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Comparison of DNA extraction protocols to detect *Mycobacterium bovis* in bovine tissue by PCR

Comparação de protocolos de extração de DNA para detectar *Mycobacterium bovis* em tecido bovino por PCR

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Abstract

The current scenario of international beef trading has increased the pressure for better and faster diagnosis of bovine tuberculosis. Although traditional culture remains the gold standard method to confirm *Mycobacterium bovis* infection, it is exceedingly time consuming, and demands viable mycobacteria. Molecular methods overcome the flaws of the bacteriological methods with faster detection and identification. However, mycobacterial features like a complex cell wall and pathogen-host interaction make the molecular detection a challenge. Three protocols for DNA extraction (A, B and C) from bovine tissues were tested to verify the most suitable technique for routine diagnostic assessment of their specificity and sensitivity. Thirty culture-positive and thirty culture-negative granulomatous lesions were included in the trial. From each sample, three tissue suspensions at different dilutions (10^{-1}, 10^{-2} and 10^{-3}) were prepared and submitted to DNA extraction. PCR procedures targeting IS*6110* were performed, employing two volumes of DNA: 5 µL of all three dilutions, and 2.5 µL of the 10^{-1} dilution. Protocol A was able to detect members of the *M. tuberculosis* complex in most samples. The sensitivity of the test decreased with increase in tissue-suspension dilution. Although Protocol A presented the highest sensitivity followed by C and B, it showed the lowest specificity, which can be due to a failure in primary isolation caused by the lack of viable organisms or incubation time. Regardless classical bacteriological methods are still recommended by OIE, after evaluating the sensitivity of DNA extraction protocols and PCR procedures, we conclude that the best strategy for *M. bovis* detection is to follow Protocol A on concentrated tissue suspensions.

Key words: DNA extraction. Bovine tissue. Bovine tuberculosis. *Mycobacterium bovis*. PCR.

Resumo

O atual comércio internacional de carne tem aumentado a pressão para haver um melhor e mais rápido diagnóstico de tuberculose bovina. O tradicional cultivo continua a ser o método padrão ouro para confirmar a infecção por *Mycobacterium bovis*, apesar de ser excessivamente demorado e necessitar de micobactérias viáveis. Métodos moleculares representam a superação de todos os defeitos dos métodos bacteriológicos com detecção e identificação mais rápidas. Entretanto, características das micobactérias, como uma complexa parede celular e a interação patógeno-hospedeiro, torna-os um desafio. Três

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protocolos de extração de DNA (A, B e C) foram testados em tecidos bovinos para verificar qual técnica é mais adequada para diagnóstico de rotina, avaliando sua especificidade e sensibilidade. Trinta lesões granulomatosas positivas no cultivo e 30 lesões granulomatosas negativas no cultivo foram utilizadas no experimento. A partir de cada amostra, três homogeneizados com diferentes diluições (10^{-1}, 10^{-2} e 10^{-3}) foram preparadas e submetidas à extração de DNA. A PCR para o gene alvo IS*6110* foi realizada empregando-se dois volumes de DNA: um com 5 µL para todas as três diluições e outro com 2,5 µL da diluição 10^{-1}. O Protocolo A foi capaz de detectar membros do complexo *M. tuberculosis* na maior parte das amostras. À medida que a diluição dos homogeneizados aumentou, a sensibilidade diminuiu. Embora o Protocolo A tenha apresentado a maior sensibilidade, seguido por C e B, este revelou a menor especificidade, que pode ser devido à insuficiência de organismos viáveis ou tempo de incubação no primo isolamento. Apesar de os métodos bacteriológicos clássicos ainda serem recomendados pela OIE, através da avaliação da sensibilidade dos protocolos de extração de DNA e dos procedimentos de PCR, concluímos que a melhor estratégia para a detecção de *M. bovis* é usar o Protocolo A em homogeneizados mais concentrados.
Palavras-chave: Extração de DNA. Tecido bovino. Tuberculose bovina. *Mycobacterium bovis*. PCR.

Introduction

Bovine tuberculosis, caused by *Mycobacterium bovis*, is a chronic zoonosis responsible for elevated losses in a livestock-based economy (POLLOCK et al., 2006). Recent studies in 13 states of Brazil that contain 75% of the Brazilian cattle population, revealed prevalence of tuberculosis-infected herds between 0.36%, in the Federal District, and 9.0%, in São Paulo (BAHIENSE et al., 2016; BARBIERI et al., 2016; DIAS et al., 2016; GALVIS et al., 2016; GUEDES et al., 2016; LIMA et al., 2016; NÉSPOLI et al., 2016; QUEIROZ et al., 2016; RIBEIRO et al., 2016; ROCHA et al., 2016; SILVA et al., 2016; VELOSO et al., 2016; VENDRAME et al., 2016).

Current trading and transportation between countries have increased the pressure for better and faster diagnosis. The definitive diagnosis requires detection of *M. bovis* through bacteriological examination, as traditional culture remains the gold standard method for routine confirmation of infection (OIE, 2009). The most significant disadvantage of this method is the time consumed, since isolation may demand more than 12 weeks (CORNER et al., 2012), and biochemical identification can extend this time even further (COLLINS et al., 1982). Moreover, the number of viable mycobacteria might decrease through inadequate handling of tissues (WARDS et al., 1995) and decontamination procedures (CORNER et al., 2012), which may lead to false-negative results.

As faster alternatives, tests based on polymerase chain reaction (PCR) have been widely employed for mycobacterial identification (TELENTI et al., 1993; PARSONS et al., 2002; SALES et al., 2014a, 2014b), mostly using cultured isolates.

The application of molecular methods on clinical specimens encounters a number of problems, including a complex mycobacterial cell wall, the presence of the bacterial cell within a phagosome, a host cell and a granuloma, and the low amount of bacterial cells or DNA (KUMAR et al., 2010; RADOMSKI et al., 2013). Additionally, inhibitory substances present in the samples or the reagents used during DNA extraction, and an excessive amount of host DNA compared to the target DNA of the pathogen, also make accurate pathogen detection in clinical specimens difficult (PARK et al., 2014).

High sensitivity and specificity, simplicity, and reasonable cost are the necessary requirements for a routine diagnostic procedure. Despite the variety of available methods, there are no standards for bacterial DNA extraction from tissue samples. The aim of this study was to assess the specificity and sensitivity of three DNA extraction protocols to detect members of the *M. tuberculosis* complex (MTC) in bovine tissues.

Material and Methods

From previously cultured granulomatous lesions (ROSALES RODRIGUEZ, 2005), we selected 30 samples identified as *M. bovis* and 30 samples that were negative for isolation. To confirm the prior negative results, the latter group was submitted to culture for a second time. The samples were decontaminated with 0.75 % 1-hexadecylpyridinium chloride (AMBROSIO et al., 2008), inoculated into Stonebrink's and Löwenstein-Jensen's media, and incubated at 37 °C for up to 90 days (CENTRO PANAMERICANO DE ZOONOSIS, 1985).

Tissue suspensions were prepared by macerating one gram of each sample in 9 mL sterile 0.85 % saline solution (10^{-1}), and two 10-fold dilutions, 10^{-2} and 10^{-3}, were then made from the original suspension.

The DNA extraction methods were first tested using 1-2 colonies of *M. bovis* AN5 pure culture. DNA extraction procedures started with 400 µL of tissue suspensions. The three dilutions of all 60 samples were submitted to the following protocols:

Protocol A

The tissue suspensions were pretreated with 100 µL of lysozyme (100 µg mL^{-1}) and 20 µL of proteinase K (20 mg mL^{-1}) at 37°C for 1 h. DNA extraction was then carried out as described by Boom et al. (1990).

Protocol B

The procedure was carried out as described by Zumárraga et al. (2001), except for the preparation of tissue suspensions described previously.

Protocol C

The DNA extraction procedure described by Santos et al. (1995) was modified. All tissue suspensions were centrifuged and the supernatant was discarded. The samples were incubated in 50 µL of 0.5 N NaOH for 10 min, and neutralized with 50 µL of 1 M NaH_2PO_4. The suspension was centrifuged, and the pellet was resuspended in 60 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1% Triton X-100 and 50 µL of proteinase K (10 mg mL^{-1}), followed by overnight incubation at 37°C. Finally, the samples were submitted to three cycles of boiling for 10 min and freezing in liquid nitrogen for 2 min.

All extracted DNA samples were stored at –20°C until PCR assay. Detection of mycobacterial DNA was performed using the primers INS-1 (5'-CGTGAGGGCATCGAGGTGGC-3') and INS-2 (5'-GCGTAGGCGTCGGTGACAAA-3'), which target the insertion element IS*6110* (also known as IS*986*), and amplify a 245bp fragment that is observed in members of the MTC (HERMANS et al., 1990).

PCR mixture contained 1.25 U of *Platinum Taq* DNA Polymerase (Invitrogen, Carisbad, California, USA), 5X Green Go Taq® reaction buffer (Promega Corporation, Madison, WI, USA), 0.2 mM dNTPs, 10 pmol of each primer, and sterilized ultrapure water to a final volume of 50 µL.

Two different volumes of DNA samples were used. Five microliters of all three dilutions and 2.5 µL of the 10^{-1} dilution were submitted to PCR. The amplified DNA was analyzed by 1% agarose gel electrophoresis and ethidium bromide staining (0.5 µg mL^{-1}).

Results

In order to compare each DNA extraction protocol, the tissue sample was considered as positive when the DNA template was amplified in at least one out of the four PCR procedures. Among the culture-positive tissue samples group, Protocol A presented 30 (100%) positive results for MTC amplification, while Protocol B and C showed 14 (46.66%) and 15 (50%) positive results, respectively (Table 1). For the culture-negative group, Protocols A, B and C exhibited 20 (66.66%), 12 (40%), and 11 (36.66%) positive samples, respectively (Table 2).

Table 1. *M. tuberculosis* complex detection by PCR for *M. bovis* culture-positive group, according to DNA extraction protocols and homogenate dilutions.

	Protocol A				Protocol B				Protocol C			
DNA volume	2.5 µL		5 µL		2.5 µL		5 µL		2.5 µL		5 µL	
dilutions	10^{-1}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-1}	10^{-2}	10^{-3}
samples												
47/03	[I]P	[II]-	-	-	-	-	-	-	-	-	-	-
51/03	P	-	-	-	-	-	-	-	P	-	-	-
54/03	P	-	-	-	-	-	-	-	P	-	-	-
67/03	P	-	-	-	-	-	-	-	P	-	-	-
74/03	P	-	-	-	-	-	-	-	-	-	-	-
75/03	P	-	-	-	-	-	-	-	-	-	-	-
80/03	P	P	P	-	-	-	-	-	-	-	-	-
81/03	P	-	-	-	-	-	-	-	-	-	-	-
85/03	P	-	-	-	-	-	-	-	-	-	-	-
92/03	P	-	P	-	-	-	-	-	P	-	-	-
112/03	P	P	-	-	P	-	-	-	-	P	-	-
126/03	P	P	-	-	-	-	-	-	-	-	-	-
127/03	P	-	P	-	-	-	-	-	-	-	-	-
129/03	P	P	-	-	-	-	-	-	-	-	-	-
142/03	P	P	-	-	P	-	-	-	-	-	-	-
167/03	P	P	P	-	P	P	P	P	P	P	-	-
209/03	P	P	-	-	-	-	-	-	-	-	-	-
215/03	P	P	P	-	P	P	P	P	P	P	-	-
224/03	P	P	P	-	-	-	P	P	-	-	-	-
256/03	-	P	-	-	P	-	-	-	-	-	-	-
278/03	P	P	P	-	-	-	P	-	-	-	-	-
288/03	P	P	P	P	P	P	-	-	-	P	-	-
295/03	P	P	-	-	-	-	-	-	P	P	-	-
319/03	P	P	P	-	P	P	P	-	P	-	-	-
325/03	P	P	P	-	P	-	P	-	P	-	-	-
338/03	P	P	-	-	P	P	-	-	P	P	-	-
339/03	P	P	-	P	P	P	-	-	P	-	-	-
356/03	P	P	P	P	-	-	-	-	-	-	-	-
407/03	P	P	-	-	P	P	-	-	P	-	-	-
414/03	P	P	P	-	P	P	P	P	P	-	-	-
positives	29/30	20/30	12/30	03/30	12/30	08/30	07/30	04/30	13/30	06/30	0/30	0/30

I) positive; II) negative.

Table 2. *M. tuberculosis* complex detection by PCR for *M. bovis* culture-negative group, according to DNA extraction protocols and homogenate dilutions.

	Protocol A				Protocol B				Protocol C			
DNA volume	2.5 µL		5 µL		2.5 µL		5 µL		2.5 µL		5 µL	
dilutions samples	10^{-1}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-1}	10^{-2}	10^{-3}
05/02	II –	IP	-	-	P	P	-	-	P	P	-	-
17/03	P	P	-	-	P	P	-	-	P	P	-	-
97/03	–	P	-	-	–	-	-	-	–	-	-	-
103/03	P	P	-	-	–	-	-	-	–	-	-	-
120/03	–	P	-	-	–	-	-	-	–	-	-	-
146/03	P	P	-	-	–	-	-	-	–	-	-	-
157/03	P	P	-	-	P	P	-	-	–	-	-	-
166/03	P	P	-	-	P	P	-	-	–	P	-	-
183/03	P	P	-	-	P	P	-	-	P	P	-	-
185/03	P	P	-	-	P	P	-	-	–	P	-	-
187/03	P	P	-	-	P	-	-	-	P	P	-	-
200/03	–	-	P	-	–	-	-	-	P	-	-	-
211/03	P	P	-	-	–	-	-	-	–	-	-	-
222/03	P	P	-	-	–	-	-	-	–	-	-	-
228/03	–	-	-	-	–	-	-	-	–	-	-	-
276/03	–	-	-	-	–	-	-	-	–	-	-	-
284/03	–	-	-	-	–	-	-	-	–	-	-	-
318/03	–	-	-	-	–	-	-	-	–	-	-	-
359/03	–	-	-	-	–	-	-	-	–	-	-	-
368/03	–	-	-	-	–	-	-	-	–	-	-	-
423/03	P	P	-	-	P	P	-	-	–	-	-	-
431/03	P	P	-	-	P	P	-	-	–	-	-	-
454/03	P	P	-	-	P	P	-	-	P	-	-	-
455/03	–	-	-	-	–	-	-	-	–	-	-	-
483/03	–	-	-	-	P	P	-	-	–	-	-	-
490/03	–	P	-	-	–	-	-	-	P	-	-	-
525/03	P	P	-	-	–	-	-	-	–	P	-	-
535/03	–	-	-	-	–	-	-	-	–	-	-	-
242/03	–	-	-	-	–	-	-	-	–	-	-	-
18/04	P	P	-	-	–	P	-	-	P	P	-	-
positives	15/30	19/30	01/30	0/30	11/30	11/30	0/30	0/30	08/30	08/30	0/30	0/30

I) positive; II) negative.

As shown in Table 1 and 2, sensitivity decreased according to dilution, as the 10^{-1} dilution yielded better results than 10^{-2} and 10^{-3} dilutions. Overall, Protocol A presented the highest sensitivity, followed by Protocol C and B. Nevertheless, it displayed the lowest specificity (Table 3).

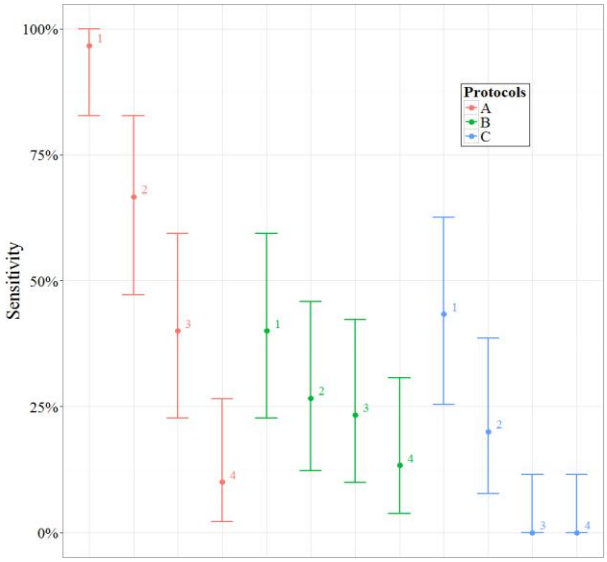
Regarding the DNA extraction protocol for tissue suspensions, Protocol A applied on a 10^{-1} dilution stood out from the others in terms of sensitivity (Figure 1).

Table 3. Sensitivity and specificity of DNA extraction protocols for *M. bovis*.

	Sensitivity (%)			Specificity (%)		
	Point Estimate	Lower 95% CL*	Upper 95% CL	Point Estimate	Lower 95% CL	Upper 95% CL
Protocol A	100	88.43	100	33.33	17.29	52.81
Protocol B	46.67	28.34	65.67	60	40.60	77.34
Protocol C	50	31.30	68.70	63.33	43.86	80.07

*confidence level.

Figure 1. Sensitivity of DNA extraction protocols and PCR procedures for *M. bovis*.



PCR procedures: 1) 2.5 µL of 10^{-1} dilution; 2) 5 µL of 10^{-1} dilution; 3) 5 µL of 10^{-2} dilution; 4) 5 µL of 10^{-3} dilution.

Discussion

Rapid detection of *M. bovis* infection has always been a matter of great importance, not only with respect to public health but also for animal health and economic issues. Several studies have been conducted to provide reliable and faster techniques to achieve this goal (LIÉBANA et al., 1995; WARDS et al., 1995; TAYLOR et al., 2007; CARDOSO et al., 2009).

The aftermath of PCR is subjected to the failure of extracting all the *M. bovis* organisms from tissue material, failure to extract DNA from *M bovis*, presence of PCR inhibitors, or a combination of these (WARDS et al., 1995).

Despite the protocol described by Boom et al. (1990) was not developed to extract DNA from mycobacteria or mycobacteria from tissue, Protocol A revealed excellent sensitivity, possibly because of the modification of enzymatic addition, as observed by Wards et al. (1995).

Zanini et al. (2001) presented PCR-positive results for all culture-positive samples and for 60% of samples that failed to grow in culture. These results are almost identical to those obtained by Protocol A, yet questionable in terms of sensitivity and specificity, since the samples in this study were incubated for only 45 days, which could have considerably decreased their chances of being culture-positive.

On the other hand, Liébana et al. (1995) incubated all samples for five months, and found 71.4% PCR-positive samples among the culture-positive and 6.25% among the culture-negative samples. In contrast to Protocol A and the one described by Zanini et al. (2001), Liébana et al. (1995) used only proteinase K and boiling for DNA extraction, which may have contributed to their PCR-negative results in culture-positive samples.

The low specificity observed in our findings was likely caused by the lack of an incubation period. Although the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2015 of the World Organization for Animal Health (OIE) recommend 90 days of incubation, studies have shown that longer incubation periods increase the sensitivity of isolation (LIÉBANA et al.; 1995; CORNER et al., 2012).

Protocols B and C were performed as described by Zumárraga et al. (2001) and Santos et al. (1995), respectively, with few modifications. Both protocols were designed to extract mycobacterial DNA from tissues. Although our study reproduced similar results as reported by Santos et al. (1995), our findings were not similar to those found by Zumárraga et al. (2001), which observed *M. bovis* PCR-positive and culture-positive from all tissue samples.

Some tuberculous tissues contain considerable numbers of *M. bovis*, but it is common to find tissues with few of these organisms (WARDS et al., 1995). The low sensitivity observed in Protocol B may be due to lower tissue concentration, once the homogenates were prepared differently from the original (ZUMÁRRAGA et al., 2001).

Despite the number of *M. bovis* colonies seen after primary culture, and the efficiency of amplification had little relationship in some cases (WARDS et al., 1995), a minimum amount of DNA is still necessary for detection by PCR methods (TAYLOR et al., 2007), and most of PCR procedures with 10^{-2} and 10^{-3} dilutions of DNA exhibited poor sensitivity.

In addition, PCR sensitivity can be affected by the inhibitors present in the clinical samples, those from the reagents used in the extraction method, or the amount of extracted DNA (CARDOSO et al., 2009; RADOMSKI et al., 2013). Cardoso et al. (2009) demonstrated that the potential effect of PCR inhibitors can be reduced by diluting (1:2) or increasing the amount of DNA in the samples. In our study, the volume of 2.5 µL for the 10^{-1} dilution of DNA submitted to PCR showed more positive samples than the volume of 5 µL for the 10^{-1} dilution of DNA in the culture-positive group, and almost no difference was observed in the culture-negative group.

Molecular techniques have demonstrated high potential to identify more cases of *M. bovis* infection when compared with the conventional methods, especially for detecting nonviable organisms (WARDS et al., 1995). Direct PCR from lesion samples represents an additional tool in the routine diagnosis of bovine tuberculosis, as it is faster and is able to detect infection when culture methods are not sufficient (CARDOSO et al., 2009).

Conclusion

The findings of the present study indicate that Protocol A, as described by Boom et al. (1990) with the modification of prior enzymatic addition, should be the chosen DNA extraction protocol in the routine of detection of members of the *M. tuberculosis* complex in bovine tissues by PCR.

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