Comparative study on PCR and microbiological culture performance for identifying *Mycobacterium tuberculosis* complex and *Mycobacterium bovis* specie in bovine samples

Comparação entre o cultivo microbiológico e a PCR na identificação do complexo *Mycobacteirum tuberculosis* e da espécie *Mycobacterium bovis* em amostras bovinas

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RESUMO

Foi avaliado o desempenho das técnicas de cultivo microbiológico e de PCR na análise de amostras de órgãos bovinos com lesões suspeitas de tuberculose. Cinquenta e duas amostras, colhidas em abatedouros, foram analisadas pelo cultivo microbiológico e o DNA extraído foi amplificado por PCR, utilizando-se os *primers* NZ1 e NZ2, que identificam micobactérias do complexo *M. tuberculosis***,** e o par de *primers pnc*A que diferencia as espécies *M. bovis* e *M. tuberculosis*. As 30 amostras de colônias isoladas foram suspensas, e a amplificação do DNA extraído foi feita por PCR, empregando-se os mesmos pares de *primers*. Embora a concordância entre as técnicas de cultivo microbiológico e de PCR realizado diretamente nas amostras clínicas com os *primers* NZ1 e NZ2 tenha sido fraca (k=0,175), os dois pares de *primers* utilizados amplificaram os genes alvos quando aplicados em 100% do DNA extraído das 30 colônias isoladas. Pela PCR com par de *primers pnc*A houve identificação de *M. bovis* nas colônias isoladas em curto intervalo de tempo, quando comparado aos testes bioquímicos. O uso concomitante de ambas as técnicas reduz o tempo para efetuar a confirmação do agente isolado, fator essencial nos estudos epidemiológicos e nas medidas de controle preventivas.

Palavras-chave. tuberculose, bovino, *Mycobacterium bovis*, PCR.

ABSTRACT

The present study aimed at evaluating the concordance between PCR and microbiological culture techniques for analysing organs samples from cattle with suspected lesions of tuberculosis. Fifty-two samples collected from slaughterhouses were analyzed by microbiological culture, and the extracted DNA was amplified by PCR using NZ1 and NZ2 primers**.** These primers identify the mycobacteria belonging to *M. tuberculosis* complex, and the primers pair *pnc*A differentiate the *M . bovis* from *M. tuberculosis* species. The colonies isolated from 30 samples were suspended, and the extracted DNA was amplified by PCR using the same primer pairs. Although the agreement has been considered weak (k = 0.175) between microbiological culture and PCR performed directly in clinical samples using NZ1 and NZ2 primers, the two pairs of primers could amplify the target genes when 100% of the extracted DNA from 30 isolated colonies were used. Thus, PCR employing *pnc*A primer pair enabled to identify *M.bovis* in the isolated colonies at a short time when compared with the biochemical assays. The concomitant use of PCR and bacteriologic culture techniques hastens the confirmation of detected agent, which is essential in conducting the epidemiological studies and in taking preventive control measures.

Keywords. tuberculosis, bovine, *Mycobacterium bovis*, PCR.

INTRODUCTION

Tuberculosis is a zoonosis, and among the etiologic agents, detaches the species belonging to *M. tuberculosis* complex, which comprises: *M. tuberculosis, M. bovis, M. africanum, M. microti, M. canettii, M.caprae and M. Pinnipedii*[1]. *M. tuberculosis and M. bovis* species can be pathogenic for man. *M. bovis*, the causative agent for bovine tuberculosis, is a serious public health issue because it can potentially infect humans[2]. In Brazil, bovine population is estimated in 167 million animals, and still nowadays there is no correct estimated prevalence of animal tuberculosis in the country, the last data refers to an official notification and indicates an average national prevalence of 1.3% infected animals[3]. Bacteriological culture is the confirmatory diagnosis for mycobacteria detection, and is considered the gold standard test by World Organization for Animal Health (OIE). Due to dysgonic and very low growth of mycobacteria, isolation and identification of *M. bovis* by conventional biochemical methods is laborious, and may take several weeks. PCR is an efficient and fast method that can be used to detect the agent in clinical samples, with a 48 hours' time processing[4,5,6]. Two PCR reactions with specific primers hybridizing the gene encoding the pyrazinimidase (*pnc*A) reported by Shah et al.[7] to differentiate colonies between *M. bovis* and *M. tuberculosis* were one of the methodologies carried out in the present work for this differentiation.

Tuberculosis is still very frequent in Brazil and the specific diagnostic takes a long time period, it would be useful a methodology able to shorten this period. For this purpose, the present trial evaluated the use of PCR for detection of *M. tuberculosis* complex and specific differentiation (*M. bovis* and *M. tuberculosis*) directly from suspect clinical samples and also from mycobacteria isolates, in comparison with microbiological culture.

MATERIAL AND METHODS

Sampling

Samples from 52 bovine (38 lymphnodes, 11 lungs, 3 liver) (see table 1) with suggestive lesions of tuberculosis (caseous lesions) were collected at slaughterhouse from São Paulo State and were transported in sterile bags at 4º C until further analysis.

Microbiological culture

Samples were cultured in Stonebrink and Petragnani media after decontamination by means of Petroff method[8] incubating two tubes of each medium at 37 ºC. The cultures were examined every week, during 90 days, for observation of mycobacteria growth, confirmed through Ziehl-Neelsen staining as described by Bier[9].

PCR reactions detection threshold

PCR detection threshold was obtained by artificial contamination of 1 ml of a healthy macerated lung with 2.3 x 10^5 CFU of *M. bovis* (AN5). Then, serial dilutions were prepared from this first suspension until 2.3 x 10^{-3} CFU/mL. All the dilutions were processed for DNA extraction and PCR assays cited in the following topics to determine the analytical sensitivities.

Still, PCR analytical sensitivity was also evaluated for colonies suspension, starting from 2.3 x 10^5 CFU of *M. bovis* (AN5)/mL of ultrapure water with serial dilutions until 2.3 x 10^{-3} CFU/mL. The suspensions were boiled (100 ºC/5 minutes) for DNA liberation and further PCR reactions.

DNA extraction

DNA was extracted from the clinical samples and from the suspensions of macerated lung artificially contaminated, and were firstly submitted to proteinase K digestion (20 mg/mL) at 56 °C for 2 hours, and then processed with commercial reagent TRIZOL (Invitrogen)[10] according to the manufacturer's instructions.

Isolated colonies were suspended in 0,85% sterile saline solution and submitted to boiling (100 °C/5 minutes) for DNA exposure[11]. Extracted DNA from clinical samples and from colonies suspension were submitted to PCR for complex *M.tuberculosis* determination and, afterwards, for identification of *M. bovis* and *M. tuberculosis* differentiation described in the next topic.

PCR Assay

PCR for *M. tuberculosis* complex detection was performed as described by Collins and Stephens[12] with primers NZ1 (5'CGACAGCGAGCAGCTTCTCGCTG 3') and NZ2 (5'GTCGCCACCACGCTGCTGGTCAGTG 3') that target IS-1081, specific insertion sequences from *M. tuberculosis* complex, which amplify a 306 bp fragment.

Amplification was performed in a total volume of 50 µL, with 200 µM of each dNTP, 1X PCR buffer (10 mM Tris-HCL, pH 8.0; 50 mM KCL), 2 mM $MgCl_2$, 25 p-mole of each primer (NZ1 and NZ2), 1.25 U Taq DNA polymerase and 10 µl of DNA. The PCR reaction consisted of an initial denaturation of 95 °C for 4 min., 30 cycles including 94 °C for 1 min., 63 °C for 90 s and 72 °C for 1 min. followed by final extension at 72 °C for 10 min.

PCR for *M. bovis* and *M. tuberculosis* identification

For *M. bovis* identification it was carried out the system described by De Los Monteros et al.[13], being one specific reaction for *M. bovis* detection and another one for *M. tuberculosis*. The amplification reactions were similar, only differing in the reverse primer. The protocol was carried out adding 10 µL of DNA to a PCR mix of 50 µL containing dNTP, buffer, $MgCl_2$ and Taq DNA polymerase as described above and 30 pmol of the forward primer *pnc*ATB-1.2 (5' ATGCGGGCGTTGATCTCGTC 3') complementary to part of *pnc*A gene that is present in both *M. tuberculosis* and *M. bovis* species, *M. bovis*-specific reverse primer *pnc*AMB-2 (5' CGGTGTGCCGGAGAAGCCG 3') or *M. tuberculosis* specific reverse primer *pnc*AMT-2 (5´ CGGTGTGCCGGAGAAGCGG 3´). The reverse primers varies only in one nucleotide in the *pncA* gene (a cytosine residue at position 169), and this substitution was enough for *pnc*AMB-2 and *pnc*AMT-2 specifically anneal to *M. bovis* or *M. tuberculosis*, respectively. PCR reactions with primers *pnc*ATB-1.2 and *pnc*AMT-2 or *pnc*AMB-2 amplify specific 185 bp products in *M. tuberculosis* or *M.bovis*, respectively. After initial denaturation at 95ºC for 10 min., DNA amplification was set with 30 cycles of 94ºC for 1 min., 67ºC for 1 min. and 72ºC for 1 min., followed by a final extension step of 72ºC for 10 minutes. Positive PCR controls were *M. bovis* AN5 (Instituto Biológico-SP) and *M. tuberculosis* $H_{37}RV$ (ATCC 27294), and ultra-pure water was used as negative control. Amplification reactions were carried out in a Peltier Thermal Cycler-100 (MJ Research).

Analysis of the amplified products

Analysis of the amplified products was performed by means of electrophoresis in 1.3% agarosis gel with TBE 0.5 X running buffer (0.045 M TRIS-Borate and 1 mM of EDTA pH 8.0). Gel was stained with ethidium bromide, visualized with a UV transiluminator (300-320 nm) and photographed by a photo-documentation system (Kodak Digital Camera DC/120 Zoom).

RESULTS

Microbiological culture

From 52 bovine samples (38 lymph nodes, 11 lungs, 3 livers) with suggestive lesions of tuberculosis (caseous lesions), 30 (57.7%) allowed the growth of mycobacterium by means of microbiological culture, and 1 (sample 11) (0.52%) was contaminated with other microorganisms and 22 (42%) were negative. From thirty isolated colonies 100% were confirmed as *Mycobacterium* spp. by Ziehl-Nielsen staining. The results are presented in Table 1.

PCR reactions detection threshold

PCR assays threshold detection for the artificially contaminated lung revealed that under conditions cited above, the reaction with primers NZ1 and NZ2 could detect up to 2.3 x 10^1 CFU/mL, the same analytical sensitivity obtained for the colonies suspension. With primers for *pnc*A gene, detection threshold was 2.3 x 10^3 CFU/mL either in the contaminated lung or colonies suspension.

DNA extracted from clinical samples

When PCR was applied in DNA extracted directly from the 52 clinical samples analyzed revealed that 6/52 (11.5%) were positive for mycobacteria from *M. tuberculosis* complex with primers NZ1 and NZ2. No sample 0/52 was positive for *pnc*A gene (*M. bovis* species) reactions for the same samples. The identified 22 negative samples from microbiological culture were confirmed negative by PCR with primers NZ1 and NZ2 and *pnc*A. The results are presented in Flowchart 1.

DNA extracted from isolated colonies

The 30 isolated colonies were submitted to boiling (100 °C/ 5 minutes) for DNA exposure and PCR methodology with primers NZ1 and NZ2, which classified 30 (100%) the colonies as belonging to *M. tuberculosis* complex. The same DNA samples were also submitted to PCR methodology with primers *pnc*ATB-1.2 and *pnc*AMB-2 which confirmed 100% as *M. bovis* species with no signal observed with primers *pnc*ATB-1.2 and *pnc*AMT-2 that identified for *M. tuberculosis*.

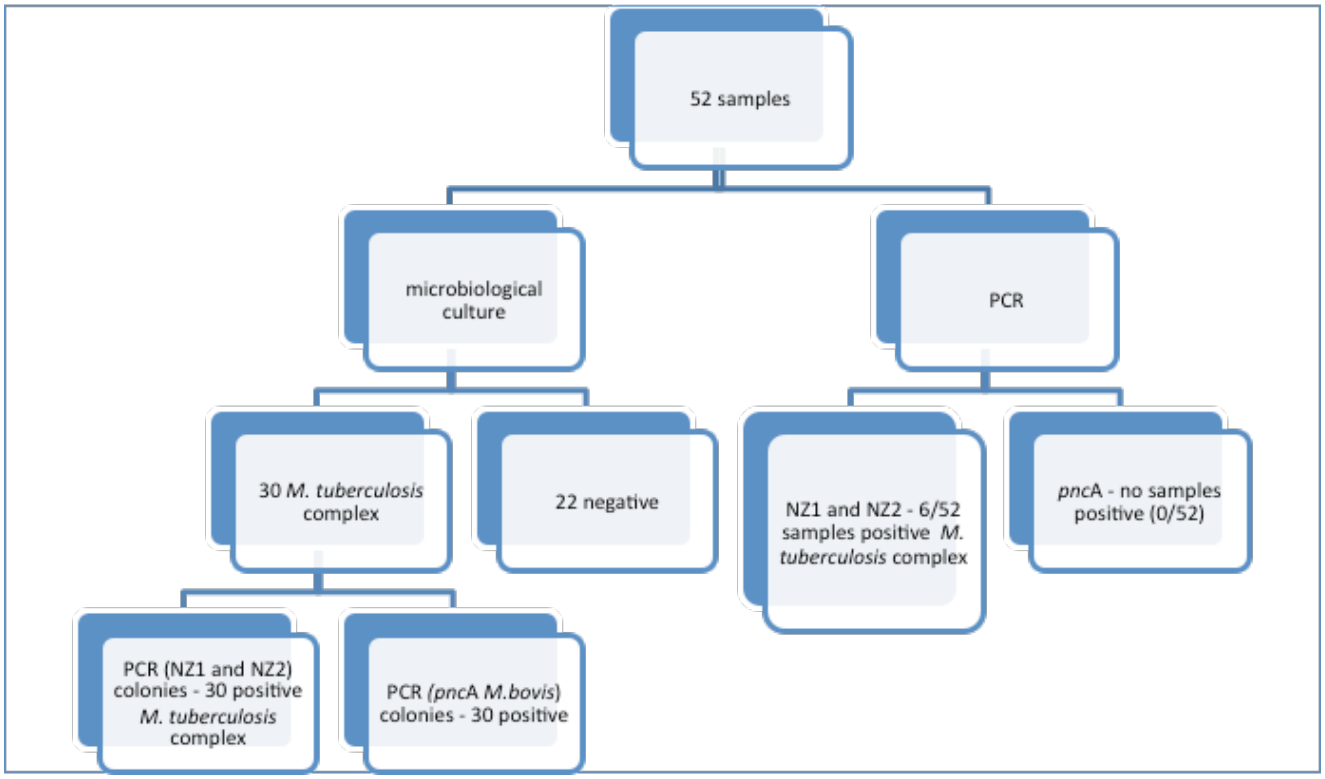
Statistical analysis

Concordance between microbiological culture and PCR with primers NZ1 and NZ2 (*Mycobacterium tuberculosis* complex) detection when applied to DNA extracted directly from clinical samples was calculated

Table 1. Isolated and PCR reaction to identify M. tuberculosis complex

Identification	Samples	Clinical Samples				Isolated Colonies		
		Culture (Baar)	Primer Nz (Nz1 And Nz2)	Primer: *pnc*amb-2**	Primer: *pnc*amt-2*	Primer Nz (Nz1 And Nz2)	Primer: *pnc*amb-2**	Primer: *pnc*amt-2*
1	Lymphonode	Positive	Positive	Negative	Negative	Positive	Positive	Negative
2	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
3	Lymphonode	Positive	Positive	Negative	Negative	Positive	Positive	Negative
4	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
5	Lymphonode	Positive	Positive	Negative	Negative	Positive	Positive	Negative
6	Lymphonode	Negative	Negative	Negative	Negative	-	-	-
7	Liver	Negative	Negative	Negative	Negative	-	-	-
8	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
9	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
10	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
11	Lymphonode	Contaminated	Positive	Negative	Negative	-	-	-
12	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
13	Lymphonode	Positive	Positive	Negative	Negative	Positive	Positive	Negative
14	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
15	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
16	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	-
17	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
18	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
19	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
20	Liver	Positive	Negative	Negative	Negative	Positive	Positive	Negative
21	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
22	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
23	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
24	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
25	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
26	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
27	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
28	Liver	Positive	Positive	Negative	Negative	Positive	Positive	Negative
29	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
30	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
31	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
32	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
33	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
34	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
35	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
36	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
37	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
38	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
39	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
40	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
41	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
42	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
43	Lymphonode	Positive	Negative	Negative	Negative	Positive	Positive	Negative
44	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
45	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
46	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
47	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
48	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
49	Lung	Positive	Negative	Negative	Negative	Positive	Positive	Negative
50	Lymphonode	Negativo	Negative	Negative	Negative	-	-	-
51	Lung	Negativo	Negative	Negative	Negative	-	-	-
52	Lung	Negativo	Negative	Negative	Negative	-	-	-

* primer pncAMT-2: specific primer to identify *M. tuberculosis*
**primer pncAMB-2: specific primer to identify *M. bovis*



Flowchart 1. Results obtained from microbiological culture and PCR of clinical samples and colonies

by MEDCALC with a 95% confidence interval[14], and was classified as minor (Kappa = 0.175).

DISCUSSION

In the present study, from 52 bovine samples analyzed, 30 (57.7%) were positive by microbiological methodology for *Mycobacterium* spp. and all the isolates (100%) could be identified as belonging to *M. tuberculosis* complex with primers NZ1 and NZ2, and classified as *M. bovis* species with *pnc*A PCR reactions.

Araujo et al.[15] reported that among 72 bovine lymph node slaughterhouse samples, 17 were positive for mycobacterium, and 13 were confirmed by PCR with primers JB21 and JB22 as *M. bovis*. However, Shah et al.[7] reported that out of twenty *M. tuberculosis* isolates, 12 (60%) amplified a specific fragment with JB21 and JB22 primers showing that this set of primers is not *M. bovis* specific. Still, they proved that *pncA* reaction could specifically differentiate all the isolates between *M. bovis* and *M. tuberculosis*. In concordance with these results, Nassar et al.[16] reported that from 42 cultivated samples, 27 (64.3%) were positive by culture method, and all isolates were confirmed by PCR to belong to *M. tuberculosis* complex with NZ1 and NZ2 primers and were identified as *M. bovis* with primers *pnc*AMB-1.2.

The concordance between microbiological and PCR methodologies for *Mycobacterium tuberculosis* complex detection was minor (k = 0.175), what could be explained due to the different aspects present in the techniques. While microbiological culture depends on microorganism availability, the quality of the analyzed sample and the timing from collection and its processing, DNA detection by PCR do not depend on neither microorganism availability, nor the contamination of the sample with other microorganisms, or the timing for its processing. However, there are external factors such as the presence of inhibitors, low quantity of tuberculosis bacillus in the sample that could interfere with the PCR results[17,18]. The low concordance between microbiological culture and PCR achieved in this study differs from the one described by Suffys et al.[18] when utilizing primers that hybridize the IS6110 of the *M. tuberculosis* genome with clinical samples, and reported 67% of positivity rate, while the current work showed a positivity of 11.5% with primers NZ1 e NZ2. This difference can be due to

the fact that the IS 6110 are present in multiple copies (8 at 20) all through the genome, while the IS 1081 used in detection of *M. tuberculosis* complex presents only a couple (2 to 6) copies[19]. Anyway, both reactions did not present an analytical sensitivity low enough to substitute the microbiological culture.

In the current work, the growth of suggestive colonies of *Mycobacterium* spp. in selective culture media (Stonebrink e Petragnani) was observed in 30 samples, and they were all confirmed as *M. bovis* by means of PCR methodology with the primers *pnc*A. The present work showed the non-applicability of PCR assay for detection of *M. tuberculosis* complex diagnosis in clinical samples using the primers NZ1 and NZ2 due to its lack of analytical sensitivity in the current conditions. However, the set of primers for *pnc*A gene showed to be able to differentiate isolates between *M. tuberculosis* and *M. bovis* species, allowing a shorter period for this identification if compared with biochemical tests, since such confirmation is needed for epidemiological studies and for tracing preventive and control measures.

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