Evaluation of polymerase chain reaction (PCR) for identifying *Mycobacterium bovis* isolates from the modified Middlebrook 7H11 agar thin layer technique

Avaliação da técnica de reação de polimerase em cadeia (PCR) para identificação de *Mycobacterium bovis* em microcolônias isoladas em camada delgada de ágar Middlebrook 7H11 modificado

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ABSTRACT

The associated use of the modified Middlebrook 7H11 agar thin layer technique and the Polymerase Chain Reaction (PCR) assay enabled to perform the early identification of microcolonies of *Mycobacterium bovis* from 12th to 25th day of culture. In order to reduce the time for performing the *Mycobacterium bovis* identification, the combined use of these two techniques was evaluated by analyzing the microcolonies of mycobacteria at the 8th day after culturing. Until the last day of analysis, all of the PCR-positive samples already showed the microcolonies**.** Therefore, the early diagnosis of bovine tuberculosis is feasible, without an apparent macroscopic colonies growth**.**

Keywords. tuberculosis, *Mycobacterium bovis*, diagnostic techniques and procedures, Polymerase Chain Reaction, early diagnosis.

RESUMO

A associação da técnica de cultivo em camada delgada no meio de ágar Middlebrook 7H11 modificado com a Reação de Polimerase em Cadeia (PCR) possibilitou a identificação precoce de *Mycobacterium bovis* em colônias macroscópicas entre o 12º e o 25º dia de crescimento. Com o objetivo de diminuir o tempo necessário para efetuar a identificação de *Mycobacterium bovis*, avaliou-se o uso combinado dessas duas técnicas, em microcolônias de micobactérias, no oitavo dia pós-semeadura. Até o último dia de observação, todas as amostras com positividade no ensaio da PCR já apresentavam crescimento microscópico, possibilitando-se a realização de diagnóstico precoce da tuberculose bovina mesmo sem aparente crescimento macroscópico das colônias.

Palavras-chave. tuberculose, *Mycobacterium bovis*, técnicas e procedimentos diagnósticos, PCR, diagnóstico precoce.

INTRODUCTION

Mycobacterium bovis is the causative agent of bovine tuberculosis, a disease responsible for losses to agriculture worldwide that amount to 3 billion dollars, and that has serious repercussions in public and animal health[1].

Early detection of the causative agent in food-producing animals is important to cull infected animals in a herd, and to adopt adequate measures to locate outbreaks and, consequently, reduce the risk of products of animal origin serving as sources of infection to humans[2]. Infection is confirmed by the isolation and identification of *M. bovis*[3,4]. Isolation of *M. bovis* may be carried out by Petroff's traditional method and culture with egg-based media, such as Stonebrink medium, or agar-based media, such as modified Middlebrook 7H11 medium, both of which have sodium pyruvate as the carbon source. However, identification by biochemical or molecular methods[3] is done only after growth is visible. This occurs after 3 to 5 weeks of incubation, and may take more than 12 weeks in Stonebrink medium, or between 12 to 26 days in modified Middlebrook 7H11 medium[3,5].

In order to decrease the time to *M. bovis* detection, several molecular methods, such as Polymerase Chain Reaction (PCR), have been employed in the diagnosis of bovine tuberculosis using samples of suspect tuberculosis lesions. This technique, however, has low sensitivity when carried out straight in tissue samples, due to the difficulty in extracting DNA from the samples, the variable amount of bacilli found in the lesions, the presence of PCR inhibitors, contamination with DNA from the host and other microorganism species[3,6-8], making it necessary to use PCR in colonies obtained by isolation, which is the gold standard for diagnosis.

In an attempt to reduce time to diagnosis, in a previous study modified Middlebrook 7H11 medium thin layer technique was used for the isolation of *M. bovis* in suspect bovine tuberculosis lesions collected from cattle and buffaloes in commercial slaughterhouses, followed by PCR for the identification of the colonies. Thus, the number of days to diagnosis was reduced to 12-25, compared with 42 to 91 days required for visible colonies in Stonebrink medium. As diagnosis confirmation by PCR was carried out after macroscopic growth of the colonies, the present study evaluated the association of modified Middlebrook 7H11 medium thin layer technique and PCR before macroscopic colonies were visible. Plates were observed for no more than 12 days to achieve the earliest possible diagnosis in samples with lesions suggestive of tuberculosis.

MATERIAL AND METHODS

A total of 10 samples of bovine tuberculosis suspect lesions. Eight of them were considered positive in a previous study, after processing and culture in Stonebrink medium and modified Middlebrook 7H11 medium thin layer culture[9], followed by confirmation by PCR (Table 1).

Table 1. Growth of colonies in plates with Middlebrook 7H11 and Stonebrink tubes with respective PCR results according to the experimental groups

SAMPLES		PLATES				TUBES			
Number	Identification	A[a]	B	*Status*	PCR[f]	A	B	*Status*[g]	PCR
1	12	+[b]	-[c]	POS[d]	POS	+	+	POS	POS
2	16	+	+	POS	POS	+	+	POS	POS
3	32	+	+	POS	POS	+	+	POS	POS
4	34	+	+	POS	POS	+	+	POS	POS
5	36	-	+	POS	POS	+	+	POS	POS
6	37	+	+	POS	POS	+	+	POS	POS
7	39	+	-	POS	POS	+	+	POS	POS
8	40	+	+	POS	POS	+	-	POS	POS
9	47	-	-	NEG[e]	**	-	-	NEG	NEG
10	49	-	-	NEG	POS	-	-	NEG	NEG

a - = plate ou tube A/plate ou tube B; b - presence of colonies in plates or tubes; c- absence of colonies in plates or tubes; d - positive sample; e - negative sample; f - Polimerase Chain Reaction; g - Result of the sample in plates, tubes or PCR; ** - Not executed.

The ten samples were decontaminated by Petroff's traditional method[10] and cultured in plates with modified Middlebrook 7H11 medium, in duplicate, in a total of 20 plates. The attempt was to have an earlier perception of the colonies and to identify them by PCR, no matter the macroscopic growth of the colonies.

Readings in optical microscope were carried out on days 5, 8 and 12 after culture. On the 13th day, plates were removed from the incubator and opened in a level 3 microbiological safety cabinet. All the content on the surface of the medium was scraped with a disposable loop, and resuspended in 1.5-mL tubes with sterile ultrapure water. Inactivation was carried out by boiling at 100 °C for five minutes, followed by freezing at -20 °C for at least one hour before use[11].

Thawed samples were submitted to PCR using primers JB-21 (5' TCGTCCGCTGATGCAAGTGC 3') and JB-22 (5' CGTCCGCTGACCTCAAGAAG 3'), described by Rodriguez et al.[12] for the identification of *Mycobacterium bovis*, generating a 500-bp final product.

Amplification with primers JB-21 and JB-22 was carried out in a thermocycler, with initial treatment at 94 °C for two minutes, followed by 35 cycles at three different temperatures: denaturation at 94 °C for 30 seconds, hybridization at 64 °C for 30 seconds, and extension and annealing at 72 °C for 60 seconds. After the last cycle, samples were kept at 72 °C for five minutes, and the amplified product was analyzed in a horizontal electrophoresis system. Agarose gels 1.5% containing 0.01% ethidium bromide were analyzed under ultraviolet light and photographed.

RESULTS AND DISCUSSION

From the ten samples in modified Middlebrook 7H11 medium thin layer culture, nine were considered positive and submitted to confirmation by PCR. As no macroscopic growth was observed, this stage was interrupted on day 12 after culture. The only sample considered negative in the thin layer plates was also negative in PCR. However, two plates considered positive were not confirmed in PCR

Table 2. Results of the observation by optical microscopy of the positivity of the samples on plates containing modified Middlebrook 7H11 thin layer and confirmed by polymerase chain reaction (PCR)

SAMPLES		PLATES			PCR[f]
Number	Identification	A[a]	B	Status[g]	Result
1	12	+[b]	+	POS[d]	POS
2	16	+	+	POS	POS
3	32	+	+	POS	POS
4	34	+	+	POS	POS
5	36	+	+	POS	POS
6	37	+	+	POS	NEG
7	39	+	+	POS	POS
8	40	+	+	POS	POS
9	47	-[c]	-	NEG[e]	NEG
10	49	+	+	POS	NEG

[a] - = plate ou tube A/plate ou tube B; [b] - presence of colonies in plates or tubes; [c]- absence of colonies in plates or tubes; [d] - positive sample; [e] - negative sample; [f] - Polimerase Chain Reaction; [g] - Result of the sample in plates, tubes or PCR.

Although the nine samples positive in this second stage were also positive in the first one, negative PCR results for samples 37 and 49 may be partially related to the amount of colonies collected from the plates to be used in PCR. Although PCR may detect very small amounts of live and dead bacilli in the sample[13], as there was no macroscopic growth in the marked areas at the moment of colony collection, only the location marked during reading in the microscope was considered. Thus, it is possible that, in this case, culture medium collection with the loop was not adequate.

In spite of the fact that the colonies were visually similar to those observed by Marcondes et al.[5] and Dib et al.[14], at initial stages, they may also be similar to those of other bacteria, leading to negative results in PCR. Bacteria of the genus *Nocardia* may have filamentous and bacillary forms[15], and may sometimes be mistakenly considered mycobacteria in the initial stages of growth, reinforcing the importance of the association of methods with PCR in the identification of *M. bovis.*

Results for the period of observation for presence or absence of mycobacteria growth in microscopic colonies in modified Middlebrook 7H11 thin layer culture showed seven of the plates with positive results in isolation and PCR. On the 8th day after culture, all samples that were confirmed to be positive in PCR had already been considered positive in culture, but observation continued until the 12th day because there were expectations for visible macroscopic growth that would confirm microscopic findings.

It is believed that this stage led to greater accuracy in the observation of the colonies due to the visual training carried out in the first stage of the experiment, which enabled the identification of the initial growth stages and the differentiation of cell debris[5]. Thus, at 5 days after culture, it was possible to safely state that the three plates considered positive in the second stage showed initial colony growth.

It was observed that, when reduction of time to reading is prioritized, the absence of macroscopic growth may lead to errors in the technique or insufficient amounts of sample for PCR, increasing the probability of having false positive results. However, the association of PCR for earlier confirmation of the results (12 days instead of the 36 days observed in previous studies) seems to be advantageous, as microscopic findings may be confirmed without the apparent need for observing macroscopic colony growth.

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