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Evaluation of optimal storage time of tuberculous lesions stored in sodium borate

Avaliação do tempo de armazenamento do borato de sódio na conservação de lesões tuberculosas

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Abstract

Preservation of specimens during transportation between abattoirs and diagnostic laboratories defines a critical stage in the definitive diagnosis of bovine tuberculosis by the isolation of *Mycobacterium bovis*. A 2-step study was designed to verify the maximum time that tissue samples can be stored in saturated sodium borate solution (SSB) with the highest detection of *M. bovis* isolates. Ninety hamsters were inoculated intraperitoneally with a suspension of *M. bovis* strain AN5 and were humanely euthanized after 40 days. Their spleens were collected and stored in SSB during four distinct periods (30, 60, 90 and 120 days) with incubation at two temperatures (27°C and 37°C). The control group was cultured on the day of euthanasia. Sixty-nine suspected tuberculous lesions samples were collected in the abattoir and were stored in SSB for three periods (30, 60 and 90 days) at 27°C in the laboratory. The bovine control group was cultured on the day of entry in the laboratory. Both experiments were analyzed separately based on the growth proportion of isolates and the number of colonies. SSB was able to maintain the viability of most *M. bovis* at high temperatures for up to 30 days. A progressive decline was observed with other storage periods at 27°C, and no growth was obtained after 60-day storage at 37°C. Despite the loss in viability of *M. bovis*, SSB is the most favorable choice to preserve specimens during transportation across a large country with high variation in environmental temperature. The sensitivity of *M. bovis* detection by bacteriological examination is inversely proportional to storage time. Therefore, the storage of tuberculous lesion specimens in SSB is recommended to not exceed 30 days at 27°C before cultivation.

Key words: Bovine tuberculosis. *Mycobacterium bovis*. Preservative. Sodium borate. Transportation.

Resumo

A conservação de espécimes durante o transporte entre abatedouros e laboratórios de diagnóstico define uma etapa crítica no diagnóstico definitivo da tuberculose bovina pelo isolamento de *Mycobacterium bovis*. Um estudo de duas fases foi delineado para verificar o tempo máximo de armazenamento que amostras teciduais podem ser mantidas em solução saturada de borato de sódio (SSB) com a mais alta detecção de isolados de *M. bovis*. Noventa hamsters foram inoculados com suspensão de *M. bovis*

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cepa AN5 por via intraperitoneal e, após 40 dias, submetidos à eutanásia humanitária. Os baços foram coletados, armazenados em SSB por quatro períodos distintos (30, 60, 90 e 120 dias) e incubados a duas temperaturas (27 e 37°C). O grupo controle foi cultivado no mesmo dia da eutanásia. Sessenta e nove amostras de lesões suspeitas de tuberculose foram coletadas em abatedouro e armazenadas em SSB por três períodos (30, 60 e 90 dias) a 27°C no laboratório. O grupo controle bovino foi cultivado no dia da entrada no laboratório. Ambos os experimentos foram analisados separadamente baseados na proporção de crescimento de isolados e no número de colônias. A SSB foi capaz de manter a maioria de *M. bovis* viáveis em altas temperaturas por até 30 dias. Houve um declínio progressivo nos outros períodos de armazenamento a 27°C, e não houve crescimento a partir de 60 dias a 37°C. Apesar da perda de viabilidade de *M. bovis*, a SSB é a escolha mais favorável para preservar as amostras durante o transporte em um grande país com alta variação de temperatura ambiente. A sensibilidade de detecção de *M. bovis* por exames bacteriológicos é inversamente proporcional ao tempo de armazenamento. Portanto, é recomendado que o armazenamento de amostras de lesões tuberculosas em SSB não exceda 30 dias a 27°C antes do cultivo.
Palavras-chave: Tuberculose bovina. *Mycobacterium bovis*. Conservante. Borato de sódio. Transporte.

Introduction

Bovine tuberculosis is a well-known chronic zoonosis caused by *Mycobacterium bovis*. This disease is responsible for elevated economic losses and public health issues. In Brazil, recent studies carried out in 13 states that hold 75% of the Brazilian cattle population, showed the prevalence of tuberculosis-infected herds among 0.36% in the Federal District and 9% 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). Thus, the success of the Program of Control and Eradication of Brucellosis and Tuberculosis relies on the surveillance system based on the isolation of *M. bovis* from suspected tuberculous lesions obtained from abattoirs (LAGE et al., 2006).

Bacteriological examination is required for the definitive diagnosis, given that traditional culture remains the gold standard method for infection routine confirmation (OIE, 2009). Culture media, decontamination procedures and incubation conditions have direct impact on the primary isolation of *M. bovis* (CORNER, 1994). Molecular techniques have also assisted detection and identification of this infectious agent (SALES et al., 2014a, 2014b).

Nevertheless, the preservation of tissue and lesion samples during transportation between abattoirs and diagnostic laboratories defines a critical stage that precedes all other procedures. The number of viable mycobacteria can decrease when specimens are handled inadequately (WARDS et al., 1995).

Immediate delivery of samples to the laboratory enhances the chances of *M. bovis* recovery. If the collected samples are cultured within 24 to 48 hours, they should be maintained between 4°C to 6°C. If this period is exceeded, specimens must be stored frozen until be thawed for cultivation (CORNER, 1994; OIE, 2009).

Brazil has a high diversity of warm environmental conditions, infrastructural availability and large distances from abattoir to laboratory, thus the use of preservative agents are required to overcome these issues. Chemical agents, such sodium carbonate, cetylpyridinium chloride (BOBADILLA-DEL-VALLE et al., 2003), chloramine-T (sodium *p*-toluenesulfonchloramide) and sodium borate (RICHARDS; WRIGHT, 1983), have been assayed for preserving mycobacteria and delaying the growth of contaminants.

Sodium borate is the most recommended (RICHARDS; WRIGHT, 1983; OIE, 2009) and employed preservative in routine abattoir inspection (CORNER, 1994; BROWN; HERNÁNDEZ DE ANDA, 1998; MILIÁN et al., 2000; MILLER et

al, 2002; RODRIGUEZ et al., 2004; THACKER et al., 2013). Although the final concentration of sodium borate varies from 0.5% (OIE, 2009) and 6% (MILIÁN et al., 2000) to a saturated solution (RICHARDS; WRIGHT, 1983; CORNER, 1994), the latter is more widely applied (BROWN; HERNÁNDEZ DE ANDA, 1998; MILLER et al., 2002; RODRIGUEZ et al., 2004; THACKER et al., 2013).

Due to the range of environmental temperature in the country and the demand for well-preserved specimens, the aim of the present study is to verify the maximum time that tissue samples can be stored in saturated sodium borate solution (SSB) with the highest detection of *M. bovis* isolates.

Material and Methods

The study was performed in two steps. The first step was based on experimental infection in animal models to produce standard lesions. The second step consisted of a trial with lesion samples collected from an abattoir.

Tissue samples

Experimental infection in Hamsters (*Mesocricetus auratus*)

The present study was carried out in accordance with ethical and biosafety regulations, and was certified by the "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of the University of São Paulo (protocol number 1415/2008).

Ninety male hamsters, weighing between 70 and 160 grams, were housed on wood-shavings bedding with commercial food and water provided *ad libitum*. *Mycobacterium bovis* strain AN5 colonies were grown in Middlebrook 7H9 broth (according to the manufacturer's instructions; Becton, Dickinson and Company, Sparks, MD, USA) and the suspension was adjusted to match the 0.5 McFarland turbidity standard by adding sterile 0.85% saline solution. Through culturing tenfold dilutions on Stonebrink medium, colony forming units (CFU) score at 30 days of incubation was used to establish the suspension chosen for inoculation. Each animal received 3,000 CFU intraperitoneally.

After 40 days, all animals were humanely euthanized. The spleens were collected and stored in non-sterile glass vials containing 25 mL SSB ($Na_2B_4O_7.10H_2O$, 14 g in 100 mL of distilled water, RODRIGUEZ et al., 2004). Forty specimens were incubated at 27°C and 40, at 37°C.

Ten specimens were used as control group and were freshly cultured on the day of euthanasia determining day zero (D.0). Of the specimens stored in SSB, ten spleens from each temperature were cultured after 30 (D.30), 60 (D.60), 90 (D.90) and 120 (D.120) days of incubation.

Bovine samples from abattoir

Sixty-nine suspected tuberculous lesions samples were collected from an abattoir. These included 29 lymph nodes, 23 lungs, 13 livers, 3 muscles and 1 heart. In the abattoir, the samples were placed in 500 mL plastic flasks and were immersed in SSB to be transported to the laboratory, where they were incubated at 27°C.

Samples were cultured after 5 (D.5), 30 (D.30), 60 (D.60) and 90 (D.90) days from collection. Given the impossibility of culturing the fresh specimens, D.5 was considered as the control group.

Cultivation

For culture, the whole spleen was used in the first step and one gram of each specimen was used in the second. The tissue was macerated in 5 mL sterile 0.85% saline solution using the Stomacher® 80 Biomaster (Seward, Port Saint Lucie, Florida, USA). The suspension was decontaminated with 0.75% 1-hexadecylpyridinium chloride at

room temperature (RT) for 20 minutes, and then centrifuged at 2,300 × *g* for 20 minutes (AMBROSIO et al., 2008). The supernatant was discarded and the sediment was resuspended in 1 mL sterile 0.85% saline solution. Each suspension was inoculated into duplicate slopes of Stonebrink medium in a volume of 0.1 mL, and incubated at 37ºC (CENTRO PANAMERICANO DE ZOONOSES, 1973).

The slopes were examined for growth every seven days. The CFU counts were scored at 90 days of incubation (OIE, 2009). For analysis, the average number of colonies from the two slopes was calculated. The total of 300 colonies was adopted when the number of colonies was too large to be counted (BRASIL, 1993).

Molecular identification

The isolates were identified by PCR-restriction fragment length polymorphism analysis (PRA) of the *hsp65* gene (TELENTI et al., 1993) and a multiplex PCR method based on the genomic regions of difference (RD) (WARREN et al., 2006).

Statistical analysis

The growth proportion of *M. bovis* isolates was analyzed using comparison of proportions test. The number of CFUs from hamster spleens (in D.0, D.30, D.60, D.90 and D.120) was analyzed using the Kruskal-Wallis' test, and the Dunn's test was used for multiple comparisons. The number of CFU from bovine specimens (in D.5, D.30, D.60 and D. 90) and the time of incubation in SSB were analyzed using the Friedman's test, and the Dunn's test was used for multiple comparisons. Differences were considered as statistically significant at $p<0.05$. The comparisons were performed using the computer programs Minitab 16 and GraphPad Instat.

Results

Experimental infection in hamsters (*Mesocricetus auratus*)

At the time of necropsy, the experimentally infected animals presented prostration and stubbly hair. The spleens were enlarged with yellow punctiform lesions on their surface. The morphological aspects of the isolates were identical to those of the *M. bovis* strain AN5. There was no growth in specimens cultured on D.60, D.90 and D.120 at 37°C.

No difference was observed between D.0 and D.30, or D.60, D.90 and D.120 on the growth proportion of isolates, at both temperatures ($p>0.05$). At 27°C, D.0 and D.30 samples showed more isolates than D.60 ($p=0.015$), D.90 and D.120 ($p=0.0003$). On the other hand, at 37°C, D.0 displayed better detection ($p=0.0001$) than D.30 ($p=0.005$), when compared to the other storage times. The median CFU number of D.0 was higher than D.30 at 27°C, but not significantly different. Table 1 shows the growth proportion of *M. bovis* and the mean and median CFU numbers.

Table 1. Growth proportion of *M. bovis* strain AN5 isolates, median and mean of CFU numbers from hamster spleens according to the days of storage in SSB and the incubation temperatures.

Storage time (days)	D.0	D.30		D.60		D.90		D.120	
Incubation temperature	-	27°C	37°C	27°C	37°C	27°C	37°C	27°C	37°C
M. bovis isolates (%)	100[a]	100[a]	70[a]	40[b]	0[b]	10[b]	0[b]	10[b]	0[b]
CFU median	300[a]	300[a]	115	109[b]	8	0[b]	0	0[b]	0
CFU mean	300	147,4	47,7	10,9	0	5	0	20	0

[a, b] different letters mean statistical difference.

Bovine samples from abattoir

From the results of experimental infection in hamsters, bovine samples were subjected to the suitable SSB storage time and incubation temperature.

The specimens were immersed in SSB for the maximum of 5 days when they arrived at the laboratory. Almost every collected tissue presented enlargement and yellow round shaped tuberculous-like lesions. Caseous lesions replacing almost the entire tissue were also observed in some specimens. The isolates that showed morphological aspects compatible with mycobacteria were classified as member of the *M. tuberculosis* complex by PRA and were identified as *M. bovis* by the RD multiplex PCR method.

Along the advance of each storage time, the growth proportion of isolates decreased ($p<0.001$) along with the CFU number ($p<0.001$), except the CFU at D.60 and D.90 ($p>0.05$). Table 2 presents the growth proportion of *M. bovis* and the median and mean of the CFU number.

Table 2. Growth proportion of *M. bovis* isolates, median and mean of CFU numbers from bovine samples incubated at 27°C according to the days of storage in SSB.

Storage time (days)	D.5	D.30	D.60	D.90
M. bovis growth (%)	100[a]	82,6[b]	26,1[c]	3[d]
CFU median	115[a]	8[b]	0[c]	0[c]
CFU mean	136,9	44,9	1,6	0,1

[a, b, c, d] different letters mean statistical difference.

Discussion

Preservation of suspected tuberculous lesions is directly related to the success of *M. bovis* detection. Maintenance of viable mycobacteria and inhibition of contaminants are important desirable factors in a preservative agent.

SSB has been used in routine procedures in Australia, where the environmental temperature can reach 40°C (CORNER, 1994). Similar climatic conditions can be observed in Brazil. In order to refine the storage conditions in a controlled environment, hamsters were used as biological models to obtain standardized tuberculous lesions.

The present study showed a decline in the growth of isolates and in the CFU number when the temperature increased from 27°C to 37°C, and when the storage time in SSB was extended. In both trials, the results from both hamster and bovine samples mostly suffered from 30 to 60 days of storage.

According to OIE (2009), boric acid in low concentrations can be used as a bacteriostatic agent, but only for limited periods, and no longer than one week. Milián et al. (2000) observed that 6% sodium borate solution was able to not interfere with *M. bovis* diagnosis of bovine lesions that were stored for up to 150 days at RT from 25°C to 35°C, even though the CFU number decreased by 98% from 30-day to 150-day storage.

Richards and Wright (1983) analyzed bovine specimens stored in SSB at RT (approximately 23°C) and observed no isolation after 14 weeks of storage (98 days), nor over 24 weeks of storage (168 days). Nevertheless, *M. bovis* was isolated from one specimen stored for 17 weeks (119 days) and from all nine specimens stored for 8 weeks (56 days) or less.

When the specimen presents high quantities of mycobacteria, the concentration of the sodium borate solution and the storage temperature might not affect *M. bovis* detection. However, if the number of viable mycobacteria is low, it may display in false-negative results.

The type of specimen preserved in SSB also plays an important role in terms of maintaining mycobacteria viability, since it determines the proximity of the bacteria to the preservative solution. An *in vitro* evaluation of preservatives that were in direct contact with culture suspensions showed that exposure to SSB for 7 days at 23°C or RT, displayed a discrete reduction in the number of viable *M. bovis* cells (RICHARDS; WRIGHT, 1983). Through constant temperature, the storage of hamster spleens in SSB at 27°C and 37°C for 150 days was harmful to *M. bovis* survival (MORATO[5]; unpublished data).

No growth of contaminants was observed in the present study, while Richards and Wright (1983) needed to reculture 6.1% of the specimens due to contamination issues.

Despite the increased loss in viability of *M. bovis* in sodium borate rather than upon chilling at 4-6°C (RICHARDS; WRIGHT, 1983; CORNER, 1994) and the decrease in growth of isolates, immersion in SSB is the most favorable choice to preserve specimens during transportation in a large country with high environmental temperature variation.

Conclusions

The storage of tuberculous lesion specimens in SSB is recommended to not exceed 30 days at 27°C before cultivation. The shorter the time they are maintained in preservative agents, the lower will be the sensitivity loss of *M. bovis* detection by bacteriological exams, which are an important part of the surveillance system in Brazil.

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[5] MORATO, F. unpublished data obtained in October, 2010.

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