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Use of the polymerase chain reaction for diagnosing bovine tuberculosis in Panama

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Summary

In addition to causing large losses to the cattle industry, *Mycobacterium bovis*, the causative agent for bovine tuberculosis, is a serious public health issue because it can potentially infect humans. Diagnosis based on isolation and identification of the bacillus is tedious and may take weeks. The diagnosis of *M. bovis* by polymerase chain reaction (PCR), using species-specific primers, is fast, highly sensitive and of great value in epidemiological studies. In this study, deoxyribonucleic acid (DNA) was extracted from 60 nasal mucus samples collected from three different farms, all located in an area where *M. bovis* is endemic. Two farms tested negative for an antibody response to the *M. tuberculosis* purified protein derivative (PPD) antigen, whereas the other farm gave a positive result.

The amplified fragment of DNA was 460 base pairs with a sequence similar to that previously reported. Only 5% of the samples from the third farm tested positive for the presence of antibodies against PPD, whereas 65% of samples (from all three farms) gave a positive result when PCR was used. Thus, the authors suggest the use of the PCR species-specific primers test to support the programme against bovine tuberculosis in Panama.

Keywords

Bovine tuberculosis – Bovines – Central America – Diagnosis – Mycobacterium bovis – Panama – Polymerase chain reaction – Purified protein derivative – Republic of Panama – Tuberculosis.

Introduction

Bovine tuberculosis caused by *Mycobacterium bovis* is considered a re-emergent zoonotic disease in many developing countries. However, in Latin America, this zoonosis is causing large economic losses to the cattle industry and infects a considerable number of reservoirs (3). Transmission between bovines occurs principally by the aerial route (90%), however, ingestion of infected material also contributes to the spread of the disease (11). Humans usually become infected through the digestive system, i.e. by drinking non-pasteurised milk from infected cows (17). Approximately 7,000 new cases of human tuberculosis are caused by bovine tuberculosis each year in Latin America (14). The increasing number of human tuberculosis cases caused by the *M. tuberculosis* complex requires a re-evaluation of veterinarian public health measures worldwide, so that the possible contribution of *M. bovis* can be analysed. The World Health Organization declared tuberculosis a worldwide emergency in 1995.

At present, two diagnostic techniques are used to identify bovine tuberculosis in Panama, as follows:

- the purified protein derivative (PPD) test
- isolation and identification of the causative agent.

The isolation through culture and biochemical characterisation of *M. bovis* can take up to eight weeks, and at least 100 viable organisms are required before a result is likely (2). Although these tests are considered classical, molecular biology enlarges the bovine tuberculosis field, through the use of polymerase chain reaction (PCR) as a basis for epidemiological studies. The PCR test displays sensitivity ranging from moderate to good (65% to 90%), and has a relatively high specificity (98% to 99%) (10).

Bocas del Toro province (Fig. 1) is the only region of Panama which has reported the presence of bovine tuberculosis. The test used in Panama is the intradermal delayed type hypersensitivity (DTH) test, using PPD from the culture of *M. bovis*, which is the only internationally recognised official test for diagnosing tuberculosis. This test is generally from Australia and comes in a concentration of 1 mg/ml. The dose consists of a single intradermal injection of 0.1 ml of tuberculin into the caudal fold. However, avian PPD is also applied to PPD bovine reactors for confirmation. Various factors, such as environmental stress, active infection, age-related factors, errors in technique and cross-reactions may contribute to false-positive and/or false-negative results, causing a loss in the diagnostic sensitivity and specificity of this test (18).

Data obtained from the Epidemiology Department of the Ministry of Agricultural Development indicate that the prevalence for bovine tuberculosis in farms in the Bocas del Toro region is 15.9%. Panama reported its first bovine tuberculosis outbreak in 1997, which was related to an importation of buffalo from Asia. The outbreak was contained by the use of PPD for diagnosis and the slaughter of infected animals. However, animals which tested negative for the presence of antibodies to PPD often displayed lesions which were compatible with tuberculosis lesions on post-mortem examination (L. Solano, personal communication).

This study is based on the standardisation of PCR and the amplification of fragment sequences to provide a suitable test for identifying *M. bovis* in nasal mucus samples. The PCR methodology has proved highly sensitive and specific (Fig. 2), and thus offers a great contribution to programmes attempting to eradicate bovine tuberculosis in Panama.

Materials and methods

Numbers of samples

This study was conducted on samples from three different farms, located in an area where bovine tuberculosis is endemic. The farms are 20 km apart and involved primarily in fattening and selling cattle. Sixty nasal mucus samples were collected at random from the three farms. One of the three selected farms tested positive, by DTH, for the presence of antibodies to *M. bovis*, at a prevalence of 0.82%.

Reference strain of *Mycobacterium bovis*

An autochthonous strain of *M. bovis*, isolated from cattle in a tuberculosis-endemic area of Panama during 1997, was used as a positive control. Isolation of the bacterium was performed on the Stonebrink medium (free of glycerol) from the lymph nodes of cattle with bovine tuberculosis

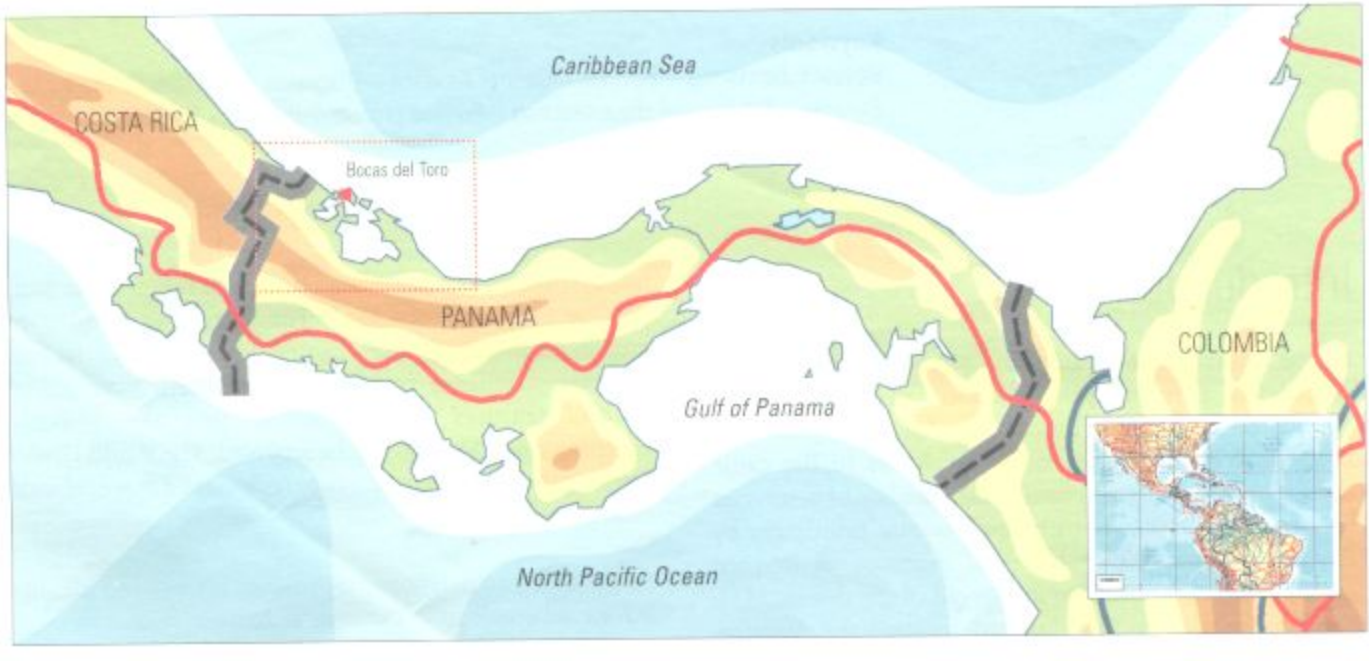
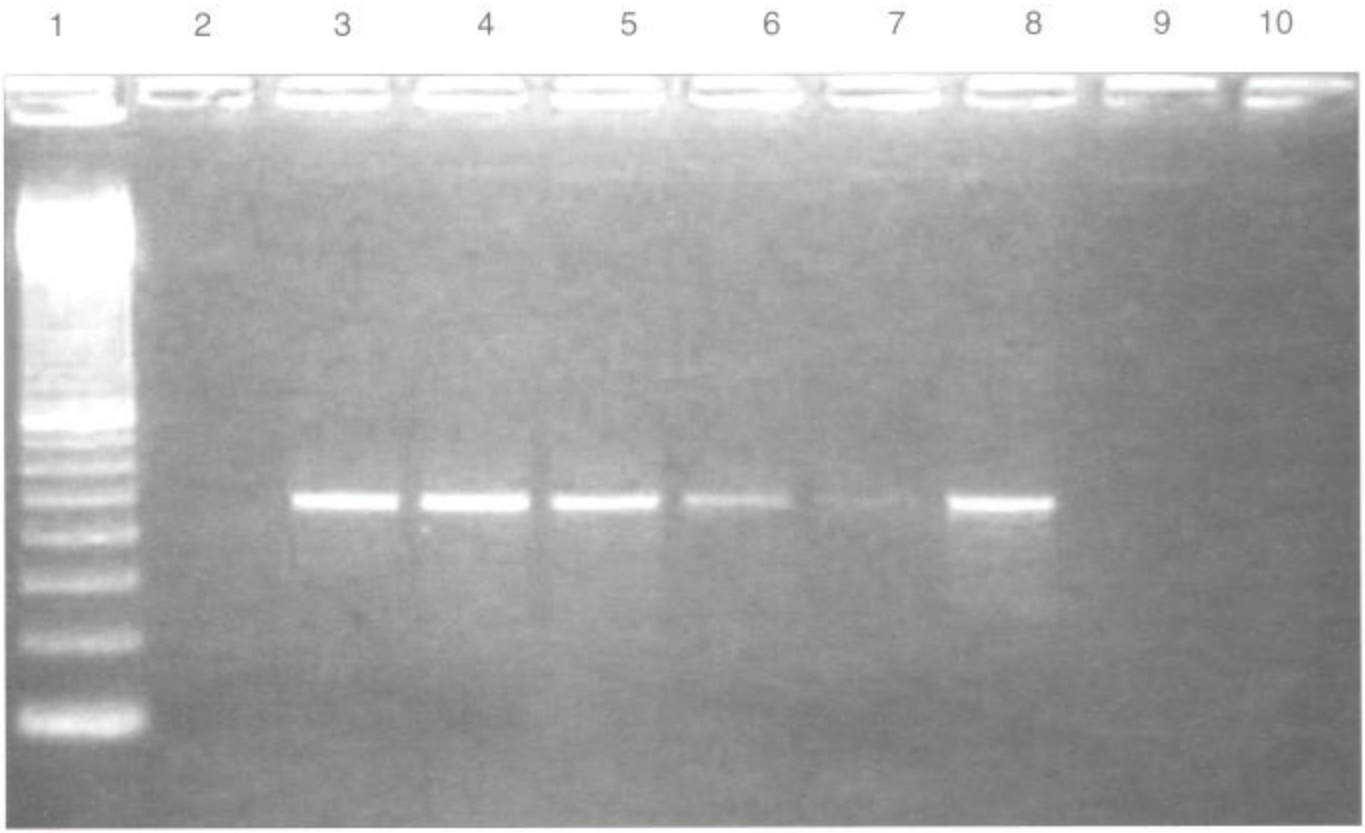


Fig. 1
Map of the Republic of Panama, showing the location of Bocas del Toro Province, at 83° 60'–80° 30' W 9°17'–7°11' N



1. Molecular weight marker
2. Negative control
3. *M. bovis* positive control (350 ng)
4. DNA *M. bovis* (35 ng)
5. DNA *M. bovis* (350 µg)
6. DNA *M. bovis* (35 pg)
7. DNA *M. bovis* (350 fg)
8. DNA *M. bovis* positive control (350 ng)

Fig. 2
The sensitivity of the polymerase chain reaction as determined by using serial dilutions of *Mycobacterium bovis* deoxyribonucleic acid concentrations, in a range between 350 nanograms and 350 femtograms

lesions. The National Institute for Food and Zoonosis Protection in Argentina confirmed the isolate type. This strain was also used in the sensitivity test.

Next, *M. bovis* colonies in culture were processed, using a modified protocol of Ross *et al.* (16). Modifications included suspension of one loopful of *M. bovis* into 500 µl of twice-distilled water, and heating at 85°C for 1 h to break down the cellular membrane of *Mycobacterium*, due to its high lipid content (4). The suspension was found to be suitable for the extraction and detection of *Mycobacterium* DNA (9), when PCR was performed with species-specific primers. The DNA concentration was determined by the use of a spectrophotometer at 260 nm.

Samples

Nasal mucus samples were collected using individual sterile swabs, placed in a tube containing 3 ml of sterile saline solution (0.85%), then shipped to the laboratory by plane. During transportation, samples were kept at 18°C and delivered to the laboratory facilities within 8 h. After arrival, all nasal mucus samples were centrifuged at 10,000 revolutions per minute (rpm) for 10 min at room temperature. Sediments were rinsed three times with phosphate-buffered saline (0.1 molar [M] pH 7.2) before being suspended in 200 µl of Tris (hydroxymethyl aminomethane)-ethylenediamine tetra-acetic acid (TE) buffer.

Deoxyribonucleic acid extraction

Two methods were used to isolate DNA from the samples. First, nasal mucus samples were suspended in 200 µl of lysis buffer and the suspension incubated in a water bath at 80°C for 1 h. The DNA extraction and purification were performed using a commercial kit.

A second methodology required phenol-chloroform-isoamyl alcohol (25:24:1) (19). Modifications to the original protocol included suspension in lysis buffer (sodium dodecyl sulphate [SDS] 20%; protein K 10 mg/ml

[final concentration of 0.5 mg/ml]; Tris 1 M pH 8.2 and ethylenediamine tetra-acetic acid [EDTA] 0.5 M). The samples were then incubated in a water bath at 85°C for 1 h. After incubation, one volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. The mixed suspension was centrifuged at 12,000 rpm for 5 min and the aqueous phase transferred into a microtube. Two volumes of absolute alcohol and 0.1 of a volume of sodium acetate (0.3 M) were added. The DNA was incubated at 20°C for 2 h, and then centrifuged at 14,000 rpm for 15 min. The supernatant was then discarded, and the sediment was further diluted with 1 ml of 70% alcohol, and kept overnight at 20°C. Samples were then centrifuged at 12,000 rpm for 15 min and the sediment was allowed to dry at 37°C, after which 50 µl of TE buffer was added.

To avoid possible cross-contamination, no more than ten samples were handled at one time. The laboratory facilities comprised various different working areas.

Deoxyribonucleic acid extraction from visceral organs

For this study, the organs of five bovines, which had tested negative by PPD, were collected from the municipal slaughterhouse of Bocas del Toro.

To standardise the methodology for the PCR, a 1 cm piece of tissue was used for DNA extraction. The tissue was triturated and placed in lysis buffer (SDS 20%, protein K 10 mg/ml [final concentration of 0.5 mg/ml], Tris 1 M pH 8.2 and EDTA 0.5 M) for 48 h. A commercial kit was used for the DNA extraction.

Primers and amplification by polymerase chain reaction

Oligonucleotide L1 (5'CCCGCTGATGCAAGTGCC 3') and L2 (CCCGCACATCCCAACACC 3') sequences were used for amplification (15). These primers amplified a DNA segment of *M. bovis* of 460 bp. Amplifications were conducted from a lyophilised mixture of Taq polymerase, templates and magnesium chloride into 25 µl containing 10 µl blank DNA and 15 µl mixed primers in de-ionised water. A thermocycler was used (30 cycles) for the amplification. The parameters employed were as follows:

- 95°C for 5 min, followed by denaturation at 95°C for 1 min
- annealing at 65°C for 1 min
- extension at 72°C for 1 min, followed by 72°C for 10 min.

An inverted hot start was used to amplify the samples. For analysis, 10 µl of the amplified sample were electrophoresed in 2% agarose gels containing 0.5 µg/ml ethidium bromide and further visualised with an ultraviolet transilluminator.

Positive and negative controls

The DNA from *M. bovis* was used as a positive control. Sterile water for molecular use, containing primers but no DNA, was used as a negative control.

Sequences

Amplified gene products were sequenced from five samples which had tested positive for tuberculosis by PCR. An amplified segment of appropriate size from the reference strain was used as a control. A mixture of all the buffers employed during the test was used for a negative control. Amplified products were cut from the agarose gel and purified with a gel extraction kit. All primers used during the DNA amplification were employed to obtain sequences. An automatic sequence apparatus was used to sequence the DNA. Sequences were then lined up and analysed with a sequencer program.

Statistical analysis

The results from animals which tested positive and/or negative by PCR and PPD were evaluated using Kappa statistical calculation (5). Percentages of positive and negative predictive values were determined as an average for all three farms examined in the study.

Results

The study sites, located 500 km west of Panama City, could only be reached by air or sea. Out of the 60 animals studied, only three tested positive by DTH, while the remaining 57 gave negative results for *M. bovis*.

As recommended (20), the processes of DNA extraction, PCR, amplification and electrophoresis were conducted in completely separate working areas. The PCR analytical sensitivity was calculated in 350 femtograms DNA, using serial dilutions of DNA concentrations.

Thus, the number was estimated in quantities of approximately 70 bacilli (Fig. 3) (15). Amplified fragments with species-specific PCR from some of the nasal mucus samples were compared to those of the reference strain of *M. bovis*. The same results were obtained independently

of the method of DNA extraction, thus indicating that either of the two methodologies described can be used.

A total of 60 nasal mucus samples were used to establish the presence or absence of *M. bovis* by PCR, using species-specific primers. Results indicated that 39 of the 60 (65%) animals studied were found positive by PCR, while only 5% were found to be positive by PPD. Table I shows a positive predictive value for PPD of 2.5% (1/40), and a negative predictive value of 90% (18/20). This negative predictive value (90%) seems appropriate when examining apparently healthy animals. Nevertheless, the low positive predictive value (2.5%) could represent a high proportion of PPD false-negative results when compared to PCR, indicating that PPD is less accurate in detecting sick animals. Further analysis showed that there is no statistical concordance between results.

The visceral organs from five animals slaughtered at an abattoir (two lung samples and three from lymph nodes) were also studied. The lung samples collected from two animals which had tested negative by PPD did not present typical bovine tuberculosis lesions. As per the regular protocol, no culture was performed for these animals. Nevertheless, one of these lung samples yielded positive results by PCR.

Lymph node samples were also collected from three animals which had tested negative by PPD. One of these animals showed a typical bovine tuberculosis lesion. Two out of the three samples yielded positive results by PCR (no data shown), including the sample from the animal with the typical lesion. In fact, *M. bovis* was isolated from the lymph node presenting the characteristic lesion, and the isolate was characterised biochemically. To corroborate

Table I

Statistical analysis determines a positive predictive value for purified protein derivative of 1/40, and a negative predictive value of 18/20

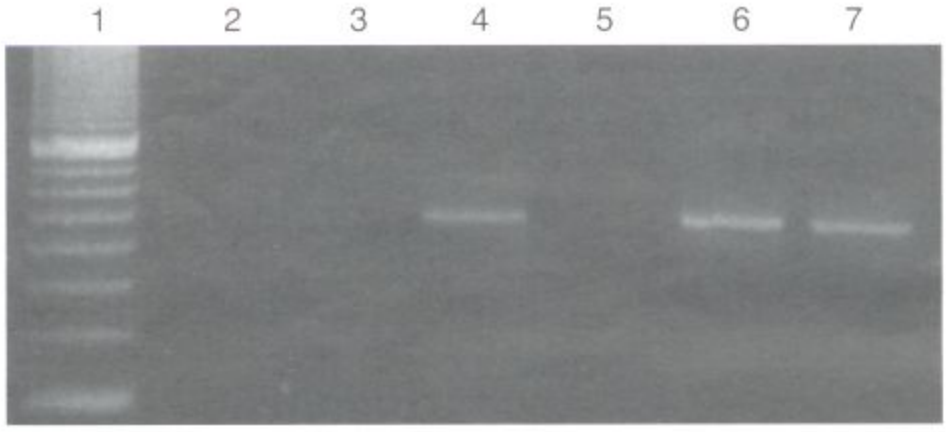
In this study, polymerase chain reaction (PCR) seems useful in identifying animals which had originally tested negative for the presence of antibodies to *Mycobacterium bovis* purified protein derivative (PPD) antigen

Results of PCR	Positive to PPD bovine antigen	Negative to PPD bovine antigen	Total
Tested positive by PCR	1	39	40
Tested negative by PCR	2	18	20

that the amplified gene product corresponded to the *M. bovis* sequence, five indicated length bands were chosen randomly for DNA amplification. As a control sample, a band of similar length from the *M. bovis* reference strain was also amplified. The sequence reported by Patarroyo *et al.* (14) was used as a further reference. Sequence analysis demonstrated 100% similarity of the five analysed samples. This sequence was deposited at the GenBank (www.ncbi.nlm.nih.gov/).

Discussion

The first official case of bovine tuberculosis was reported in Bocas del Toro, on the west side of the Republic of Panama, in 1997. Control measures comprised intensive field diagnosis, using PPD bovine antigen, and culling of animals in the affected farms and surrounding areas. These



1. Molecular weight marker
2. Negative control
3. *M. tuberculosis*
4. Positive nasal mucus sample
5. Negative nasal mucus sample
6. Positive nasal mucus sample
7. Deoxyribonucleic acid *M. bovis* positive control

Fig. 3

Amplified fragments from some of the nasal mucus samples compared to control *Mycobacterium bovis* and *M. tuberculosis* strains

measures succeeded in controlling the outbreak. The farm where the bacillus was first found raised bovines and buffalo for human consumption.

Transmission of *M. bovis* is mainly aerial (12). As a result, it seemed appropriate to use samples of nasal mucus to diagnose the disease by PCR. Sample collection is simplified and the possibility of culling healthy animals is avoided. Susceptibility to infection varies from one animal to another, but genetically pure animals are considered to be more susceptible to the disease (17). Human tuberculosis originating from bovines has been extensively documented (6).

There is no information concerning the actual situation in the Republic of Panama at present. Several methodologies and tests for identifying *Mycobacterium* are in use, but some require a substantial time period (13, 20). Using PCR, however, results can be obtained within a day. Thus, the PCR test, as described in this paper, has the potential to become a powerful tool for controlling bovine tuberculosis in the agricultural sector. The use of PCR to amplify genome fragments of *M. bovis* also seems to be an efficient way of detecting samples contaminated with this bacterium.

Fragment sequences of amplified DNA confirmed that the amplified gene sequences corresponded to *M. bovis*, and not to an artefact of a non-specific amplification.

This study highlighted two important issues, which must be considered in any control and eradication programme. The first concerns the large number of wild animal species that may be acting as reservoirs for *M. bovis* infection, particularly in regions where bovine tuberculosis has been controlled but outbreaks of the disease still occur, as described by other researchers (1). The high concentration of sequences rich in guanine and cytosine (G + C), and their distribution within the *M. bovis* genome, is very valuable in identifying isolates from different farms. This, in turn, enables workers to differentiate strains that are nonetheless geographically related (7), as in the case of the farms in this study. It is important to include other molecular techniques, such as analysing restriction fragment length polymorphism sizes or the Southern blot technique, which also aid in differentiating isolates.

The second issue is the importance of evaluating external factors which may influence the results of the DTH. A false-negative reaction can occur due to numerous factors, such as the following:

- an early stage of infection
- the end of the gestation period
- post-delivery immunosuppression
- incorrectly applying the test
- misinterpreting the skin test
- other adverse environmental factors.

On the other hand, a false-positive reaction to DTH may be due to cross-reaction with other *Mycobacterium* species (18). Predictive values for PPD had no concordance with the results obtained from the PCR test. Therefore, the sensitivity and specificity of the PPD field test should be re-evaluated (8). Factors such as transportation, appropriate storage temperature (between 2°C and 8°C), test application and interpretation of the results must be standardised. A training programme must be implemented to overcome most of these problems. The PCR test could be used alongside the programme, to aid in evaluating its success.

The presence of *M. bovis* in organs without macroscopic lesions is suggestive of chronic or sub-clinical infection (4). For this reason, the need for a more sensitive and specific test, such as PCR, is clear. With such a test, animals testing positive for *M. bovis* could be identified before slaughter.

Conclusions

Panama has reported the presence of *M. bovis* among cattle since 1997, without being able to achieve total eradication. Although PCR is a costly technique, this epidemiological tool must be considered a valuable test method in areas of high risk, due to its high level of accuracy.

This study demonstrates that other factors which may be affecting the PPD test in the field should be evaluated. The PCR test, with the use of species-specific primers, allows the amplification of an exclusive fragment of the *M. bovis* genome and excludes the possibility of cross-reactions with other mycobacteria. This highly sensitive test is also quickly conducted. Thus, using the PCR test in the official control programme should contribute to better monitoring and control of bovine tuberculosis in the Republic of Panama and, ultimately, to its eradication.

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L'amplification en chaîne par polymérase appliquée au diagnostic de la tuberculose bovine au Panama

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Résumé

Outre les pertes considérables qu'il cause au secteur de l'élevage, *Mycobacterium bovis*, l'agent causal de la tuberculose bovine, représente une grave menace pour la santé publique, dans la mesure où il peut affecter l'être humain. Le diagnostic reposant sur l'isolement et l'identification du bacille peut prendre plusieurs semaines. En revanche, l'amplification en chaîne par polymérase (PCR) utilisant des amorces spécifiques d'espèce pour le diagnostic de *M. bovis* s'avère une technique rapide, très sensible et extrêmement utile pour les études épidémiologiques. La présente étude a utilisé des extraits d'ADN issus de prélèvements nasaux provenant de trois exploitations, toutes trois situées dans une zone où *M. bovis* est endémique. Les prélèvements provenant des deux premières exploitations n'ont pas révélé d'anticorps vis-à-vis de l'antigène de la tuberculine purifiée (PPD). Un résultat positif a été obtenu parmi les prélèvements provenant de la troisième exploitation.

Le fragment amplifié d'ADN de 460 paires de bases portait une séquence comparable à celle observée préalablement. Seulement 5 % des prélèvements de la troisième exploitation possédaient des anticorps vis-à-vis de la PPD, tandis que 65 % des prélèvements (en provenance des trois exploitations) ont donné des résultats positifs en PCR. Les auteurs recommandent donc l'utilisation de la PCR à amorces spécifique d'espèce pour renforcer le programme de prophylaxie de la tuberculose bovine au Panama.

Mots-clés

Amérique centrale – Amplification en chaîne par polymérase – Bovin – Diagnostic – Mycobacterium bovis – Panama – Tuberculine purifiée – Tuberculose – Tuberculose bovine.

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Uso de la reacción en cadena de la polimerasa para diagnosticar la tuberculosis bovina en Panamá

I. Cedeño, R. de Obaldía, O. Sanjur, V. Bayard, E. Ortega-Barría & C. Escobar

Resumen

Además de las cuantiosas pérdidas que causa a la industria ganadera, *Mycobacterium bovis*, el agente etiológico de la tuberculosis bovina, plantea importantes problemas de salud pública por su potencial para infectar al ser humano. El diagnóstico basado en el cultivo y la caracterización del bacilo puede llevar semanas. La reacción en cadena de la polimerasa (PCR) con cebadores específicos de esa bacteria, en cambio, ofrece un medio de diagnóstico de gran rapidez, sensibilidad y utilidad en estudios epidemiológicos. Los autores describen una experiencia en la que se extrajo ADN de 60 muestras de muco nasal de ejemplares de tres explotaciones, situadas todas ellas en una zona en la que *M. bovis* es endémico. Dos de esas tres explotaciones dieron resultado negativo a las pruebas de detección de una respuesta inmunitaria contra el antígeno PPD (derivado proteico purificado) de *M. tuberculosis*, mientras que la tercera granja dio un resultado positivo.

El fragmento de ADN amplificado tenía 460 pares de bases y presentaba una secuencia similar a la descrita anteriormente. Sólo el 5% de las muestras de la tercera explotación dieron positivo a la presencia de anticuerpos contra el PPD, mientras que el 65% de las muestras de las tres explotaciones dieron un resultado positivo con la aplicación de una PCR. Los autores proponen, en consecuencia, que se utilice la PCR con cebadores específicos de *M. bovis* como técnica de apoyo al programa de lucha contra la tuberculosis bovina en la República de Panamá.

Palabras clave

Bovinos – Centroamérica – Derivado proteico purificado – Diagnóstico – Mycobacterium bovis – Panamá – Reacción en cadena de la polimerasa – Tuberculosis – Tuberculosis bovina.

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