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Microbiological analysis in the fixation and preservation of dog cadavers with ethyl alcohol and sodium chloride solution

Análise microbiológica na fixação e conservação de cadáveres de cães com álcool etílico e solução de cloreto de sódio

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Abstract

There are several fixative or preservative solutions for use on cadavers, and formaldehyde is the most widely used. However, this chemical may present negative effects for professionals who manipulate it. Therefore, this study aimed to identify and quantify the main microorganisms related to the fixation and preservation of dog cadavers using an alternative and formaldehyde-free solution. After arterial injection (120 mL kg^{-1} 95% 96° GL ethyl alcohol and 5% pure glycerin), cadavers were placed in 96° GL ethyl alcohol for 30 (group 1), 60 (group 2), 90 (group 3), and 120 days (group 4). After the fixation period, they remained under preservation in a 30% aqueous sodium chloride solution for 120 days. Bacterial quantification was performed by the pour plate method. The bacterial population was present in all groups during fixation, except for group 1, but never exceeded 9×10^1 CFU mL^{-1} in total aerobes and 7×10^1 CFU mL^{-1} in total anaerobes. The microbial population was present in all groups in at least two moments during preservation and never exceeded 7×10^1 CFU mL^{-1} in total aerobes and anaerobes. The presence of fungi was observed in 8 out of 34 analyses. *Pseudomonas* sp., *Escherichia coli*, and *Bacillus* sp. were identified in the analyzed samples. Microbiological counting was low, and no signs of contamination were observed in the vats at visual inspection.
Key words: Anatomy. Animal. Bacterium. Surgery. Microbiological counting.

Resumo

Várias são as soluções fixadoras ou conservantes de cadáveres, e o formaldeído é o mais utilizado. Entretanto, esse agente pode apresentar efeitos negativos para os profissionais que o manipulam. Portanto, o objetivo deste trabalho foi identificar e quantificar os principais microrganismos relacionados a fixação e conservação de cadáveres de cães utilizando-se solução alternativa e livres de formaldeído. Após injeção arterial (120 mL kg^{-1}, de 95% de álcool etílico 96° GL e 5% de glicerina pura), foram colocados em álcool etílico 96° GL por 30 dias (grupo 1), 60 dias (grupo 2), 90 dias (grupo 3) e 120 dias (grupo 4). Após o período de fixação, permaneceram sob conservação em solução aquosa de cloreto de

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sódio 30% por 120 dias. A quantificação bacteriana foi realizada por plaqueamento em profundidade (Pour Plate). Durante a fixação, houve presença de população bacteriana em todos os grupos, exceto no grupo 1, e nunca foi ultrapassado o valor de 9×10^1 UFC mL^{-1} (Unidade Formadora de Colônia) nos aeróbios totais e 7×10^1 UFC mL^{-1} nos anaeróbios totais. Durante a conservação, houve presença de população microbiana em todos os grupos em pelo menos dois momentos, e nunca foi ultrapassado o valor de 7×10^1 UFC mL^{-1} nos aeróbios totais e nos anaeróbios totais. A presença de fungos foi observada em 8 das 34 análises. Houve identificação de *Pseudomonas* sp., *E. coli* e *Bacillus* sp. nas amostras analisadas. A contagem microbiológica foi baixa e não foram observados, à inspeção visual, sinais de contaminação nas cubas.
Palavras-chave: Anatomia. Animal. Bactéria. Cirurgia. Contagem microbiológica.

Introduction

Fixation of anatomical parts for studies in laboratories is essential since it avoids their deterioration, maintains the firmness and insolubility of tissues, besides promoting their protection (RODRIGUES, 2010). Fixation and preservation of this material also aim to reduce pathogen proliferation, minimizing the risk of infections in professionals working in direct contact with them, both in veterinary and in human medicine (EISMA; WILKINSON, 2014).

In addition to the microbiological issue, it is important to evaluate the muscular texture of anatomical parts used for teaching. The use of 10% formaldehyde for the preservation of chicken breast muscles over a year proved to make them 4.4 to 5.0 times more rigid when compared to unfixed ones, with increased rigidity in the first week of preservation (GUASTALLI et al., 2012). The very rigid muscular texture of anatomical parts can make difficult the teaching of surgical technique since it distances itself from the normal muscular rigidity of a living animal, impairing the training of students. When chicken pectoral muscles are subjected to fixation and preservation at 96° GL alcohol, there is a decrease in softness, making them almost five times more rigid to cut during the first six months and three times more rigid after one year of immersion in the preservative agent, showing better results regarding the use of formaldehyde (NUNES et al., 2011).

Chemically preserved cadavers or by freezing techniques may be effective for teaching veterinary surgery as they present few changes in color and texture of tissues (SILVA et al., 2007). Veterinary medicine students have demonstrated a better acceptance of preserved cadavers for ethical reasons, allowing a reduction of euthanasia of healthy animals due to the use of animals that died naturally in shelters and clinics (MATHEWS et al., 2010).

There are several examples of fixative solutions capable of preserving cadavers for surgical use, such as Thiel, Klotz, Jores (RODRIGUES, 2010), and modified Larssen solutions (SILVA et al., 2004), and all of them contain formaldehyde in their formulation. Studies have tested the efficiency of modified saline solution and formaldehyde in the fixation and preservation of dog cadavers, demonstrating the presence of bacteria of the genera *Enterococcus* sp., *Staphylococcus* sp., *Bacillus* sp., and *Clostridium* sp. in anatomical parts fixed with modified saline solution, in addition to the occurrence of *Pseudomonas* sp. in the use of formaldehyde (JANCZYK et al., 2011). Fungi also multiply in fixed cadavers, being more resistant than bacteria. *Aspergillus* sp. is the most commonly found fungus in indoor environments and contaminated structures, remaining viable through spores (SOLOMON, 1975).

Formaldehyde has been shown a good fixative and preservative of anatomical parts. However, there is a high risk of toxic effects such as eye irritation, alterations in gene expression, and association with several types of cancer due to the constant exposure of professionals to this chemical, in addition to environmental contamination (NTP, 2010).

Based on these factors, this study aimed to analyze microbiologically glycerinated ethyl alcohol and 30% formaldehyde-free sodium chloride solutions for the preparation of dog cadavers for surgical training.

Material and Methods

Thirty-two cadavers of 8 male and 24 female adult dogs, who died due to causes that did not involve evident morphological alterations, such as large tumor masses, extensive lacerating traumas or bone fractures, from the Zoonosis Control Center of Ribeirão Preto, SP, were used after approval by the Municipal Legal Department (process 02.2014/000027-1). The animals were frozen (freezer at −18 °C) shortly after death and transported to the Laboratory of Animal Anatomy of UNESP Jaboticabal, SP.

The selected animals had a body weight between 4.3 and 12.5 kg and a body score from 4 (ribs easily palpable, with minimal fat covering; waist easily noted, viewed from above; abdominal tuck evident) to 5 (ribs palpable without excess fat covering, waist observed behind ribs when viewed from above; abdomen tucked up when viewed from side). A body condition score of 4 to 5 is considered ideal by Laflamme (1997) on a scale of 1 to 9.

The animals were thawed in a horizontal refrigerator at 6 °C, weighed and then divided randomly into four groups (Table 1), fixed, and preserved. After the period of cadaver fixation with ethyl alcohol (EA), which ranged from 30 to 120 days according to the studied group, the preservation phase was carried out with a 30% aqueous sodium chloride solution (ASCS) for 120 days. Sodium chloride was placed in similar plastic boxes (with a 310-liter total capacity), and the same volume (180 liters) was used for each group in the fixation phase.

Table 1. Groups fixed and preserved in ethyl alcohol (EA) and 30% aqueous sodium chloride solution (ASCS), respectively.

Group	Number of animals	Days fixed in EA	Days preserved in 30% SACS
1	8	30	120
2	8	60	120
3	8	90	120
4	8	120	120

Counting of total mesophilic aerobic and anaerobic bacteria

Monthly microbiological analyses were carried out in the tanks used to store the animals in alcohol and later in ASCS. In each analysis, 100 mL of the preservative solution was collected in previously sterilized vials. The quantification of viable mesophilic aerobic and anaerobic facultative bacteria was performed by the pour plate technique. In this technique, the sample was diluted up to 5 times; from each dilution, 1.0 mL was used as inoculum in duplicate and distributed on previously sterilized plates. The nutrient agar medium, molten and cooled to 45 °C in a water bath, was then poured into the Petri dish containing the diluted suspension of the sample. The material was homogenized by rotating the plate in circular, clockwise and counterclockwise movements, repeated for about ten times. After medium solidification, the plates were incubated in chambers under the appropriate temperature and atmosphere.

To this end, plates for counting aerobic microorganisms were stored directly in a bacteriological oven, while plates for counting anaerobic microorganisms were stored in anaerobic jars using Aneroback (Probac), both incubated at 37 °C for 24 h. After this period, the counting of colony-forming units (CFU) was performed (VANDERZANT; SPLITTSTOESSER, 1992).

After counting, five colonies with different phenotypic characteristics were isolated from each plate on BHI (brain heart infusion) agar, which were incubated at 37 °C for 24 hours for later identification of the genera *Bacillus* sp., *Clostridium* sp., *Pseudomonas* sp., and *Streptococcus* sp. and species *Escherichia coli*.

Bacterial identification

Each culture was sown on selective media for the analyzed genera, as follows: MacConkey agar, for evaluation of *Pseudomonas* sp. and *E. coli*; SPS (sulfite-polymyxin-sulfadiazine) agar, for identification of the genus *Clostridium* sp.; MYP (mannitol-yolk-polymyxin) base agar, for isolation of *Bacillus* sp.; and azide blood agar, for identification of *Streptococcus* sp.

After incubation at 37 °C for 24 hours, the cultures were evaluated for cell morphology, Gram classification, presence of spores, and some specific biochemical tests to confirm each genus or species (BARROW; FELTHAM, 1993).

Results and Discussion

The identification and microbiological quantification of the main microorganisms in the alcohol tanks used to fix anatomical parts and in the ASCS tanks used as preservatives are shown in Tables 2 to 5.

Cadavers of various animal species can be preserved in 30% aqueous sodium chloride solution, as it provides good preservation of anatomical parts for five years, demonstrating no contamination of tissues or development of bad odor (OLIVEIRA, 2014). As in veterinary medicine, saturated sodium solutions are efficient preservatives for human cadavers used in surgical training, presenting bactericidal and fungicidal effect for a period of study of fourteen days, which suggests a low risk of infection (HAYASHI et al., 2014).

The success in the use of a 30% sodium chloride solution may be due to the creation of an environment that hinders the survival of microorganisms, requiring a greater osmoregulation capacity (HAYASHI et al., 2016). Several concentrations of fixatives and preservatives for anatomical parts have been evaluated, and the use of saline solution at a concentration below 20% has failed to preserve them for use in tissue dissection (FRIKER et al., 2007). However, in our study, the 30% aqueous sodium chloride solution was effective to preserve whole dog cadavers even with contamination of *Pseudomonas* sp., the most common bacterium in cases of contamination of anatomical parts (SPICHER; PETERS, 1976), in addition to *E. coli*, *Bacillus* sp., *Streptococcus* sp., and *Clostridium* sp.

Table 2. Identification and microbiological quantification of group 1, with animals fixed for 30 days in ethyl alcohol and preserved for 120 days in 30% aqueous sodium chloride solution.

Group 1 – alcoholic solution			
Time (days)	Total aerobes (CFU mL^{-1})	Total Anaerobes (CFU mL^{-1})	Isolate
0	neg.	neg.	
30	neg.	neg.	
Group 1 – saline solution			
Time (days)	Total aerobes (CFU mL^{-1})	Total Anaerobes (CFU mL^{-1})	Isolate
0	neg.	neg.	
30	$< 1.0 \times 10^1$	neg.	
60*	3.0×10^1	4.0×10^1	*E. coli*, *Streptococcus* sp.
90	neg.	2.0×10^1	
120	$< 1.0 \times 10^1$	7.0×10^1	

neg. = negative microbiological growth; * = presence of fungus in the sample.

Table 3. Identification and microbiological quantification of group 2, with animals fixed for 60 days in ethyl alcohol and preserved for 120 days in 30% aqueous sodium chloride solution.

Group 2 – alcoholic solution			
Time (days)	Total aerobes (CFU mL^{-1})	Total Anaerobes (CFU mL^{-1})	Isolate
0	neg.	neg.	
30*	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	*Pseudomonas* sp., *E. coli*, *Bacillus* sp.
60	unc.	unc.	
Group 2 – saline solution			
Time (days)	Total aerobes (CFU mL^{-1})	Total Anaerobes (CFU mL^{-1})	Isolate
0	5.0×10^1	$< 1.0 \times 10^1$	
30*	2.3×10^2	$< 1.0 \times 10^1$	
60	2.0×10^1	2.0×10^1	*E. coli*, *Streptococcus* sp.
90	$< 1.0 \times 10^1$	neg.	
120	4.0×10^1	2.0×10^1	

neg. = negative microbiological growth; * = presence of fungus in the sample; unc. = uncountable.

Table 4. Identification and microbiological quantification of group 3, with animals fixed for 90 days in ethyl alcohol and preserved for 120 days in 30% aqueous sodium chloride solution.

Group 3 – alcoholic solution			
Time (days)	Total aerobes (CFU mL^{-1})	Total Anaerobes (CFU mL^{-1})	Isolate
0	neg.	neg.	*Pseudomonas* sp., *E. coli*, *Bacillus* sp.
30*	3.0×10^1	$< 1.0 \times 10^1$	
60*	6.0×10^1	$< 1.0 \times 10^1$	
90*	6.0×10^1	$< 1.0 \times 10^1$	
Group 3 – saline solution			
Time (days)	Total aerobes (CFU mL^{-1})	Total Anaerobes (CFU mL^{-1})	Isolate
0	6.0×10^1	$< 1.0 \times 10^1$	*E. coli*, *Streptococcus* sp., *Clostridium* sp.
30	$< 1.0 \times 10^1$	1.2×10^3	
60	neg.	3.0×10^1	
90	neg.	neg.	
120	neg.	neg.	

neg. = negative microbiological growth; * = presence of fungus in the sample.

Table 5. Identification and microbiological quantification of group 4, with animals fixed for 120 days in ethyl alcohol and preserved for 120 days in 30% aqueous sodium chloride solution.

Group 4 – alcoholic solution			
Time (days)	Total aerobes (CFU mL^{-1})	Total Anaerobes (CFU mL^{-1})	Isolate
0	neg.	neg.	*Pseudomonas* sp., *E. coli*, *Bacillus* sp.
30	9.0×10^1	7.0×10^1	
60	4.0×10^1	$< 1.0 \times 10^1$	
90	4.0×10^1	1.0×10^2	
120	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	
Group 4 – saline solution			
Time (days)	Total aerobes (CFU mL^{-1})	Total Anaerobes (CFU mL^{-1})	Isolate
0	1.5×10^2	1.6×10^2	*E. coli*, *Streptococcus* sp., *Bacillus* sp.
30*	3.0×10^1	$< 1.0 \times 10^1$	
60*	$< 1.0 \times 10^1$	3.0×10^1	
90	7.0×10^1	7.0×10^1	
120	neg.	$< 1.0 \times 10^1$	

neg. = negative microbiological growth; * = presence of fungus in the sample.

Conclusions

The anatomical technique used by the association of ethyl alcohol and 30% sodium chloride solution was effective in the preservation and microbiological control of dog cadavers used for teaching in anatomy and veterinary surgery.

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