmentos de pulmão e baço de paciente que morreu com o diagnóstico de tuberculose miliar foram usados para avaliar a influência do tipo de fixador e dos protocolos de fixação e inclusão em parafina na performance da PCR. Fragmentos de tecido foram fixados por quatro h a 48 h, usando formalina não tamponada a 10% ou formalina tamponada a 10% e incluídos em parafina pura ou misturada a cera de abelha. As amostras foram submetidas a PCR para amplificação do gene da beta-actina humana e, separadamente, para amplificação da sequência de inserção IS6110, específica do complexo *M. tuberculosis*. O resultado da amplificação do gene da beta-actina foi positivo em todas as amostras. Não foram gerados amplicons na PCR-IS6110 em amostras de tecido pulmonar fixadas usando formalina não tamponada a 10% e incluídas em parafina com cera de abelha. Em conclusão, fatores inibitórios combinados interferiram na detecção de *M. tuberculosis* em material de arquivo. É importante controlar estes fatores inibitórios para poder implementar o diagnóstico molecular em laboratórios de patologia.

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