



POTENCY AND STABILITY OF VARIOUS MODERN RABIES VACCINES  
FOR VETERINARY USE<sup>1</sup>

In order to assure national services responsible for implementation of dog vaccination campaigns, as well as the people living in the areas where these operations are carried out, of the efficacy of their efforts, the rabies vaccine used should have a potency of at least 1 I.U. per dose when injected into the dog. For this purpose, laboratories should produce vaccines:

- having a potency higher than 1 I.U. per dose on the day of manufacture; and
- which will retain a satisfactory level of potency of at least 1 I.U. for a period of at least six months after its date of manufacture, under normal storage conditions (+2-+8°C).

Within the framework of its assistance programme for technology transfer related to rabies vaccine production, WHO requested its Collaborating Centre for Reference and Research on Rabies in Paris to carry out this study.

This document describes the equipment and methods used for the production of modern rabies vaccines, i.e. inactivated, concentrated, stabilized and adjuvanted vaccines, and gives the results of potency tests performed 8 and 17 months after processing on the various vaccine preparations differing by their formula of the stabilizing media and the aluminium hydroxide gel concentrations.

1. Material and methods

The study comprised the following steps:

- Production of rabies virus in BHK-21 cell cultures. Clarification and immediate inactivation of the harvests.
- Mixing and concentration of the inactivated harvests.
- Dilution of the inactivated concentrated antigen in four stabilizing media.
- Adsorption of each of the four preparations on aluminium hydroxide gel at two different concentrations of the adsorbent.

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- Checking of the potency of the vaccines at the end of the manufacturing process.
- Checking of the potency of the vaccines after 8-9 months' storage at +4°C.
- Checking of the potency of the vaccines after 17-18 months' storage at +4°C.

## 2. Production of the virus in cell cultures and its harvesting and inactivation

The BHK-21 cells (Pasteur Institute/Paris seed) were dispensed into 850 cm<sup>3</sup> roller bottles and infected in the exponential growth phase (multiplicity of infection = 1/3).

The supernatant (Stoker medium + 0.5% bovine albumin) was harvested on the 4th and 7th days following infection of the cells.

The harvests were immediately clarified by cold centrifugation and 5% sucrose added. They were then inactivated with betapropiolactone 1:4000 and shaken slowly for 24 hours at +4°C and then for 2 hours at +37°C.

Altogether, eight successive lots were obtained:

- 4 lots with strain PV-BOB or PM(PV2)<sup>1</sup>
- 4 lots with strain PV-IP Paris (PV4)<sup>1</sup>

Each lot was analysed by two methods: specific haemagglutination of goose erythrocytes and ELISA titration of the rabies glycoprotein (G), using as a reference preparation the vaccine prepared in fetal calf kidney cells, purified (lot 04 with a titer of 15,000 ng of G per dose, equivalent to a potency of 10 I.U./dose).

The analysis revealed a great difference in the yields of rabies antigen in the harvests, depending on the inoculum. This difference is clearcut in the figures for inactivated blended harvests (see Table 1): the yield is about 18 times greater in cultures infected with strain PV4 (PV-IP Paris) than in those infected with strain PV2 (PV BOB - PM).

TABLE 1. YIELD OF GLYCOPROTEIN (G), DEPENDING ON INOCULUM

Viral inoculum used for infecting cells	Mixture of inactivated harvests		
	Haemagglutinating activity, 50%	Amount of glycoprotein produced	
		per 10 <sup>6</sup> infected cells	Total Harvests
PV2(PV BOB - PM)	1/16	170 ng	2 400 000 ng
PV4 (PV IP Paris)	1/256	3 130 ng	41 400 000 ng

<sup>1</sup>Report of German Green Cross/WHO Workshop on Monoclonal Antibody in Rabies Diagnosis and Research, Marburg, 27-28 May 1984 (WHO/Rab.Res./84.20).

### 3. Concentration of the inactivated harvests

The harvests obtained with the two virus strains were pooled in a single container in order to obtain a volume great enough for concentrating the rabies antigen in an industrial-type filtration apparatus; 14.1 litres of mixed harvests were thus obtained, containing a total of 43,800,000 ng of rabies glycoprotein, or 3100 ng/ml.

The concentration factor was calculated on the basis of the reference vaccine values - a titer of 15,000 ng of G for a potency of 10 I.U. (or 1500 ng per I.U.). To obtain 5 units per 1 ml dose of vaccine after twofold dilution of the concentrate in stabilizing media plus adsorbents, the titer of the antigen must be  $1500 \times 5 \times 2 = 15,000$  ng G/ml. Hence the concentration factor is  $15,000/3100 = 4.8$ .

This concentration was obtained by ultrafiltration on a semipermeable membrane with a cut-off point of 100,000 (Millipore PTHK cartridge).

The volume of the harvests was reduced to 1.1 litres to allow for a thorough washing of the membranes with the rinsing solution and thereby an almost complete recuperation of the antigen. The membranes had been washed with the recirculating rinsing solution (culture medium with 5% sucrose added to it) until the pre-established concentration factor was reached. Thus the 3 litres of concentrated antigen were obtained, the concentration factor being 4.7.

The antigen yield was 100% (calculated figure 14,600 ng/ml approximately; experimental figure 15,500 ng/ml).

### 4. Dilution of the concentrate in stabilizing media

Three formulas were established (see Table 2) on the basis of the known protective effect of certain sugars and amino acids on enveloped RNA virus suspensions.

TABLE 2. FORMULA OF STABILIZING MEDIA

Ingredients	Medium A (per litre)	Medium B (per litre)	Medium C-D (per litre)
Glycine	7.0 g	7.0 g	7.0 g
Histidine HCl	8.4 g	8.4 g	8.4 g
Arginine L	2.5 g	2.5 g	2.5 g
Alanine L	3.6 g	3.6 g	3.6 g
Dextran 20 000	12.0 g	12.0 g	12.0 g
Lactose	54.0 g	54.0 g	None
Sorbitol	30.0 g	30.0 g	None
Sucrose	None	60.0 g	60 g
Tween 80 at 1/200	10 ml	10 ml	10 ml

(pH adjusted to 7.4)

An Na<sub>2</sub>K phosphate buffer (see Table 3) was used in preparing the stabilizing media and to make up the volume of the stabilized and adsorbed vaccines.

TABLE 3. FORMULA OF THE DILUTION BUFFER PER LITRE

NaCl	3.3 g
KCl	0.12 g
Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O	1.2 g
KH <sub>2</sub> PO <sub>4</sub>	0.12 g
CaCl <sub>2</sub>	0.08 g
SO <sub>4</sub> Mg 7 H <sub>2</sub> O	0.06 g

Stabilizing media were prepared in the dilution buffer with a concentration factor of 5.4. The composition (in percentage of the final product) of the four stabilizing media is shown in Table 4 below.

TABLE 4. COMPOSITION OF STABILIZING MEDIA (IN %)

Glycine	0.13	0.13	0.13	0.13
Histidine	0.15	0.15	0.15	0.15
Arginine	0.04	0.04	0.04	0.04
Alanine	0.07	0.07	0.07	0.07
Dextran 20 000	0.22	0.22	0.22	0.22
Lactose	1.0	1.0	--	--
Sorbitol	0.55	0.55	--	--
Bovine albumin	7.5	--	7.5	--
Human albumin	--	--	--	7.5
Sucrose	2.5	3.6	3.6	3.6

After division of the concentrated virus into four equal fractions of 750 ml, four vaccine lots were prepared as indicated in Table 5. The concentration factor of the virus was thus brought to 2.8 when compared to the pooled inactivated harvests.

TABLE 5. FORMULA OF DIFFERENT STABILIZED VACCINES

Ingredients	Stabilized vaccines			
	A	B	C	D
Concentrated antigen	750 ml	750 ml	750 ml	750 ml
Medium A	280 ml	--	--	--
Medium B	--	280 ml	--	--
Medium C-D	--	--	280 ml	280 ml
10% bovine albumin	200 ml	--	200 ml	--
20% human albumin	--	--	--	100 ml
Dilution buffer	--	200 ml	--	100 ml
Merthiolate 1/200	26 ml	26 ml	26 ml	26 ml
Final volume	1256 ml	1256 ml	1256 ml	1256 ml
pH	7.4	7.2	7.4	7.4

5. Adsorption of the vaccines

"ALHYDROGEL" aluminium hydroxide gel, containing 2 mg of aluminium per ml was added to the stabilized vaccines. This gel was chosen for its purity and stability as shown when used as an adjuvant and adsorbent in vaccines for human use, such as influenza, hepatitis B and other vaccines.

Before it was used, the pH of the gel was adjusted to pH 8 by adding 40 ml of glycine buffer (pH 9.2 per litre of gel). It was then autoclaved at 150°C.

Each of the vaccines A, B, C and D was divided into two equal parts for adsorption at two different concentrations of aluminium hydroxide, as indicated in Table 6, below.

TABLE 6. FORMULAS OF ADJUVANTED VACCINES

	Final concentration of adsorbant							
	5%				12.5%			
	A1	B1	C1	D1	A2	B2	C2	D2
Stabilized vaccine	625 ml	625 ml	625 ml	625 ml	625 ml	625 ml	625 ml	625 ml
Aluminium hydroxide gel	38 ml	38 ml	38 ml	38 ml	95 ml	95 ml	95 ml	95 ml
Dilution buffer	97 ml	97 ml	97 ml	97 ml	40 ml	40 ml	40 ml	40 ml
Final volume	760 ml	760 ml	760 ml	760 ml	760 ml	769 ml	760 ml	760 ml
pH	7.4	7.7	7.4	7.6	7.3	7.6	7.3	7.5

The gel was added gradually, the bottles being shaken slowly. After the volume had been brought up to 760 ml with the dilution buffer, the vaccines were shaken for 18 hours at +4°C.

The final concentration of the adsorbent in the vaccines was 5% in A1, B1, C1 and D1, and 12.5% in A2, B2, C2 and D2.

The concentration factor of rabies virus in the adsorbed vaccine was 2.3 in relation to the inactivated harvest pool (see Table 7).

After the aluminium hydroxide had been added, there still remained some non-adsorbed glycoprotein in the liquid phase of the vaccines: 30-35% in the A1, B1, C1 and D1 portions containing 5% aluminium gel and 12-25% in the A2, B2, C2 and D2 portions containing 12.5% aluminium gel. The finding confirms the results of several previous experiments at Institute Pasteur Production which had indicated that there is always a residue of rabies antigen in the liquid phase whatever the adsorption procedure used.

6. Testing of the vaccine potency

The results of tests carried out during and on completion of manufacture are summarized in Table 7, as follows:

TABLE 7. RESULTS OF VACCINE POTENCY TESTING

Production stage n = concentration factor	Volume in ml	Haemagglutination dilution, HA 50%	Glycoprotein ng/ml	I.U.'s in NIH test	
				Calculated	Experimental
Mixture of harvests	14 100	1/128	3 100	2	ND
Concentrated inactivated virus; n = 4.7	3 000	1/512	15 500	10	ND
Dilutions of stabilizing media n = 2.8					
A	1/256	1/256	8 000	5	ND
B	1/256	1/256	7 600	5	4.3
C	1/256	1/256	6 100	4	6.0
D	1/256	1/128	6 200	4	ND
Lots of stabilized & adsorbed vaccines n = 2.3					
A1	760	1/64*	2 300*	4	3.05
A2	760	1/32*	1 600*	4	3.08
B1	760	1/64*	2 300*	4	3.15
B2	760	1/32*	900*	4	3.4
C1	760	1/128*	2 400*	3	4.5
C2	760	1/32*	1 600*	3	3.3
D1	760	1/64*	2 100*	-	ND
D2	760	1/32*	1 100*	-	ND

at t 0

ND = No data

\* = Titration of HA and G in the adsorption supernatant

This work confirms the value of titrating the rabies glycoprotein (G) during the various stages of the manufacturing process. The G content enables a very good estimate to be made of in vivo potency by means of the NIH test, "equivalent" I.U.'s being calculated by comparison with the reference vaccine.

The findings also demonstrate that the glycoprotein from virus cultivated on diploid cells (fetal calf kidney) and that obtained in BHK-21 cell cultures have similar potency.

The glycoprotein content of the stabilized vaccines before adsorption lies between 6000 and 8000 ng/ml and is in line with anticipated figures. There was no significant difference in potency between the two B and C lots subjected to the NIH test. The absence of albumin in lot B therefore had no effect on the vaccine at the end of the manufacturing process.

The NIH test was carried out on the adsorbed vaccines, except for lots D1 and D2, which contained human albumin and were therefore of no interest for veterinary production.

There were no significant differences between the six lots tested which showed levels ranging from 3 to 4.5 I.U./ml. If the variations inherent in the method are taken into account these figures are in accordance with the "calculated" values before the addition of aluminium hydroxide gel. Contrary to what is usually asserted, there is therefore no appreciable fall in the titer expressed in international units under the effect of the adsorbent (see Table 7 above).

## 7. Stability of the vaccines

### 7.1 Schedule of stability tests

Retesting of vaccine potency by means of the NIH test, initially scheduled to be carried out after six months' storage at +4°C, was not in fact done until the vaccines had been in store in a cold room for 8-9 months.

Two series of tests were carried out, the first in July 1987 (t + 8 months) on lots A2, B2 and C2, the second in August 1987 (t + 9 months on lots A1, B1, C1 and D1). The results of the tests initially scheduled to be carried out after 12 months were performed only after 17-18 months' storage.

### 7.2 Results of the NIH tests

In view of the fact that only one reading was available for each vaccine from each consecutive test, and bearing in mind the high degree of variability of the NIH test, the results must be interpreted with caution. The variations between the potencies in I.U.'s of the different vaccines at t 0 and between the potencies of the same vaccines at t + 8 or t + 9 months, are always within the limits of variation of the NIH test and are not significant.

However, all the vaccines but one (B2) vary in the same direction, i.e. the figures for potency decrease between t 0 and t + 8 or t + 9 months. It is therefore reasonable to suppose that the potency of these vaccines actually does drop during the nine months following their manufacture. The drop in potency is, however, moderate. None of the values obtained during the second test was below 1.3 I.U.

After 17 and 18 months' storage, only vaccine lots A2, B2 and C2 were titrated. For each lot the NIH value represents the mean of two tests. A very slight decrease of potency is observed for these three vaccines vis à vis the potency values found at the date of manufacture (see Table 8 below). However, even after 17 months' storage the potency of the three vaccines was at least 1 I.U./ml.

TABLE 8. STABILITY OF LIQUID, ADJUVANTED RABIES VACCINES AFTER 17 MONTHS' STORAGE AT +4°C.

Vaccine lot	Aluminium hydroxide	Potency in I.U./ml		
		t 0	t 8/9 months	t 17 months
A1	0.10	3.05	2.30	NT
A2	0.25	3.08	1.69	1.17
B1	0.10	3.15	1.54	NT
B2	0.25	3.40	3.50	1.01
C1	0.10	4.40	1.30	NT
C2	0.25	3.30	1.67	1.21

NT = Not tested

If the vaccines are compared in relation to the amount of aluminium hydroxide gel added, it will be seen that in two groups out of three (B and C), the higher concentration of gel produces the better results (see Table 9).

TABLE 9. STABILITY OF ADJUVANTED RABIES VACCINE

Vaccine lot	Adjuvants wt Al/ml	Potency at	
		t 0	t 8/t 9
A1	0.10 mg/ml	3.05	2.30
A2	0.25 mg/ml	3.08	1.69
B1	0.10 mg/ml	3.15	1.54
B2	0.25 mg/ml	3.40	3.50
C1	0.10 mg/ml	4.40	1.30
C2	0.25 mg/ml	3.30	1.67
D1	0.10 mg/ml	4.00	2.90

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#### 8. Conclusions

The potency of lots A2, B2 and C2 measured by NIH tests was higher than 1 I.U./ml after 17 or 18 months' storage. We may conclude that a reasonable expiry date is one year as from the date of manufacture of these inactivated, liquid, stabilized and adjuvanted vaccines. This duration should satisfy most of the vaccine producers and vaccine users.

From the results obtained, it is not possible to say that one stabilizing medium is better than the other. However, stabilizing medium B, which contains neither albumin nor serum, and which costs less and is easier to manufacture, is a good candidate for any laboratory contemplating the production of inactivated, liquid, adjuvanted veterinary vaccines.

An aluminium hydroxide gel concentration of 0.25 mg/ml leads to the adsorption of a greater quantity of rabies antigen at the end of the manufacturing process.

Additional studies aiming at determination of the protection given in the target species by such a vaccine should be undertaken by the laboratories interested in its preparation.

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