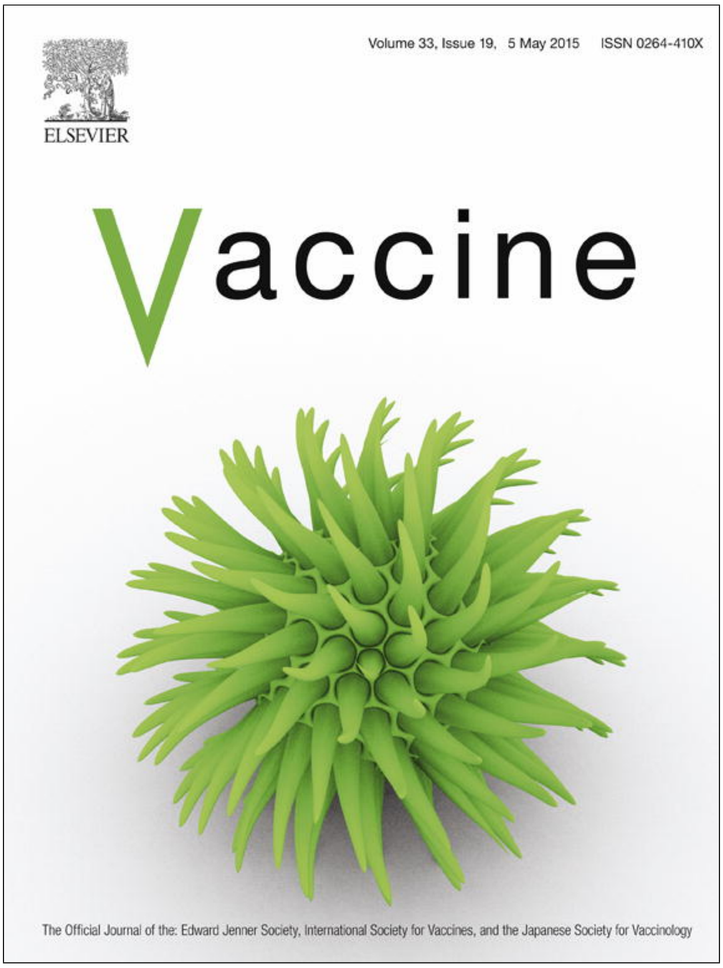
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Brief report

Rabies vaccine preserved by vaporization is thermostable and immunogenic

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ABSTRACT

A rabies vaccine that is thermostable over a range of ambient environmental temperatures would be highly advantageous, especially for tropical regions with challenging cold-chain storage where canine rabies remains enzootic resulting in preventable human mortality. Live attenuated rabies virus (RABV) strain ERAG333 (R333E) was preserved by vaporization (PBV) in a dry, stable foam. RABV stabilized using this process remains viable for at least 23 months at 22 °C, 15 months at 37 °C, and 3 h at 80 °C. An antigen capture assay revealed RABV PBV inactivated by irradiation contained similar levels of antigen as a commercial vaccine. Viability and antigen capture testing confirmed that the PBV process stabilized RABV with no significant loss in titer or antigen content. Live attenuated and inactivated RABV PBV both effectively induced RABV neutralizing antibodies and protected mice from peripheral RABV challenge. These results demonstrate that PBV is an efficient method for RABV stabilization.

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1. Introduction

Elimination of canine rabies is possible with current methods, but novel approaches are needed to enhance vaccine availability [1]. Current rabies vaccines for humans and animals require cold-storage [2–5]. Maintenance of cold-chain is challenging in remote, high-risk rabies enzootic regions. A vaccine that is stable and potent at ambient temperatures would be advantageous for pre- and post-exposure prophylaxis (PEP) in humans and animals.

Preservation by vaporization (PBV) is a foam drying technique. PBV requires one to 5 h of boiling, sublimation, and evaporation at $\geq -10\,^{\circ}$C and ≤ 3 Torr [6]. PBV is scalable, reproducible, and cost-effective. Proteins, live bacterial vaccines, and live attenuated virus vaccines have been prepared using foam drying to enhance stability [7–9]. In the current study, rabies virus (RABV) PBV vaccines were characterized. These studies demonstrated that RABV PBV was thermostable, immunogenic, and protected mice from peripheral challenge.

2. Materials and methods

Fixed RABV Evelyn–Rokitnicki–Abelseth (ERA) strain was attenuated as previously described [10–13]. The recovered virus was sequenced and had only the desired change (R333E). The resulting virus, referred to as ERAG333, was grown as described [14].

ERAG333 supernatant was mixed (1:2) with 30% sucrose and 15% methylglucoside in phosphate buffer (pH = 7.0). 0.5 ml of mixture was distributed into crimp vials and dried using Genesis and Virtis Ultra freeze-dryers (SP Scientific, Warminster, PA, USA) that were modified for vacuum pressure control [6]. After 2 h of processing, the solid material formed stable dry foam. Secondary drying was performed under vacuum at 35 °C and 45 °C for 20–24 h. RABV PBV in crimp vials at 22 °C with desiccant was irradiated by electron beam at various doses. Viability of irradiated RABV was measured as described below except in 96-well plates on 4 consecutive days post-infection.

RABV PBV in crimp vials was placed at 22 °C with desiccant, in a dry incubator at 37 °C, in mineral oil bath at 80 °C and 90 °C for viability, or in a water bath at 80 °C for electrochemiluminescent (ECL) assays. Vials were removed at different time points and reconstituted with 0.4 ml PBS (0.01 M, pH 7.4). Virus titers were measured in an 8-well chamber slide as described [15]. The mean focus

Abbreviations: ECL, electrochemiluminescent; ERA, Evelyn–Rokitnicki–Abelseth; ffu, focus forming units; GMT, geometric mean titer; G, glycoprotein; IM, intramuscular; IU, international units; MAb, monoclonal antibody; PEP, post-exposure prophylaxis; PBV, preservation by vaporization; RABV, rabies virus; rVNA, rabies virus neutralizing antibodies; RFFIT, rapid fluorescent focus inhibition test.

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Table 1
Viability of RABV after PBV and storage at different temperatures.

Temperature (°C)	Rabies virus titer ($\log_{10}$ ffu/ml)									
	Initial	1 h	2 h	3 h	16 h	1 Month	2 Months	3 Months	15 Months	23 Months
22	7.91 ± 0.05	ND[a]	ND	ND	ND	7.51 ± 0.07	7.40 ± 0.09	7.39 ± 0.12	7.42 ± 0.04	7.33 ± 0.05
37	7.56 ± 0.14	ND	ND	ND	ND	7.13 ± 0.17	6.99 ± 0.01	ND	6.1 ± 0.09	5.58 ± 0.22
80	7.51 ± 0.07	7.56 ± 0.09	7.48 ± 0.15	7.46 ± 0.05	6.53 ± 0.05	ND	ND	ND	ND	ND
90	7.51 ± 0.07	6.07 ± 0.09	ND	ND	ND	ND	ND	ND	ND	ND

[a] Not determined (ND).

forming units (ffu)/ml and standard deviation were calculated from at least three statistical replicates.

The Meso Scale Discovery platform (Meso Scale Discovery, Gaithersburg, MD, USA) was used to perform RABV antigen capture ECL assays as described [14]. RABV glycoprotein (G) monoclonal antibody (MAb) 62-80-6 was used at 1 μg/ml for capture and 0.5 μg/ml for detection.

Approved animal use protocols were established with CDC IACUC. On day 0, 14, and 30 blood was collected as described previously from 4-week-old, female, CD-1 mice (Charles River Laboratory, Wilmington, MA, USA) assigned to groups of 10, and the geometric mean titer (GMT) of RABV neutralizing antibodies (rVNA) in international units (IU)/ml was determined using a rapid fluorescent focus inhibition test (RFFIT) or a modified RABV neutralization test for small volumes [14,16,17]. Live attenuated RABV PBV vaccine, placebo, and inactivated RABV PBV, stored 36 days at 22 °C in the dark with desiccant, were reconstituted with 0.4 ml of sterile PBS (0.01 M, pH 7.4) without calcium or magnesium (Mediatech, Inc. Manassa, VA, USA). Reconstituted vaccine and RABV ERAG333 from frozen stock was subsequently diluted using the same PBS. Commercially available RABV vaccine RabAvert (lot: 464011A) was purchased from Novartis Pharmaceuticals (Dorval, Quebec, Canada) and reconstituted according to the manufacturer's instructions. On day 0, mice were vaccinated intramuscular (IM) in the right leg as described [14]. Titrations of inoculum were completed as described above. For inactivated vaccines, the BCA Protein Assay (Thermo Scientific, Rockford, IL, USA) was used according to manufacturer's instructions. On day 30 all mice were challenged IM in the left leg with $10^{4.2}$ $MICLD_{50}$ of canine RABV 3374R (fox salivary gland homogenate). Animals were monitored and euthanized at first signs of rabies as previously described [14]. The brain stem was collected from euthanized animals and subjected to the direct fluorescent antibody test for rabies [18]. Endpoint was 30 days after the last death in the placebo group, surviving animals from each group were randomly selected for rabies diagnosis, and all were rabies negative. Probability values were calculated using chi-square test with a 95% confidence interval.

3. Results

The starting titer of RABV ERAG333 before PBV was 8.3 $\log_{10}$ ffu/ml. After PBV, about 0.2 $\log_{10}$ of viable virus was lost resulting in 8.11 ± 0.12 $\log_{10}$ ffu/ml. Following inactivation via irradiation, all tested doses damaged RABV and resulted in lower virus titers; no viable virus was recovered in samples treated with 12 kGy (data available upon request). The complete inactivation of RABV after treatment with 12 kGy was verified in three blind passages.

RABV PBV was stored at 22 °C with desiccant for 1, 2, 3, 15, or 23 months. After 2 months viability dropped 0.5 $\log_{10}$; then remained stable until the experiment ended, when viability only decreased approximately 0.6 $\log_{10}$ (Table 1). RABV PBV was incubated at 37 °C for 1, 2, 15, or 23 months. After 2 months, viability dropped <1 $\log_{10}$ and after 15 months dropped 1.5 $\log_{10}$. RABV PBV was placed at 80 °C or 90 °C. After 3 h at 80 °C, viability was essentially the same, and only 1 $\log_{10}$ of viability was lost after 16 h. Incubation at 90 °C was significantly more damaging, and RABV PBV lost >1 $\log_{10}$ of activity after 1 h.

MAb 62-80-6 was used for capture and detection of RABV G in an antigen capture assay and counts $\mu g^{-1}\,ml^{-1}$ were determined. In agreement with the measured virus titers, live attenuated RABV PBV had the same counts $\mu g^{-1}\,ml^{-1}$ as the original ERAG333 virus (Table 2). Inactivation of RABV PBV by irradiation resulted in a decrease in antigen content but was similar to a commercial inactivated vaccine. When inactivated RABV PBV was placed at 80 °C with high humidity for 3 h, antigen content decreased 48% while decreasing 30% in a commercial vaccine incubated under the same conditions.

Live attenuated or inactivated RABV PBV was used to vaccinate mice IM. Both live and inactivated RABV PBV effectively induced rVNA titers by day 14. RABV PBV induced rVNA titers similar to ERAG333 and commercial vaccine by day 14 and surpassed ERAG333 and commercial vaccine by day 30. Inactivated RABV PBV induced rVNA titers on day 30 similar to commercial vaccine on day 14 (Table 3).

Different dilutions of live attenuated RABV PBV induced similar rVNA titers. Only the undiluted and 10^{-1} dilution of inactivated RABV PBV vaccine induced rVNA titers. The immunogenicity of the inactivated RABV PBV is consistent with the *in vitro* antigen capture results.

On day 30 all mice were challenged IM with canine street RABV. All animals that received commercial vaccine survived (Table 3, $p < 0.01$ compared to placebo). All animals also survived in groups that received ERAG333 or live RABV PBV, consistent with the observed rVNA responses. In groups that received inactivated RABV PBV all animals survived except in the 10^{-2} group. In this group, 80% survived despite only 3 individuals (30%) having a measurable rVNA response. Survivorship in this group was significantly different compared to the placebo ($p < 0.05$) but not compared to the commercial vaccine or other inactivated RABV PBV groups.

Table 2
Antigenic G content of different RABV vaccines measured by antigen capture assay using the 62-80-6 α RABV G MAb.

Antigen	Storage conditions		
	Time	Temperature (°C)	ECL counts $\mu g^{-1}\,ml^{-1}$ [a]
ERAG333[b]	20 Months	−80	2200
Live attenuated RABV PBV	20 Months	22	2200
Commercial vaccine	25 Months	4	1400
	3 h	80	980
Inactivated RABV PBV	20 Months	22	1300
	3 h	80	680
Native ERA G[c]	18 Months	−80	9100
Denatured ERA G[c]	10 min	98	7
Placebo	20 Months	22	3

[a] Estimated from the best fit linear regression as previously described [14].
[b] Parent strain for both live attenuated and inactivated vaccines; generated by reverse genetics.
[c] Purified RABV ERA glycoprotein [14].

Table 3
Immunogenicity and efficacy of rabies vaccines in mice.

	Live Attenuated RABV PBV			ERAG333[a]	Placebo	Commercial Vaccine	Inactivated RABV PBV		
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-2}	None	None	None	10^{-1}	10^{-2}
Titer (Log_{10} ffu)	6.8[b]	5.7	4.4	7.9	NA[c]	NA	NA	NA	NA
Load (μg of total protein)	ND[d]	ND	ND	ND	300[b]	620	350	34	2.3
GMT day 14 (IU/ml)[e]	0.28	0.19	0.24	0.42	<0.05	0.092	0.096	0.077	<0.05
SD day14 (IU/ml)[f]	±0.49	±0.68	±0.94	±0.97	±0.0097	±0.41	±0.10	±0.24	±0.016
GMT day 30 (IU/ml)[e]	1.8	0.96	1.7	0.84	<0.05	0.58	0.36	0.15	0.067
SD day 30 (IU/ml)[f]	±2.4	±15	±2.9	±1.9	±0.015	±1.9	±1.4	±0.57	±0.12
Seroconversion (%)[g]	90[h]	100[h]	100	90	0	90	90	70[h]	30
Survival (%)[i]	100[h]	100	100	100	22	100	100	100	80[j]

[a] Parent strain for both live attenuated and inactivated vaccines; generated by reverse genetics.
[b] 0.1 ml dose.
[c] Not applicable (NA); cannot be determined for inactivated vaccines.
[d] Not determined (ND).
[e] Geometric mean titer (GMT) of rabies virus neutralizing antibodies.
[f] Standard deviation (SD) of rabies virus neutralizing antibody titers.
[g] Percent with >0.05 IU/ml titer on day 30; Group size = 10 except placebo $n = 9$.
[h] $p < 0.01$ compared to placebo using chi-square test with a 95% confidence interval.
[i] Group size = 10 except placebo $n = 9$.
[j] $p < 0.05$ compared to placebo using chi-square test with a 95% confidence interval.

4. Discussion

RABV ERAg333 was successfully formulated into stable, dry foam using PBV technology. Live attenuated RABV PBV was stable for 23 months at 22 °C and 2 months at 37 °C. Stability decreased as temperature increased, yet RABV PBV remained stable for at least 3 h at 80 °C. A commercial vaccine was not included for comparison because viability was used to measure stability. Only inactivation post-preservation was considered here so that the effect of PBV could be independently evaluated. Other methods of inactivation, such as β-propiolactone, could be used in the future.

An antigen capture assay was used to compare the antigen content of different vaccines. MAb 62-80-6 which binds a linear epitope in RABV G was used for both antigen capture and detection [14]. By using the same antibody for capture and detection, only trimeric G is detected as demonstrated by low ECL counts for heat denatured RABV G. While the antigen capture assay is not a substitute for potency testing, live attenuated and inactivated RABV PBV were both adequately antigenic and immunogenic.

A single dose of live attenuated or inactivated RABV PBV effectively induced rVNA and protected all mice from IM challenge. Previous challenge experiments using the same RABV, dose, and route resulted in 100% mortality in unvaccinated mice. However, the IM challenge, while more closely modeling natural infection, introduces greater variability [19].

The advantages of PBV are that live attenuated RABV can be stabilized and formulated into an oral vaccine suitable for use in domestic or wild animals. These preliminary results support further testing in target species and the evaluation of PBV technology for other vaccines, e.g. RABV-vectored ebola vaccine [20]. If formulated into a safe, potent vaccine, inactivated RABV PBV paired with a needle-less delivery system could be considered for human use. Access to safe, potent, and thermostable vaccines is paramount for canine rabies elimination and prevention of rabies in humans.

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