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SCIENTIFIC COMMUNICATION

# NEUTRALIZING ANTIBODY RESPONSE IN CATTLE ADMINISTERED EITHER REFRIGERATED OR FROZEN INACTIVATED RABIES VACCINES

**A. Albas[1], O.L. Fontolan, P.E. Pardo, F. Bernardi, L.M. Alves, A.A.B. Carvalho, P.S. Pozzetti, F.H. Ito**

[1]Instituto Biológico - Núcleo Regional de Presidente Prudente, CP 298, CEP 19100-000, Presidente Prudente, SP, Brasil.

ABSTRACT

For the storage and shipment of inactivated rabies vaccines, the recommended temperature is 2º to 8ºC (36º to 46ºF) . Forty-eight bovines were vaccinated with commercial rabies vaccines that had been stored either refrigerated or frozen. Neutralizing antibody (SN) titers, prior to vaccination at day 0 were < 5. Thirty days later, titers of animals vaccinated with vaccine-1, either refrigerated or frozen, ranged 11-439. At day 180, with vaccine-1, refrigerated, the maximum titer had decreased to 11, and to 8 at day 360. At day 30, animals vaccinated with frozen vaccine-1 presented titers ranging from 6-279; and at day 180 and 360, titers had decreased markedly. With refrigerated or frozen vaccine-2, at day 30, varying degrees of titers > 5 were detected, and two animals showed no response. With vaccine-2, refrigerated, three sera elicited titers at day 180, and at day 360, only one was reactant. With vaccine-2, frozen, at day 180 and 360, titers were < 5. The Anova test indicated that responses to either refrigerated or frozen vaccines were serologically similar, however, by the Tukey-Kramer test, significant results were found for the day 180, between vaccine-1, frozen X vaccine-2, frozen, and vaccine-1, refrigerated X vaccine-2, frozen ($p < 0.01$). The frozen avridine-adjuvanted vaccine-1 enabled detectable levels of antibody for a longer period than the other vaccines.

KEY WORDS: Rabies vaccines, bovines, storage temperature, refrigeration, freezing, neutralizing antibody.

RESUMO

FORMAÇÃO DE ANTICORPOS NEUTRALIZANTES EM BOVINOS VACINADOS COM VACINAS ANTI-RÁBICAS INATIVADAS ARMAZENADAS EM REFRIGERAÇÃO OU EM CONGELAMENTO. A temperatura recomendada para armazenamento e transporte de vacinas anti-rábicas inativadas é de 2º a 8ºC (36º a 46ºF). Quarenta e oito bovinos foram vacinados com vacinas anti-rábicas comerciais que tinham sido armazenadas em temperaturas de refrigeração ou congelamento. Os títulos de anticorpos neutralizantes (SN), antes da vacinação, no dia 0, foram todos < 5. Trinta dias após, animais vacinados com vacina-1, refrigerada ou congelada, apresentaram títulos variando entre 11-439. Aos 180 dias, com a vacina-1, refrigerada, os títulos máximos diminuiram para 11 e, para 8 aos 360 dias. Com vacina-1, congelada, títulos encontrados aos 30 dias situavam-se entre 6-279 e, aos 180 e 360 dias, os títulos diminuíram substancialmente. Animais vacinados com vacina-2, refrigerada ou congelada, aos 30 dias, apresentaram títulos > 5, exceto dois animais que não apresentaram respostas. Com vacina-2, refrigerada, três animais apresentaram títulos aos 180 dias, e aos 360 dias, somente um foi reagente. Com vacina-2, congelada, aos 180 e 360 dias, os títulos foram < 5. Pelo teste de ANOVA os resultados sorológicos proporcionados pelas vacinas refrigeradas ou congeladas foram semelhantes e o teste de Tuckey-Kramer apresentou resultados significantes ($p < 0,01$) para os dados de 180 dias, entre vacina-1, congelada X vacina-2, congelada e vacina-1, refrigerada X vacina-2, congelada. A vacina-1, congelada, contendo avridine proporcionou níveis de anticorpos detectáveis por um período mais prolongado.

PALAVRAS-CHAVE: Vacinas anti-rábicas, bovinos, temperatura de armazenamento, refrigeração, congelamento, anticorpos neutralizantes.

Vaccine production is a biological process, using living organisms or their products as raw materials and accidents with vaccine will occur during its shipment and handling, and mishaps with vaccines can result in the loss of thousands of dollars. Individuals who will be handling and administering vaccines should be informed about the specific storage requirements and stability limitations of the vaccines they use (YUAN et al.,1995).

Depending upon the vaccine, the storage requirements currently recommended by the manufacturers should be the refrigerating temperature or the so-called cold chain kept between 2º to 8ºC (36º to 46ºF) (VON HEDENSTROM & KAHLER, 1992) or the freezing temperature at -15ºC (+5ºF) or even colder (WATSON et al., 1993).

Live or attenuated vaccines are fragile because they contain live organisms and lyophilized forms should be maintained refrigerated, but they may be frozen (BURKE et al., 1999). The rinderpest virus (Kabete "O") vaccine was stored in liquid nitrogen temperature without showing any deleterius effect on the titer and its antigenicity (PADMARAJ et al.,1994).

Attenuated rabies vaccines used in routine immunization of cattle in Brazil currently include ERA and SAD strains. In Europe and in North America, attenuated strains have been tested extensively as oral vaccines in order to control rabies in wildlife. These live and recombinant vaccines resisted to several temperatures above 8ºC or higher, and stability could be maintained even after storage at freezing temperature (PASTORET et al., 1996).

Unlike attenuated vaccines, inactivated vaccines do not contain live organisms but they do contain chemical adjuvants and are not as fragile unless exposed to freezing temperatures. The inactivated rabies vaccines commercially available in Brazil nowadays are of cell-culture origin and contain chemical adjuvants like aluminum hydroxide and inactivated by binary ethylene imine (BEI). Others contain avridine, a lipoidal amine and stimulant of interferon production (NIBLACK et al., 1979). In Brazil, the vaccine manufacturers, both of live and inactivated ones, all recommend refrigerating temperatures between 2º to 8º C for their storage.

Some works refered to the relatively high stability of inactivated rabies vaccines when submitted to temperatures above 2º to 8ºC (ALBAS et al., 1992; DIAZ et al., 1988), and lyophilized human diploid-cell-strain vaccine retained its antigenicity for humans despite continuous exposure to high ambient temperature for up to 11 weeks (NICHOLSON et al., 1983).

Shipment temperature is a very important matter, vaccines should not touch the ice packs and all inactivated vaccines should be refrigerated immediately upon receiving shipment (KENDAL & GARRISON, 1997). Low storage temperatures adversely affected tetanus toxoid (TT) potency, and only one study was found on the impact on TT immunogenicity of freezing, although other studies evaluated the impact of freezing on the tetanus component of diphteria, tetanus, and Pertussis (DTP) or diphteria, and tetanus (DT), and freezing DTP at -20ºC did not reduce its potency (DIETZ et al., 1997). Vaccines for human use such as the DTP, DTaP, IPV, hepatitis A, hepatitis B, influenza, rabies, etc, are inactivated vaccines and should not be frozen at arrival condition, and freezing is not recommended for storage. Freezing destroys potency and storage outside this temperature of 2º to 8ºC may reduce potency (US Department of Health and Human Services, 1991).

Partial freezing during the production of the Fuenzalida and Palacios vaccine for human use was linked to a decrease in vaccine potency due to the appearance of fibrous particles of a proteic nature, so the recommendation is to avoid freezing the brain suspension in distilled water, keeping the temperature between 3º to 5ºC (AMASINO et al., 1986).

Most of the instructions of vaccines suppliers that accompany the shipment recommend the refrigerating temperature of 2º to 8ºC (36º to 46ºF), however, for attenuated vaccines currently available for human use, like the oral polio vaccine (OPV) and varicella vaccine, the recommended temperature for the storage is -15ºC or colder. Varicella vaccine is less stable to heat than other vaccines, thus it must be stored at -15ºC (5ºF) or less, and potency of this vaccine begins to decrease soon after being exposed to temperatures above -15ºC (WATSON et al., 1993). For the attenuated ERA and SAD vaccines commercially available in lyophilized form in the Brazilian market, the recommended storage temperature is 2º to 8ºC, but they could be maintained frozen.

A CNN news report from Internet mentioned in March 1998 that a shipment of 200,000 doses of anthrax vaccine destined for U.S. troops in the Persian Gulf was stopped because it apparently had frozen during shipment, destroying its effectiveness. The vaccines were shipped from Mechanicsburg, Pennsylvania, to Germany en route to the Middle East, and at least 20,000 vials had suffered a radical temperature change, apparently freezing and rendering the doses useless (CNN, 1998). In the hot Australian climate, freezing is the greatest threat to vaccine potency (GUTHRIDGE & MILLER, 1996) and a similar situation may occur frequently in Brazil, especially when considering shipment of vaccines of veterinary use to distant areas. And we do not know if any of the frozen vaccines could be salvaged.

The aim of this paper is to investigate the effect of freezing temperature on the potency of inactivated rabies vaccines, because information on the subject is

very scarce in the literature Many of the animal rabies vaccine manufacturers consulted said that the chemical adjuvants and additives used are the main limitation for the freezing of the inactivated vaccines.

**Vaccines:** Commercial rabies vaccines used were from two manufacturers, vaccine-1* was constituted of a PV virus, BHK-21 clone 13 replicated and binary ethylene imine (BEI) inactivated and adjuvanted with avridine, batch No. 003/97, Habel test = 4.3, adsorbed in aluminum-gel-hydroxide and recommended through intramuscular inoculation at a dose of 2.0 ml and shelf-life of two years; vaccine-2** was also a PV virus, BHK-21 clone 13 replicated and BEI-inactivated vaccine*, batch No. 002/97, Habel test = 4.51, and adsorbed in aluminum-gel-hydroxide, recommended intramuscular dose of 2.0 mL, and shelf-life of 12 months.

**Animals**: In this experiment, 48 male and female weaned nelore crossbreds, 4 to 12 months old in age and without having any records of previous vaccination were vaccinated and had been maintained at Fazenda São Manoel, in Emilianópolis, SP, until the end of the experiment.

**Mouse serum neutralization (SN) test:** The test used was according to FITZGERALD (1996), adopting the 5-fold serial dilution, using the CVS strain provided by the Laboratório de Referência Animal (LARA)-Campinas, SP, and the lethal doses used in the test were in a range of 10 to 100 $MICLD_{50}$ / 0.03 mL. Titers were calculated according to the method of REED & MÜENCH (1938) and expressed as the reciprocals of the serum dilutions and titers < 5 were considered as zero (ALBAS et al., 1998).

**Procedures**: The 48 animals were separated randomly into 4 groups of 12 animals each; animals numbered from 1 to 12 received intramuscularly, on the neck muscle, a 2.0 mL single dose of vaccine-1 that had been stored at refrigerating temperature of 2º to 8ºC; animals numbered from 13 to 24 received a 2.0 mL single dose of vaccine-1 stored overnight at a frozen temperature of -15ºC; and animals numbered from 25 to 36, and from 37 to 48 received similar treatments through intramuscular inoculation, using a single dose of 2.0 mL of vaccine-2 that was stored either refrigerated or frozen. Both vaccines were transported to the ranch in each separated icebox containing ice packs, in order to guarantee a temperature of 2º to 8ºC, and the frozen vaccines were quickly thawed with tap water and rolling the vials between the palms, and once defrosted, the vials were placed at 2º to 8ºC until use. All animals were bled shortly before the administration of the single dose of vaccines, and the subsequent bleedings were made at 30, 180, and 360 post vaccination days. Serum samples were stored frozen until use.

For statistical analysis, individual titers were submitted to logarithmic transformation, using $\log_{10}(SN + 1)$ and then analyzed by ANOVA test and SN values corresponding to 30, 180 and 360 days were analyzed using the linear regression test, adopting an $\propto = 0.05$ (ZAR, 1984). Statistical calculations were made by means of a computerized software GraphPad InStat*** tm v2.01, and by the Tukey-Kramer multiple comparisons test, when $q > 4.574$ then the corresponding P value is less than 0.05.

The results of SN test of the animals that received a single dose of rabies vaccine-1, either refrigerated or frozen, are presented in Table 1. Shortly before vaccination, i.e., at day 0, all sera tested were found without any detectable titer of rabies neutralizing antibodies or titers were < 5. At day 30, all the animals inoculated with refrigerated vaccine-1 presented titers > 5, the minimum titer was 11, and the maximum titer reached 439, with the SN geometric mean titer of 2.0615 (or antilog = 115.2126). At 180 days and 360 days, the maximun SN titers had decreased respectively to ≤ 11 and ≤ 8. Similarly, at day 30, animals that received frozen vaccine-1 were found with titers > 5, the minimum was 6 and the maximum, 279, with the SN geometric mean titer of 1.6529 (or antilog = 44.9726). At 180 and 360 days, the highest titers had decreased to ≤ 55 and ≤ 25, respectively.

The SN antibody titers found in the sera of animals that received either refrigerated or frozen vaccine-2 are illustrated in Table 2. At day 0, all titers were < 5, and at 30 days, all the sera from animals vaccinated with refrigerated vaccine-2 were found with titers ranging from 11 to 228, except the animal number 32, which showed no response to vaccination. Subsequently, titers decreased to < 25 at 180 days, and at 360 days, only one serum presented a titer = 9. With the frozen vaccine-2, at day 30, all sera were found with titers > 5, with the exception of the serum from animal number 44 that showed titer = 0. At 180 and 360 days, titers found were all < 5.

The data of SN titers, when analyzed by the ANOVA test indicated significant variations among columns means ($p < 0.0001$), however, for the data corresponding to day 30, statistical differences were not found by means of the Tukey-Kramer multiple comparisons test, for the following analyses: vaccine-1, refrigerated X vaccine-1, frozen ($q = 2.587$, $p > 0.05$);

* RABIVAC, Pfizer Ltda, Guarulhos, SP.
** BGS-CELL, Hertape S.A., Belo Horizonte, MG.
*** GraphPad Software v2.01, 10855 San Diego,CA 92121 USA.

vaccine-1, refrigerated X vaccine-2, refrigerated (q = 4.045, p > 0,05); vaccine-1, refrigerated X vaccine-2, frozen (q = 4.411, p > 0.05); vaccine-1, frozen X vaccine-2, refrigerated (q = 1.458, p > 0.05); vaccine-1, frozen X vaccine-2, frozen (q = 1.824, p > 0.05). For the data corresponding to day 180, significant results were found for the comparisons between vaccine-1, frozen X vaccine-2, frozen (q = 5.908, p < 0.01) and vaccine-1, refrigerated X vaccine-2, frozen (q = 6.279, p < 0.01). For the SN data of the day 360, no comparison was significant.

Regression analysis indicated negative linear relationships between the bleeding days and $\log_{10}$SN titers for the two vaccines, either refrigerated or frozen, with p < 0.0001, with the following equations: vaccine-1, refrigerated: Y = 2.066 - 0.005488x; vaccine-2, refrigerated: Y = 1.351 - 0.003959x; vaccine-1, frozen:

Table 1 - Results of mouse serum neutralization (SN) test performed on sera of bovines vaccinated with a single dose of rabies vaccine-1, stored at either refrigerating or freezing temperature.

| Animal number | Approx.Age (months) | SN titer*, Vaccine-1 (refrigerated, at 2º to 8ºC) | | | | Animal number | Approx.Age (months) | SN titer, Vaccine-1 (frozen, at -15ºC) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 day | 30 days | 180 days | 360 days | | | 0 day | 30 days | 180 days | 360 days |
| 1 | 10 | 0 | 279 | 11 | 0 | 13 | 4 | 0 | 163 | 45 | 25 |
| 2 | 11 | 0 | 268 | 11 | 8 | 14 | 8 | 0 | 228 | 11 | 11 |
| 3 | 7 | 0 | 279 | 11 | 0 | 15 | 12 | 0 | 19 | 0 | 0 |
| 4 | 8 | 0 | 34 | 0 | 0 | 16 | 11 | 0 | 13 | 6 | 5 |
| 5 | 10 | 0 | 41 | 0 | 0 | 17 | 11 | 0 | 55 | 55 | 0 |
| 6 | 12 | 0 | 439 | 11 | 8 | 18 | 10 | 0 | 279 | 0 | 0 |
| 7 | 12 | 0 | 152 | 11 | 0 | 19 | 7 | 0 | 163 | 45 | 25 |
| 8 | 7 | 0 | 279 | 11 | 0 | 20 | 6 | 0 | 6 | 0 | 0 |
| 9 | 9 | 0 | 365 | 11 | 5 | 21 | 4 | 0 | 11 | 0 | 0 |
| 10 | 9 | 0 | 11 | 0 | 0 | 22 | 9 | 0 | 95 | 5 | 0 |
| 11 | 11 | 0 | 55 | 5 | 0 | 23 | 10 | 0 | 11 | 0 | 0 |
| 12 | 5 | 0 | 37 | 11 | 0 | 24 | 12 | 0 | 25 | 11 | 0 |
| GMT | | 0 | 115.2126 | 6.0856 | 1.6745 | GMT | | 0 | 44.9726 | 5.4594 | 2.4581 |
| Mean age** 9.25 | | | | | | Mean age** 8.60 | | | | | |

*Reciprocal of the serum dilution, SN titer < 5 was considered as 0.
GMT = Geometric mean titer
** Arithmetical mean

Table 2 - Results of mouse serum neutralization (SN) test performed on sera of bovines vaccinated with a single dose of rabies vaccine-2, stored at either refrigerating or freezing temperature.

| Animal number | Approx. Age | SN titer*, Vaccine-2 (refrigerated, at 2º to 8ºC) | | | | Animal number | Approx. Age | SN titer, Vaccine-2 (frozen, at -15ºC) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 day | 30 days | 180 days | 360 days | | | 0 day | 30 days | 180 days | 360 days |
| 25 | 9 | 0 | 228 | 0 | 0 | 37 | 12 | 0 | 37 | 0 | 0 |
| 26 | 7 | 0 | 95 | 0 | 0 | 38 | 9 | 0 | 279 | 0 | 0 |
| 27 | 12 | 0 | 32 | 5 | 0 | 39 | 9 | 0 | 11 | 0 | 0 |
| 28 | 12 | 0 | 8 | 0 | 0 | 40 | 4 | 0 | 32 | 0 | 0 |
| 29 | 9 | 0 | 68 | 25 | 0 | 41 | 5 | 0 | 55 | 0 | 0 |
| 30 | 4 | 0 | 58 | 19 | 9 | 42 | 8 | 0 | 68 | 0 | 0 |
| 31 | 6 | 0 | 45 | 0 | 0 | 43 | 11 | 0 | 74 | 0 | 0 |
| 32 | 7 | 0 | 0 | 0 | 0 | 44 | 10 | 0 | 0 | 0 | 0 |
| 33 | 5 | 0 | 19 | 0 | 0 | 45 | 10 | 0 | 9 | 0 | 0 |
| 34 | 6 | 0 | 19 | 0 | 0 | 46 | 10 | 0 | 13 | 0 | 0 |
| 35 | 11 | 0 | 11 | 0 | 0 | 47 | 7 | 0 | 14 | 0 | 0 |
| 36 | 7 | 0 | 19 | 0 | 0 | 48 | 11 | 0 | 8 | 0 | 0 |
| GMT | | 0 | 26.4525 | 1.9551 | 1.2115 | GMT | | 0 | 23.0993 | 0 | 0 |
| Mean age** 7.90 | | | | | | Mean age** 8.80 | | | | | |

*Reciprocal of the serum dilution, SN titer < 5 was considered as 0.
GMT = Geometric mean titer
** Arithmetical mean age (months)

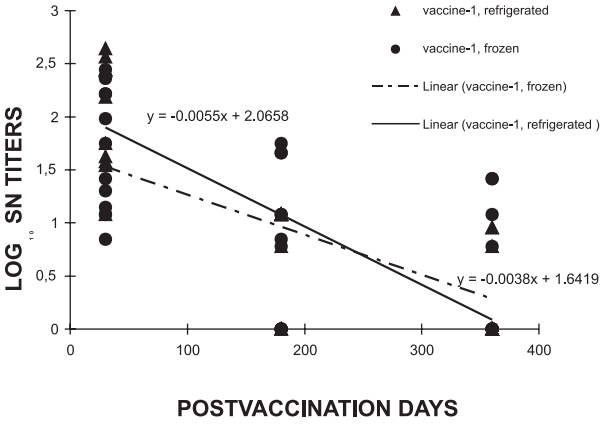


Fig. 1 – Simple linear regression between the results of mouse serum neutralization (SN) test performed on sera of bovines vaccinated with a single dose of rabies vaccine-1, stored at either refrigerating or freezing temperature and postvaccination days.

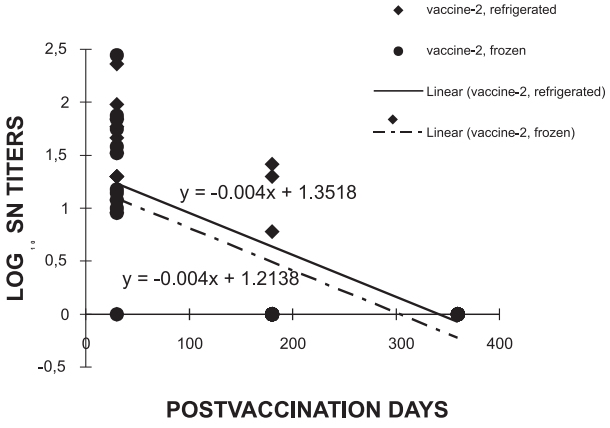


Fig. 2 – Simple linear regression between the results of mouse serum neutralization (SN) test performed on sera of bovines vaccinated with a single dose of rabies vaccine-2, stored at either refrigerating or freezing temperature and postvaccination days.

Y = 1.642 - 0.003762x; vaccine-2, frozen: Y = 1.213 - 0.003993x (Fig. 1 and Fig. 2).

In this study, the ANOVA test indicated no differences in the SN titers found in sera of animals that were injected either refrigerated or frozen rabies vaccines and bled at day 30. The vaccinated animals responded with measurable amounts of SN titers 30 days after vaccination, but a majority of them were found with low titers at 180 and 360 postvaccination days. In this aspect, all sera of animals vaccinated with vaccine-2 that had been frozen were found with titers < 5 at 180 days, this could be interpreted that freezing exerted a negative effect on the vaccine potency. However, similar interpretation cannot be made with the results of SN titers corresponding to animals vaccinated with the frozen vaccine-1 at day 180, because no statistical difference was found in SN titers in the sera of animals vaccinated with either refrigerated vaccine-1 or vaccine-2.

By the results of linear regression analysis, the prediction for $\hat{y} = 0$, the corresponding X is 436 days for frozen vaccine-1. Similar predictions found by using the regression equation, for the vaccine-1, refrigerated, the X is 376; for vaccine-2, refrigerated, the expected X is 341; and for vaccine-2, frozen, X is 303. Accepting the existence of linear relationship and a cut point titer of 5, the prediction for $\hat{y} = 0.699$, the corresponding X is 249 days, for the vaccine-1, refrigerated; for the vaccine-2, refrigerated, the X = 164 days; for vaccine-1, frozen, the X = 250 days; and for vaccine-2, frozen, the X = 128 days.

Thus, independently of being refrigerated or frozen, these vaccines could not provide a long-lasting SN antibody titer of ≥5, usually accepted as the protective level for the existence of a certain degree of immunity (Atanasiu, 1967), however, the findings of short duration of antibody titers after vaccination of bovines using commercial vaccines are in accordance to the previous work of Albas et al. (1998), who recommended a booster dose to ensure adequate protection.

In this experiment, results were somewhat ambiguous because for vaccine-1 that contained avridine, even after being frozen, its capacity to induce the formation of SN antibody apparently was not influenced and enabled a longer period of duration of antibody. However, results elicited by the vaccine-2, frozen, are controversal. The quality of vaccine, the adjuvants and additives like saponin and $Al(OH)_3$ may interfere with the vaccine stability at low temperatures (Doel & Pullen, 1990), but the question of vaccine storage at freezing temperature and consequent decrease in its potency could be revised in a more carefully designed scientific study, and the immunity of vaccinated animals should not be measured only by means of SN antibody titers.

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