



**Rocío Silvia Sandoval-Monzón¹,
Irma Cecilia Karla Arévalo-Rodríguez¹,
Aldo Alberto Carrillo-Torres¹,
Luis Felipe Ruiz-García^{1,2}**

¹Facultad de Medicina Veterinaria, Universidad Nacional Mayor de San Marcos, Lima; ²Facultad de Ciencias Veterinarias y Biológicas, Universidad Científica del Sur, Lima, Perú

Received: September 28, 2020
 Revised: January 24, 2021
 Accepted: January 25, 2021

Corresponding author:
 Rocío Silvia Sandoval Monzón, DVM
 Facultad de Medicina Veterinaria, Universidad Nacional Mayor de San Marcos, Lima, Perú
 Tel: +51-987036867, Fax: +51-987036867
 E-mail: rocio.sandoval@unmsm.edu.pe

No potential conflict of interest relevant to this article was reported.

Efficacy of physical and chemical treatments on the inactivation of bovine leukosis virus present in milk

Purpose: The objective of the present study was to evaluate the efficacy of pasteurization, freezing, the addition of formaldehyde and peroxymonosulfate on the inactivation of the bovine leukemia virus (BLV) present in milk.

Materials and Methods: A sheep bioassay was carried out in 40 sheep, which were intraperitoneally inoculated with leukocytes from milk infected by the BLV previously treated with one of the virus inactivation methods. Five study groups were evaluated: (1) control group: milk without previous treatment, (2) pasteurization group: milk treated by pasteurization, (3) freezing group: milk treated by freezing for 36 hours, (4) formaldehyde group: 0.1% formaldehyde, and (5) peroxymonosulfate group: 0.05% peroxymonosulfate. The inoculated animals were followed for 10 weeks.

Results: At week 10 post-inoculation, all the animals (8/8) of the control group and the peroxymonosulfate group were seropositive to BLV, while no animals were seropositive (0/8) to BLV in the remaining three groups. Statistically significant differences were found between the pasteurization, freezing and formaldehyde groups with respect to the control ($p < 0.001$) and peroxymonosulfate groups ($p < 0.001$).

Conclusion: The results indicate that pasteurization, freezing and formaldehyde processes are efficient in inactivating the BLV and can be used in milk to prevent the transmission of the virus.

Keywords: Bovine leukosis virus, Inactivation, Treatment, Milk

Introduction

Enzootic bovine leukosis is a disease of great economic and health care importance in dairy farming [1] and has a relevant zoonotic potential [2]. This disease is caused by the bovine leukemia virus (BLV), negatively affects the quantity and quality of milk production [3], and increases reproductive problems, premature culling of cows [4] and cases of clinical mastitis as well as other infectious diseases [3].

Most infected animals are asymptomatic carriers of the disease [5]. BLV infects the lymphocytes and integrates the DNA as a provirus into the genome of cells. Cell-free virus is rarely detected in vivo and only appears during the acute stage of infection [6]. Once infection is established, immune responses effectively suppress the viral replication cycle, and the virus replicates only by mitotic division of provirus-bearing cells [7]. The virus remains indefinitely throughout the life of the animal [8] and results in per-

sistent antibody response [9].

Most BLV transmission occurs through the transfer of provirus-infected lymphocytes, through blood, colostrum, and milk [10]. Feeding with infected milk reduces the virus seronegative calf population early [11]. Therefore, the management of milk and colostrum in the feeding of calves is critical for the implementation of a detection and segregation program, making treatments that allow the elimination of the BLV present in milk and colostrum of infected cows necessary [12,13].

Studies conducted by Baumgartener et al. [14] and Sandoval Monzon et al. [15] demonstrated that pasteurization of milk eliminates BLV. Likewise, it has been reported that freezing of colostrum inactivates the BLV in colostrum [16] and milk [15]. However, the application of chemical treatments could be a simpler alternative to inactivate this milk virus in the field. Chemical products such as formaldehyde have demonstrated viricidal action and peroxymonosulfate has shown to be safe as a viricide [11,17-19].

Formaldehyde at 0.1% has long been used as an inexpensive preservative in calf feeds of colostrum and milk [20-22]. Furthermore, Mbuthia et al. [23] found that the addition of formaldehyde to colostrum maintains immunoglobulin concentrations during 4 weeks of storage. On the other hand, potassium peroxymonosulfate has been shown to have antimicrobial activity against different types of pathogens [19]. Therefore, the aim of the study was to evaluate the efficacy of physical (freezing and pasteurization) and chemical (formaldehyde and peroxymonosulfate) treatments on the inactivation of the BLV provirus present in blood cells present in milk.

Materials and Methods

Ethical considerations

The care and use of animals in this study was approved by the Animal Ethics and Welfare Committee of the Universidad Nacional Mayor de San Marcos (approval no. 2019-5).

Experimental design

To evaluate the efficacy of physical and chemical treatments on the inactivation of BLV provirus present in blood cells in milk, a randomized, experimental study was carried out in which five experimental groups were evaluated: controls, pasteurization, freezing, formaldehyde, and peroxymonosulfate. A sheep bioassay was performed as an alternative means to study BLV transmission in 40 clinically healthy 4-month-old

male sheep [16,17]. Eight repetitions were carried out, in each repetition five sheep were inoculated, one sheep from each experimental group. For each repetition, a milk mixture with infected leukocytes was prepared. The treatment was considered effective when there is no seroconversion against BLV [16,24].

Preparation of milk with infected cells

For each repetition, 5 L of milk mixture with infected leukocytes were prepared from milk from a cow seronegative to BLV and 150 mL of blood from a seropositive cow with persistent lymphocytosis [15,25]. The seropositivity of the cows was identified using the commercial INgezim BLV Compac 2.0 kit (Eurofins, Ingenasa, Spain) and the performance of 2 blood counts with a difference of 72 days [15]. The mixture was distributed in five aliquots, which were destined to each of the experimental groups.

BLV inactivation procedures

The mixture was divided into five aliquots of 1 L each, which were subjected to the following treatments: (1) control: the mixture did not undergo any treatment, (2) pasteurization: physical treatment consisting of heating the mixture to 63°C and maintaining this temperature for 30 minutes [14], (3) freezing: physical treatment in which the mixture was frozen at -23°C for 36 hours [15], (4) formaldehyde: chemical treatment that consisted of the addition of 2.7 g of 37% formaldehyde to the mixture (0.1% formaldehyde) which was left to act at room temperature for 10 minutes, and (5) peroxymonosulfate: chemical treatment that consisted of the addition of 1 g of 50% peroxymonosulfate to the mixture (0.05% peroxymonosulfate) and allowed to act at room temperature for 10 minutes.

Inoculation of the sheep

After the BLV inactivation treatments had been carried out, the treated aliquots were centrifuged at 2,800 g for 15 minutes and the milk fat and the supernatant were removed. The pellet (cell fraction) was diluted in phosphate buffered saline, sufficient for 5 mL. Then, the inoculums were placed in syringes and labeled until the time of inoculation of the animals. It was confirmed that the number of leukocyte cells reached a minimum concentration of 3×10^8 leukocytes in each inoculum [16]. This procedure was carried out in each treatment independently and in each repetition.

Before starting the inoculation, it was verified that the sheep

were seronegative to BLV, using the INgezim BLV Compac 2.0 serological diagnostic kit (Eurofins). The sheep were inoculated intraperitoneally with the inoculum corresponding to each experimental group. The previously sedated sheep (0.3 mg/kg of xylazine) were inoculated intraperitoneally (right paralumbar fossa) with 5 mL of the inoculum, using a 16G×6.35-cm intravenous catheter [15]. Finally, yohimbine was used at a dose of 0.2 mg/kg to reverse the effect of xylazine.

Follow-up

Weekly blood samples were taken over a 10-week follow-up period. The leukocyte and lymphocyte counts were analyzed by a complete blood count. Animals with values higher than 12,000 leukocytes/μL and 9,000 lymphocytes/μL were considered to have leukocytosis and lymphocytosis, respectively. Infection with BLV in sheep was determined using the INgezim BLV Compac 2.0 serological diagnostic kit (Eurofins). A sheep was considered as infected by BLV when the antibody titers increased and remained high during the weeks following inoculation.

Statistical analysis

The statistical packages RStudio ver. 4.0 (RStudio, Boston, MA, USA), Minitab ver. 18.0 (Minitab Ltd., Coventry, UK), and IBM SPSS Statistics ver. 25.0 (IBM Corp., Armonk, NY, USA) were used for the statistical analyses. The weekly frequency of seropositive sheep with leukocytosis and lymphocytosis was calculated, as well as the weekly average of the leukocyte and lymphocyte counts. To compare the weekly frequency of seropositive sheep, the Fisher.multcomp function was used in RStudio, comparing the results obtained within each week. To evaluate the weekly leukocyte and lymphocyte counts, the BoxCox transformation was used to transform the data to the normal distribution, and then a multivariate analysis of variance of repeated measures was used. Bonferroni multiple comparison was performed to determine differences between treatments within each week. All analyses were performed with a significance level of 5%.

Results

Table 1 shows the weekly serological response of sheep inoculated with milk containing cells infected by BLV previously treated with the different inactivation procedures. The animals in the control group began to seroconvert from the first week post inoculation and maintained this condition during

Table 1. Weekly results of the enzyme-linked immunosorbent assay test of the sheep that received intraperitoneal inoculum of milk with cells infected by the bovine leukemia virus previously treated with different inactivation methods

Week	Control	Pasteurization	Freezing	Formaldehyde	Peroxy-monosulfate
0	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}
1	1/8 (12.5) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	1/8 (12.5) ^{a)}
2	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	4/8 (50) ^{a),b)}
3	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	6/8 (75) ^{b)}
4	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	8/8 (100) ^{b)}
5	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	8/8 (100) ^{b)}
6	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	8/8 (100) ^{b)}
7	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	8/8 (100) ^{b)}
8	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	8/8 (100) ^{b)}
9	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	8/8 (100) ^{b)}
10	8/8 (100) ^{b)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	0/8 (0) ^{a)}	8/8 (100) ^{b)}

Values are presented as number of seropositive animals/total number of animals (% of seropositive).

^{a),b)}Different letters indicate statistically significant differences in rows (p=0.05).

the 10-week study period. On the other hand, none of the sheep from the pasteurization, freezing, and formaldehyde treatments seroconverted along the 10 weeks of the study. However, similar to the control group, the peroxymonosulfate treatment group began to seroconvert from the first week post-inoculation. Significant statistical differences were observed among the pasteurization, freezing, and formaldehyde treatments from the second week compared to the control group (p<0.001) and from the third week compared with the peroxymonosulfate group (p=0.01).

Fig. 1 shows the mean leukocyte counts in sheep post-inoculation. At week 1 post-inoculation, there was a significant increase in the leukocyte counts of the control group compared to the formaldehyde group (p=0.04). This increase was maintained during the following week, with statistically significant differences between the control group and the pasteurization (p=0.02) and freezing (p=0.04) groups. On the other hand, the counts in the peroxymonosulfate group increased from week 3, with statistical differences compared to the freezing group (p=0.04). Likewise, the highest leukocyte counts were found in the control and peroxymonosulfate groups, coinciding with the seroconversion of the animals post-inoculation.

Fig. 1 shows that in week 3 there was a significant increase in lymphocyte counts in the peroxymonosulfate group compared to the freezing (p=0.04) and pasteurization groups (p=0.02). Likewise, at week 8, the lymphocyte count of the per-

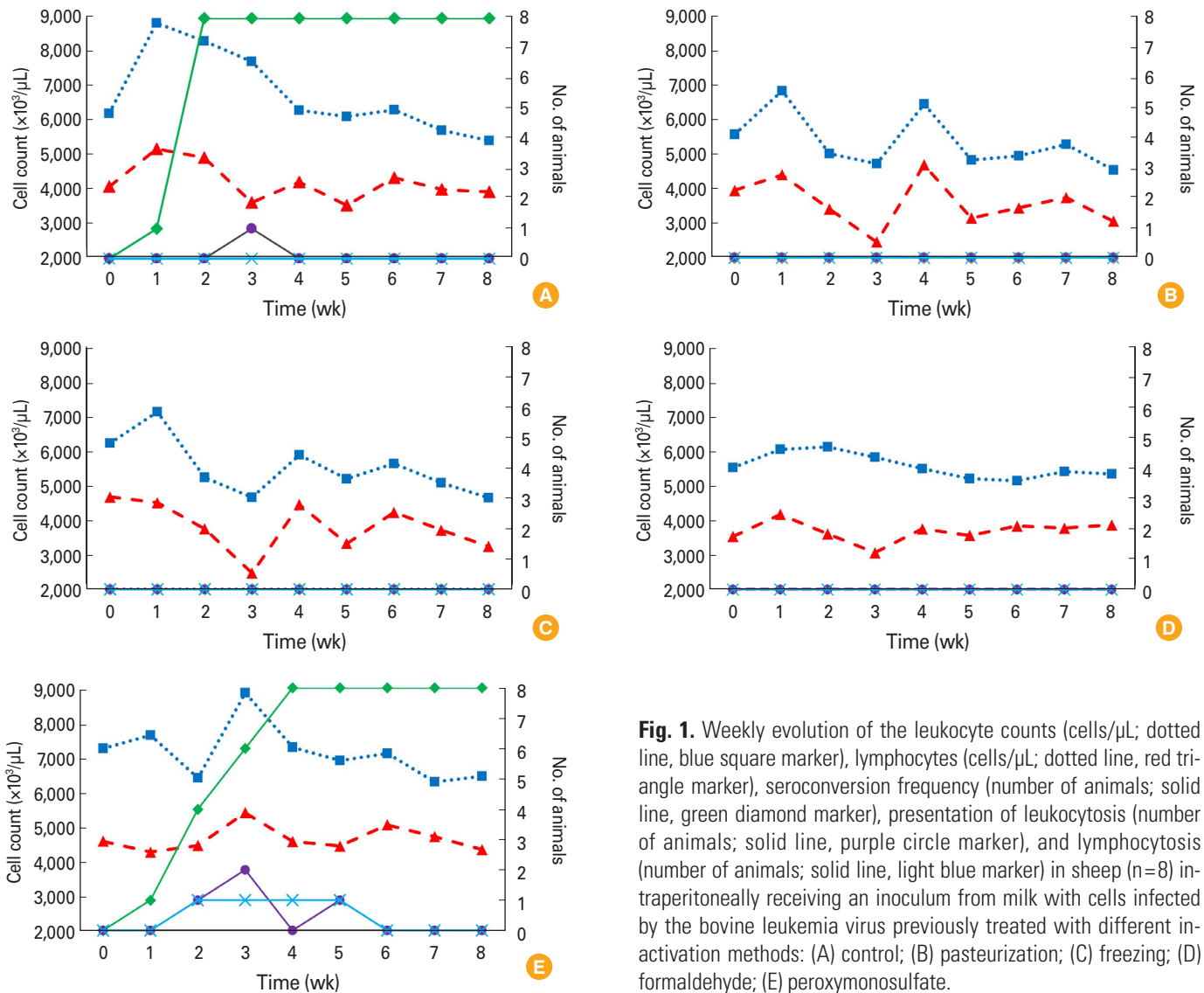


Fig. 1. Weekly evolution of the leukocyte counts (cells/μL; dotted line, blue square marker), lymphocytes (cells/μL; dotted line, red triangle marker), seroconversion frequency (number of animals; solid line, green diamond marker), presentation of leukocytosis (number of animals; solid line, purple circle marker), and lymphocytosis (number of animals; solid line, light blue marker) in sheep (n=8) intraperitoneally receiving an inoculum from milk with cells infected by the bovine leukemia virus previously treated with different inactivation methods: (A) control; (B) pasteurization; (C) freezing; (D) formaldehyde; (E) peroxymonosulfate.

oxymonosulfate group was significantly higher than the pasteurization group ($p=0.03$), with the sheep in the peroxymonosulfate presenting the highest lymphocyte counts.

None of the animals in the pasteurization, freezing or formaldehyde groups presented leukocytosis or lymphocytosis during 8 weeks post-inoculation. In the control group, one animal presented leukocytosis at week 3, and in the peroxymonosulfate group, one animal presented leukocytosis for three weeks, and another animal presented leukocytosis only at week 3. Likewise, it was found that one animal from the peroxymonosulfate group presented lymphocytosis for 4 consecutive weeks. These results coincide with the seroconversion in the peroxymonosulfate and control groups post-inoculation and the absence of seroconversion in the other groups.

Discussion

The results of this study show that the two physical treatments (pasteurization and freezing) and one of the chemical treatments (formaldehyde) inactivated the BLV provirus present in blood cells from milk, with peroxymonosulfate not achieving the same results. The sheep bioassay was also found to be an effective method for the study of BLV infectivity [26], since 100% of the sheep in the control group seroconverted in the second week after inoculation [7] and the antibodies remained present throughout the study [9], verifying the replication of the virus in the organism of inoculated animals [26].

The efficiency of pasteurization depends on critical variables such as temperature, time, and product composition [27]. Heat thermally destabilizes intermolecular interactions

between capsid proteins and the integrity of the free virus envelope. This thermal destabilization produces the loss of infectivity of the viruses [28], although Reichert et al. [29] found that pasteurization does not denature BLV provirus DNA. However, in this case, BLV was present in a proviral form within lymphocytic cells [6]. In the present study, the effect of pasteurization on BLV infectivity, which involves various cellular mechanisms, was evaluated.

Several studies have shown that pasteurization reduces the viability of cells in milk by more than 90% [30], preventing the provirus from replicating and infecting other cells. Other recent investigations have demonstrated the inactivation of BLV by pasteurization [15,31] and the inactivation of a wide range of enveloped and non-enveloped viruses [27]. While pasteurization is an effective method, unfortunately, some studies have shown that it destroys some nutritional and immune components [32].

Freezing efficiently inactivates BLV. Our results coincide with those in human studies that report that the process of freezing breast milk prevents the transmission of certain viruses, such as cytomegalovirus and human T-cell lymphotropic virus type I [33,34]. Likewise, it has been reported that freezing inactivates BLV [15,16]. Although it has been found that the freezing process does not significantly alter proviral DNA [29], it does produce intracellular ice formation, which causes the loss of membrane potential when the ice crystals break the membrane preventing the cell from surviving the process [35].

In relation to chemical treatments, formaldehyde was found to inactivate the BLV provirus, preventing the infection of inoculated sheep. Formaldehyde is a commonly used inactivator for killed vaccines [36]. There is scientific evidence showing that 0.02% formaldehyde destroys almost 50% of the glycoproteins of BLV, demonstrating its deleterious effect on the viral antigen [17].

The results of this study demonstrate the effectiveness of formaldehyde in inactivating the provirus present in blood cells. BLV inactivation may be due to the effects exerted by formaldehyde on cells that contain it. Formaldehyde has been found to have a cytostatic effect [36]. Chemical studies indicate that formaldehyde is a reactive electrophilic species that readily reacts with various functional groups of biological macromolecules such as proteins, glycoproteins, nucleic acids, and polysaccharides in a cross-linking manner [37]. Likewise, formaldehyde has been found to act on the amino and sulfate groups of proteins and the nitrogen atoms of the

purine base ring [38].

On the other hand, the results obtained with peroxymonosulfate show that, at the dose used, the treatment was not effective in inactivating the BLV provirus. Peroxymonosulfate has viricidal action and has been shown to be more effective than other compounds [18,19]. The ineffectiveness of peroxymonosulfate to inactivate the BLV provirus could be due to its viricidal action on the capsid proteins of free viruses [19,39]. Although peroxymonosulfate was ineffective in this study, it could be effective at doses higher than those used in this study.

Implementing pasteurization, freezing, or formaldehyde treatments in milk and colostrum supplied to calves can reduce the rate of transmission in neonates, which has been determined to occur in approximately 6% to 16% of animals [40]. Both pasteurization, freezing and formaldehyde are good alternatives to prevent the transmission of BLV. However, pasteurization and freezing require having the necessary infrastructure and equipment to be carried out; therefore, treatment with formaldehyde could be an option in places in which the necessary infrastructure is not available to implement the other two methods.

In conclusion, the physical treatments of pasteurization and freezing inactivated the BLV provirus present in blood cells found in milk. Of the chemical treatments only formaldehyde effectively inactivated BLV, while peroxymonosulfate did not achieve the same results.

ORCID

Rocío Silvia Sandoval-Monzón

<https://orcid.org/0000-0002-6249-9076>

Irma Cecilia Karla Arévalo-Rodríguez

<https://orcid.org/0000-0002-2435-2866>

Aldo Alberto Carrillo-Torres

<https://orcid.org/0000-0002-4543-9039>

Luis Felipe Ruiz-García

<https://orcid.org/0000-0003-1756-768X>

References

1. Nekouei O, VanLeeuwen J, Sanchez J, Kelton D, Tiwari A, Keefe G. Herd-level risk factors for infection with bovine leukemia virus in Canadian dairy herds. *Prev Vet Med* 2015;119:105-13.
2. Buehring GC, DeLaney A, Shen H, et al. Bovine leukemia virus discovered in human blood. *BMC Infect Dis* 2019;19:

- 297.
3. Yang Y, Fan W, Mao Y, et al. Bovine leukemia virus infection in cattle of China: association with reduced milk production and increased somatic cell score. *J Dairy Sci* 2016; 99:3688-97.
 4. Romero JJ, Davila G, Beita G, Dolz G. Relationship between serological status to enzootic bovine leukosis and reproductive parameters in specialized dairy herds in Costa Rica. *Agron Costa Rican* 2015;39:7-18.
 5. Juliarena MA, Gutierrez SE, Ceriani C. Determination of proviral load in bovine leukemia virus-infected cattle with and without lymphocytosis. *Am J Vet Res* 2007;68:1220-5.
 6. Evermann JF, DiGiacomo RF, Hopkins SG. Bovine leukosis virus: understanding viral transmission and the methods of control. *Vet Med* 1987;82:1051-8.
 7. Gutierrez SE, Forletti A. Bovine leukemia virus. In: Liu D, editor. *Molecular detection of animal viral pathogens*. Boca Raton, FL: CRC Press; 2016. p.157-66.
 8. Florins A, Gillet N, Asquith B, et al. Cell dynamics and immune response to BLV infection: a unifying model. *Front Biosci* 2007;12:1520-31.
 9. World Organization for Animal Health (OIE). Enzootic bovine leukosis. In: World Organization for Animal Health (OIE), editor. *Manual of diagnostic tests and vaccines for terrestrial animals 2019*. Paris: World Health Organization for Animal Health (OIE); 2019. p.1113-24.
 10. Miller JM, Van der Maaten MJ. Infectivity tests of secretions and excretions from cattle infected with bovine leukemia virus. *J Natl Cancer Inst* 1979;62:425-8.
 11. Juliarena MA, Barrios CN, Lutzelschwab CM, Esteban EN, Gutierrez SE. Bovine leukemia virus: current perspectives. *Virus Adapt Treat* 2017;9:13-26.
 12. Foley JA, Otterby DE. Availability, storage, treatment, composition, and feeding value of surplus colostrum: a review. *J Dairy Sci* 1978;61:1033-60.
 13. McGuirk SM, Collins M. Managing the production, storage, and delivery of colostrum. *Vet Clin North Am Food Anim Pract* 2004;20:593-603.
 14. Baumgartener L, Olson C, Onuma M. Effect of pasteurization and heat treatment on bovine leukemia virus. *J Am Vet Med Assoc* 1976;169:1189-91.
 15. Sandoval Monzon RS, Arevalo Rodriguez I, Carrillo Torres A, Ruiz Garcia LF. Efficacy of pasteurization and freezing on the inactivation of bovine leukosis virus present in milk. *Rev Inv Vet Peru* 2020;31:e16849.
 16. Kanno T, Ishihara R, Hatama S, et al. Effect of freezing treatment on colostrum to prevent the transmission of bovine leukemia virus. *J Vet Med Sci* 2014;76:255-7.
 17. Miller JM, Van Der Maaten MJ. Evaluation of an inactivated bovine leukemia virus preparation as an immunogen in cattle. *Ann Rech Vet* 1978;9:871-7.
 18. Eleraky NZ, Potgieter LN, Kennedy MA. Virucidal efficacy of four new disinfectants. *J Am Anim Hosp Assoc* 2002;38: 231-4.
 19. Hashizume M, Aoki K, Ohno S, et al. Disinfectant potential in inactivation of epidemic keratoconjunctivitis-related adenoviruses by potassium peroxymonosulfate. *Eur J Ophthalmol* 2019 Dec 9 [Epub]. <https://doi.org/10.1177/1120672119891408>.
 20. Kaiser AG. The use of colostrum preserved with formalin for rearing calves. *Aust J Exp Agric Anim Husb* 1977;17: 221-3.
 21. Karioki DI, Gitau GK, Munyua SJ. The use of preserved colostrum for rearing replacement dairy calves: calf performance, economics and on-farm practicability in Kenya. *Onderstepoort J Vet Res* 1995;62:167-70.
 22. Levine BS. Effect of formaldehyde on the direct microscopic count of raw milk. *Public Health Rep* 1950;65:931-8.
 23. Mbuthia EW, Klobasa F, Gachui CK, Abate A. Effect of treatment with formaldehyde and formic acid on immunoglobulin content of stored bovine colostrum. *Anim Feed Sci Technol* 1997;67:291-8.
 24. Ferrer JF, Kenyon SJ, Gupta P. Milk of dairy cows frequently contains a leukemogenic virus. *Science* 1981;213:1014-6.
 25. Roberts DH, Lucas MH, Wibberley G. Effect of heat on bovine leukosis virus-infected lymphocytes. *Br Vet J* 1983;139: 291-5.
 26. Kenyon SJ, Ferrer JF, McFeely RA, Graves DC. Induction of lymphosarcoma in sheep by bovine leukemia virus. *J Natl Cancer Inst* 1981;67:1157-63.
 27. Groner A, Broumis C, Fang R, et al. Effective inactivation of a wide range of viruses by pasteurization. *Transfusion* 2018;58:41-51.
 28. Boschetti N, Niederhauser I, Kempf C, Stuhler A, Lower J, Blumel J. Different susceptibility of B19 virus and mice minute virus to low pH treatment. *Transfusion* 2004;44: 1079-86.
 29. Reichert M, Grundboeck-Jusko J, Rulka J, Stec J, Kozaczynski W. Influence of selected technological treatments on the BLV provirus DNA occurring in the tissues of leukaemic cattle. *Bull Vet Inst Pulawy* 1994;38:52-60.
 30. Li N, Richoux R, Perruchot MH, Boutinaud M, Mayol JF,

- Gagnaire V. Flow cytometry approach to quantify the viability of milk somatic cell counts after various physico-chemical treatments. *PLoS One* 2015;10:e0146071.
31. Godden S, McMartin S, Feirtag J, et al. Heat-treatment of bovine colostrum. II: effects of heating duration on pathogen viability and immunoglobulin G. *J Dairy Sci* 2006;89:3476-83.
32. Godden SM, Smith S, Feirtag JM, Green LR, Wells SJ, Fetrow JP. Effect of on-farm commercial batch pasteurization of colostrum on colostrum and serum immunoglobulin concentrations in dairy calves. *J Dairy Sci* 2003;86:1503-12.
33. Maschmann J, Hamprecht K, Weissbrich B, Dietz K, Jahn G, Speer CP. Freeze-thawing of breast milk does not prevent cytomegalovirus transmission to a preterm infant. *Arch Dis Child Fetal Neonatal Ed* 2006;91:F288-90.
34. Ando Y, Ekuni Y, Matsumoto Y, et al. Long-term serological outcome of infants who received frozen-thawed milk from human T-lymphotropic virus type-I positive mothers. *J Obstet Gynaecol Res* 2004;30:436-8.
35. Shi Q, Xie Y, Wang Y, Li S. Vitrification versus slow freezing for human ovarian tissue cryopreservation: a systematic review and meta-analysis. *Sci Rep* 2017;7:8538.
36. Feldman MY. Reactions of nucleic acids and nucleoproteins with formaldehyde. *Prog Nucleic Acid Res Mol Biol* 1973;13:1-49.
37. Fox CH, Johnson FB, Whiting J, Roller PP. Formaldehyde fixation. *J Histochem Cytochem* 1985;33:845-53.
38. Favero MS, Bond WW. Chemical disinfection of medical and surgical materials. In: Block SS, editor. *Disinfection, sterilization, and preservation*. 4th ed. Philadelphia, PA: Lea & Febiger; 1991. p.617-41.
39. Kizu J, Takagi K, Kuroda Y, Maezawa K, Matsumoto K, Hori S. Stability and color degradation in solutions of complex-type chlorinebased disinfectant cleaner. *Jpn J Infect Prev Control* 2014;29:411-6.
40. Hopkins SG, DiGiacomo RF. Natural transmission of bovine leukemia virus in dairy and beef cattle. *Vet Clin North Am Food Anim Pract* 1997;13:107-28.