

Research Article

Detection of *Mycobacterium avium* subsp. *paratuberculosis* by a Direct In Situ PCR Method

**Fernando Delgado,¹ Diana Aguilar,² Sergio Garbaccio,¹ Gladys Francinelli,¹
R. Hernández-Pando,² and María Isabel Romano³**

¹ Instituto de Patobiología, CICVyA, INTA, De Los Reseros y Dr. Nicolás Repetto S/N, Hurlingham, 1686 Buenos Aires, Argentina

² Experimental Pathology Section, Department of Pathology, Instituto Nacional de la Nutrición “Salvador Zubirán”,

Vasco de Quiroga 15, Colonia Sección XVI, Tlalpan, 14000 Mexico City, DF, Mexico

³ Instituto de Biotecnología, CICVyA, INTA, De Los Reseros y Dr. Nicolás Repetto S/N, Hurlingham, 1686 Buenos Aires, Argentina

Correspondence should be addressed to Fernando Delgado, fdelgado@cni.inta.gov.ar

Received 12 January 2011; Revised 3 March 2011; Accepted 26 April 2011

Academic Editor: Jesse M. Hostetter

Copyright © 2011 Fernando Delgado et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In situ detection of *Mycobacterium avium* subsp. *paratuberculosis* is useful for diagnosis and research of paratuberculosis. The aim of this paper was to detect this agent in formalin-fixed, paraffin-embedded tissue samples by a direct in situ PCR. The technique was performed on ileum or ileocaecal lymph node samples from 8 naturally infected cattle and 1 healthy calf, by using p89 and p92 primers for amplification of IS900 sequence. Moderate positive signal was detected in all positive samples and not in negative control, but tissues resulted were affected in many cases due to the enzymatic treatment and the high temperature exposition. Although the technique was useful for Map detection, the signal was lower than immunohistochemistry probably because of the fixation process. In one case, signal was higher, which might be due to the detection of spheroplasts. Thus, the described method should be recommended when others resulted negative or for spheroplasts detection.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (Map) is the causative agent of paratuberculosis (PTB), also called John's disease. This affects cattle, sheep, and goats and produces losses in dairy and beef production. Clinical features include diarrhea and loss of weight, and the main pathologic changes are granulomatous inflammation of the intestine and mesenteric lymph nodes [1]. Additionally, PTB is suspected to be related to Crohn's disease (CD) in humans although this hypothesis is currently debated [2, 3].

Histopathology is used as a diagnostic method, but it is also a very important tool for researching PTB. Detection of Map in tissue samples increases the pathologic diagnosis and may be necessary when experimental infections are performed. Several techniques such as Ziehl Nielsen staining (ZN), immunohistochemistry (IHC), and in situ hybridization (ISH) were tested for detection of the agent [2, 4, 5], but their performances are different. ZN and IHC are easy

to perform and have high sensitivity [4–6], but false negative can arise when infection was recent or bacilli were scanty. Besides, their specificity may be considered low since ZN can not differentiate among acid fast microorganisms and antigens shared by different mycobacteria may affect IHC performance. When both are compared, ZN is cheaper, but IHC may detect antigens of Map even when the bacillus was digested in the cytoplasm of macrophages. On the other hand, ISH is specific but more expensive, hard to perform, and its interpretation may be difficult because of the lower signal obtained [5]. However, detection of Map by ISH is considered useful because it detects spheroplasts (forms of Map with deficiencies in the cell wall) which may be involved with the disease and may be not detected by ZN or IHC [2]. Besides, detection of DNA of Map may be useful when IHC and ZN are negative because bacilli and their antigens are damaged.

In situ PCR (ISP) consists of the amplification of one specific sequence of DNA in a tissue sample. It has been

described as a very sensitive and specific technique and is used for diagnosis or research in many diseases. Related to PTB, an ISP method followed by in situ hybridization was performed for detection of Map in sheep and mice tissue samples [7, 8]. Although it was useful for Map identification, a direct ISP (dISP) method which does not require the hybridization step should be easier to perform. To our knowledge, dISP was successfully used to detect the infection of *M. tuberculosis* in samples from affected and healthy human subjects [9], but not for Map detection in veterinary medicine. The aim of this paper was to detect *Mycobacterium avium* subsp. *paratuberculosis* in tissue samples of naturally infected cattle fixed in formalin paraffin embedded, by using a dISP method, and compare it with immunohistochemistry.

2. Material and Methods

2.1. Analyzed Samples. Samples of ileum or ileocaecal lymph node from nine cows were used. Eight corresponded to adult animals with clinical signs of PTB and isolation of Map from faeces. The other sample was from a calf belonging to a free herd with no changes or clinical signs, which was used as negative control. Tissues were fixed in 10% formalin solution and embedded in paraffin following the standard histological procedures. Compatible lesions and acid fast bacilli were previously confirmed by hematoxylin and eosin and ZN staining performed following routine techniques.

2.2. Direct In Situ PCR. Tissue sections (2 µm thickness) were obtained and mounted on positive charged slides. These were deparaffinised by keeping 18 h at 60°C and immersed in xylene (30 min at 37°C), absolute ethanol at room temperature (RT), 75% ethanol (RT), 50% ethanol (RT), 25% ethanol (RT), and water (RT). Then, they were made permeable by incubation at room temperature in 0.02 mol/L HCl for 10 min, followed by 0.01% triton X-100 for 90 s. Proteins were depleted by incubation with 1 mg/L proteinase K (Gibco, Paisley, UK) for 30 min at 37°C, which was inactivated by boiling in a microwave for 15 s. Endogenous alkaline phosphatase was inactivated by immediately immersing the slides into 20% acetic acid for 15 s.

The PCR was performed by incubation of the sections with 50 mL 1X reaction buffer (Gibco, BRL), 1.5 U Taq polymerase, 2 mmol/L MgCl₂, 40 mmol/L dNTP, 0.2 mmol/L dUTP labelled with digoxigenin (Boehringer Mannheim, Lewes, UK), and 60 pg each of IS900 *M. avium* subsp. *paratuberculosis* insertion sequence primers. The primers used were p89 (sequence 5'-CGTCGGGTATGGCTTTCATGTGGTTGCTGTG-3') and p92 (sequence 5'-CGTCGTTGGCCACC-CGCTGCGAGAGCAAT-3'), previously tested by ISH [2, 5]. The slides were sealed with the Assembly tool (Perkin Elmer, Cambridge, UK) and placed in a Touch Down thermocycler (Hybaid, Ashford, UK). PCR was undertaken with the following thermocycler conditions: 5 min at 95 8C, 35 cycles of 94 8C (1 min), 64.5 8C (1 min) and 72 8C (1 min) ending at 72 8C for 2 min. PCR products were detected with alkaline phosphatase-conjugated sheep antibodies against anti-

digoxigenin (Boehringer Mannheim) diluted 1/500. The chromogen was 5-bromo-4-chloro-3-indolyl phosphate toluidine salt and tetrazolium nitroblue (Boehringer Mannheim) diluted 1/50. Sections were counterstained with nuclear fast red. For control of false positives, each test section was subjected to PCR without the Taq polymerase.

2.3. Immunohistochemistry. Immunohistochemistry was performed following procedures previously described [5]. Briefly, 2 µm sections were obtained, mounted on positively charged slides, and deparaffinized. Endogenous peroxidase activity was blocked with 10% hydrogen peroxide in methanol (20 min), and antigenic recovery was performed by humid heat treatment (121°C, 15 min) in citrate buffer (monohydrate citrate, 10 mM, pH 6). After cooling, slides were immersed in TBS buffer (50 mM Tris-HCl, 300 mM NaCl pH 7.6) for 20 min. A blocking step was performed (BSA (Promega) 2% in TBS, 5 min), after which 40 mL of the anti-Map antibody (rabbit polyclonal, Queen's University Belfast, Northern Ireland, UK) diluted 1/100 in TBS was added and incubated at 4°C overnight. The reaction was revealed using the LSAB2[®] system (Dako Citomation System) and DAB. Slides were counterstained with Mayer Hematoxylin and coverslipped with synthetic medium.

2.4. Slides Interpretation. All preparations were observed with a conventional light microscope at 40 X, 100 X, 200 X, 400 X, and 1000 X magnification and were compared observing the same regions in all cases. The obtained results were classified as negative (-), weak (+), moderate (++), and intense (+++), according to the number of stained cells at 400 X. When abundant staining was observed at 100 X, it was classified as intense.

3. Results

3.1. In Situ PCR. All infected samples showed staining, which consisted in small blue spots inside the macrophages or Langhans giant cells. Most of them were in the cytoplasm, and few were in the nucleus of the cells (Figures 1(a) and 1(b)). The intensity of the signal was moderate in all cases (Table 1). Negative control was negative, and positive tissues which were incubated without Taq did not show any signal.

Tissue morphology was not perfectly conserved. Connective tissue was not correctly counterstained and a lot of cellular nuclei could not be clearly observed (Figure 1(d)). Areas of tissue were disrupted and removed from the slide in several cases, which made impossible the interpretation of dISP in these areas.

3.2. Immunohistochemistry. All infected tissues showed immunostaining in areas with granulomatous inflammation. Staining was inside the epithelioid and Langhans giant cells, which were distributed in the ileal mucosa and submucosa (Figure 1(c)). The signal was intense in all cases, except in sample 6, in which it was weak (Table 1). Negative control did not show immunostaining.

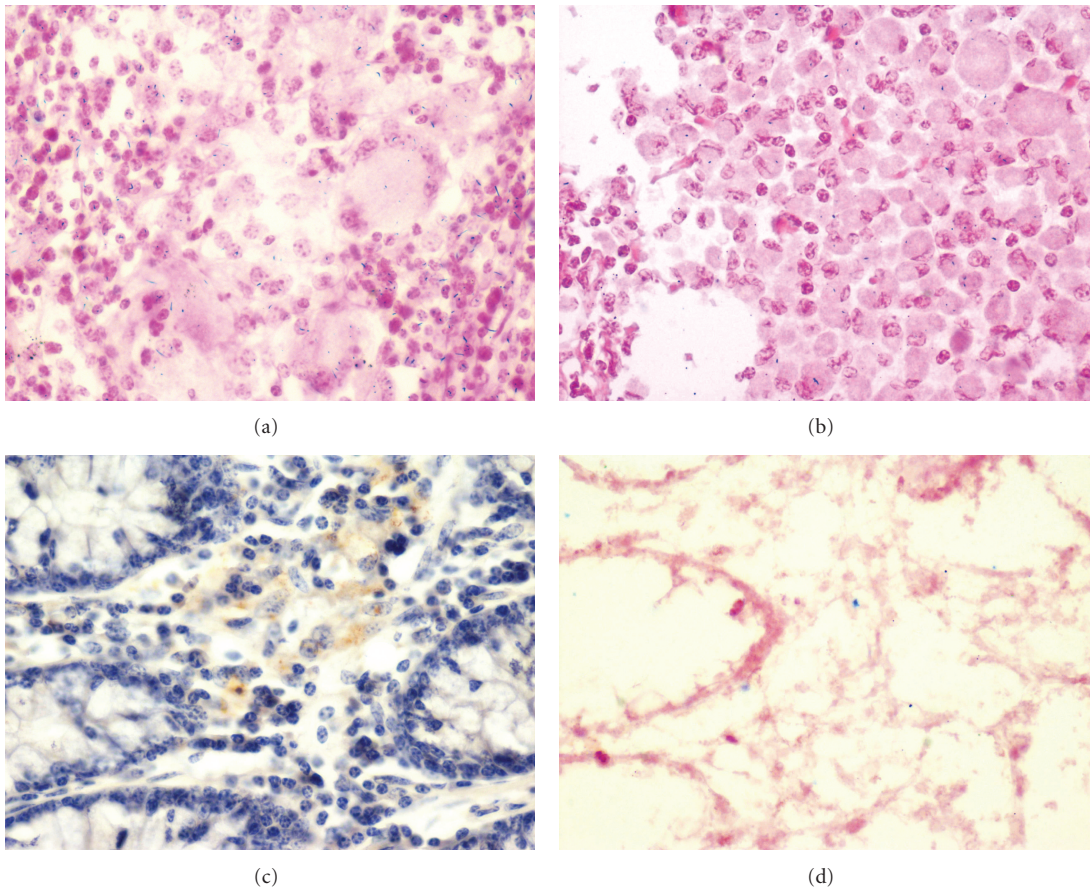


FIGURE 1: (a) Positive in situ PCR signal (small blue spots) inside the cytoplasm of epithelioid and Langhans giant cells (lamina propria, ileum). (b) Positive in situ PCR signal (small blue spots) inside the cytoplasm of macrophages (ileocaecal lymph node, cortex). (c) Positive immunostaining (brown color) inside the cytoplasm of epithelioid cells. Tissue's architecture is perfectly conserved (lamina propria, ileum). (d) Positive in situ PCR signal inside the cytoplasm of an epithelioid cell. Interpretation of this slide became very difficult due to damage on tissue's architecture (lamina propria, ileum).

TABLE 1: Analyzed samples and obtained results.

Case	Organ	Map culture	Histopathology	IHC	ISPCR
1	Ileum	Positive	Positive	+++	++
2	Ileum	Positive	Positive	+++	++
3	Ileum	Positive	Positive	+++	++
4	Ileocaecal LN	Positive	Positive	+++	++
5	Ileocaecal LN	Positive	Positive	+++	++
6	Ileum	Positive	Positive	+	++
7	Ileum	Positive	Positive	+++	++
8	Ileum	Positive	Positive	+++	++
9	Ileum	Negative	Negative	—	—

References: (IHC): immunohistochemistry, (dISP): direct in situ PCR. (+++): intense, (++): moderate, (+): weak, (—): negative.

Tissue morphology was perfectly conserved in all cases, and interpretation was easy to perform.

4. Discussion

Detection of Map in tissue samples supports pathological diagnosis and allows to determinate where the agent persists in the experimental disease [4, 8]. The obtained results indi-

cated that dISP was able to detect Map in all formalin-fixed, paraffin-embedded tissue samples from naturally infected cattle. The blue positive signal was clearly identified in areas with pathological changes, inside the macrophages or Langhans giant cells. Negative control and positive samples incubated without Taq enzyme resulted negatively, which confirmed that obtained signal in the positive samples was due to the presence of Map.

It was described that spheroplasts may be related to the development of PTB or CD, and these forms of Map can be detected by ISH [2, 7]. In a similar way, dISP would be useful for their identification since it is based in the detection of mycobacterial DNA, and this fact may improve its sensitivity when compared to IHC. In the present paper, IHC signal was intense in all cases, except in sample 6, in which it was weak but moderate for dISP. This fact may be explained by the detection of spheroplasts in this section, which was suggested in a previous study [5].

Although the sensitivity of dISP was described as very high [9], our results indicate that its sensitivity is lower than IHC since the dISP detected signal was moderate and immunostaining was intense in most of cases. However, this difference on staining results may be related with damage on DNA probably occurred during the fixation process, because variables such as time of fixation or nature of fixative solution were not considered when sampling was done, and they can alter the DNA integrity [10]. Although IHC may also be affected by fixation [11], antigen recovery may recuperate immunogenicity. Considering that the analyzed samples were not collected for dISP and fixation was not controlled, further studies will be necessary to evaluate sensitivity of dISP.

In situ hybridization was not tested in the present paper. However, the signal obtained with dISP was higher than the weak signal reported for ISH in our previous paper [5]. The cause of this difference may be related to the amplification of DNA obtained by dISP. Besides, the size of the probe, which has to penetrate the cell to hybridize the target DNA in ISH, did not affect the efficiency of dISP since primers and dNTPs are very small and constantly available.

The specificity of the dISP method is based on the amplified gene sequence. Although "IS900-like" sequences were described in other microorganisms [12, 13], IS900 is considered the gold standard in the molecular detection of Map by PCR [14]. It may be possible that these sequences affect the performance, which might decrease the specificity of the technique. The amplification of other more specific sequence of Map may reduce mismatching [7], and further studies will be necessary to determinate which sequence improves the sensitivity. However, it is critical to relate dISP staining with the histopathological changes in order to avoid false positive diagnosis.

Tissue morphology was affected when dISP was performed. This fact may be related to the enzymatic digestion and repeated exposition of the slides to high temperature in each PCR cycle since this problem was not detected with IHC. Because of this, interpretation of the latter was easier, while tissues damaged by dISP required repeating the performed test.

5. Conclusion

A dISP method against IS900 DNA sequence was successfully used for Map detection in formalin-fixed, paraffin-embedded tissue samples, which were obtained from naturally infected adult cows. Although the signal was lower

than IHC, further studies should be necessary to determinate sensitivity and specificity of the technique. As it was previously described for ISH, detection of Map DNA by dISP should be useful for the detection of spheroplasts. However, the tissue's structure was affected, and its development was more difficult than IHC. At the light of these facts, this method should be performed after IHC failed to detect Map or to detect spheroplasts in tissue samples with compatible changes.

Acknowledgments

The authors thank María del Carmen Tagle, Claudia Moreno, and Daniel Funes for the histological support, and Gimena Rodriguez, Gloria Jauregui, and Mariana Falabella for the paper review.

References

- [1] G. W. Dunkin, "Paratuberculosis of cattle and sheep," *Proceedings of the Royal Society of Medicine*, vol. 30, no. 2, pp. 83–90, 1936.
- [2] L. Sechi, M. Mura, F. Tanda et al., "Identification of *Mycobacterium avium* subsp. *paratuberculosis* in biopsy specimens from patients with Crohn's disease identified by in situ hybridization," *Journal of Clinical Microbiology*, vol. 39, no. 12, pp. 4514–4517, 2001.
- [3] F. A. K. El-Zaatari, M. S. Osato, and D. Y. Graham, "Etiology of Crohn's disease: the role of *Mycobacterium avium paratuberculosis*," *Trends in Molecular Medicine*, vol. 7, no. 6, pp. 247–252, 2001.
- [4] D. J. Brees, S. B. Reimer, N. F. Cheville, A. Florance, and C. O. Thoen, "Immunohistochemical detection of *Mycobacterium paratuberculosis* in formalin-fixed, paraffin-embedded bovine tissue sections," *Journal of Veterinary Diagnostic Investigation*, vol. 12, no. 1, pp. 60–63, 2000.
- [5] F. Delgado, D. Etchehoury, A. Gioffré et al., "Comparison between two in situ methods for *Mycobacterium avium* subsp. *paratuberculosis* detection in tissue samples from infected cattle," *Veterinary Microbiology*, vol. 134, no. 3–4, pp. 383–387, 2009.
- [6] S. A. Martinson, P. E. Hanna, B. O. Ikede et al., "Comparison of bacterial culture, histopathology, and immunohistochemistry for the diagnosis of Johne's disease in culled dairy cows," *Journal of Veterinary Diagnostic Investigation*, vol. 20, no. 1, pp. 51–57, 2008.
- [7] M. Jeyanathan, D. C. Alexander, C. Y. Turenne, C. Girard, and M. A. Behr, "Evaluation of in situ methods used to detect *Mycobacterium avium* subsp. *paratuberculosis* in samples from patients with Crohn's disease," *Journal of Clinical Microbiology*, vol. 44, no. 8, pp. 2942–2950, 2006.
- [8] E. Sanna, C. J. Woodall, N. J. Watt et al., "In situ-PCR for the detection of *Mycobacterium paratuberculosis* DNA in paraffin-embedded tissues," *European Journal of Histochemistry*, vol. 44, no. 2, pp. 179–184, 2000.
- [9] R. Hernández-Pando, M. Jeyanathan, G. Mengistu et al., "Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection," *Lancet*, vol. 356, no. 9248, pp. 2133–2138, 2000.
- [10] C. E. Greer, S. L. Peterson, N. B. Kiviat, and M. M. Manos, "PCR amplification from paraffin-embedded tissues. Effects

- of fixative and fixation time,” *American Journal of Clinical Pathology*, vol. 95, no. 2, pp. 117–124, 1991.
- [11] M. M. Arnold, S. Srivastava, J. Fredenburgh, C. R. Stockard, R. B. Myers, and W. E. Grizzle, “Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens,” *Biotechnic and Histochemistry*, vol. 71, no. 5, pp. 224–230, 1996.
- [12] D. Cousins, R. Whittington, I. Marsh, A. Masters, R. J. Evans, and P. Kluver, “Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: implications for diagnosis,” *Molecular and Cellular Probes*, vol. 13, no. 6, pp. 431–442, 1999.
- [13] A. S. Motiwala, M. Strother, A. Amonsin et al., “Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: evidence for limited strain diversity, strain sharing, and identification of unique targets for diagnosis,” *Journal of Clinical Microbiology*, vol. 41, no. 5, pp. 2015–2026, 2003.
- [14] P. Kralik, I. Slana, A. Kralova, V. Babak, R. H. Whitlock, and I. Pavlik, “Development of a predictive model for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces by quantitative real time PCR,” *Veterinary Microbiology*, vol. 149, no. 1–2, pp. 133–138, 2011.