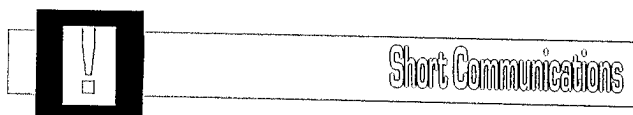# POTENCY OF VETERINARY RABIES VACCINES IN THE NETHERLANDS: A CASE FOR CONTINUED VIGILANCE

E.J.M. Rooijakkers[1,2] J.H.M. Nieuwenhuijs[3], A.A. Vermeulen[2], G. van Steenis[2], and A.D.M.E. Osterhaus[1,4]

Short Communications

SUMMARY

Commercial rabies vaccines, used by veterinarians in the Netherlands, were collected for testing in the mouse potency test. Of the six vaccines tested, two were clearly below the minimal requirements for potency of 1.0 IU. Of these six vaccines the rabies virus glycoprotein (GP) and nucleoprotein (NP) contents were determined in an antigen competition ELISA. The GP content proved to correlate well with the potency found in the mouse potency test ($r=0.95$, $p<0.01$), whereas no such correlation was found for the NP content ($r\sim0$, $p>0.05$). After the manufacturers were told about the results, one of the two vaccines that did not comply with the requirements was withdrawn from the market. Measurement of the GP content of a second lot of the remaining vaccines indicated that sufficiently high levels of GP were present in all five. Additional *in vivo* testing in mice for efficacy against intracerebral challenge with the Dutch bat rabies virus EBL1-12 resulted in acceptable levels of protection with four of these five vaccines of the second lot. The data presented illustrate the need for continued potency evaluation of veterinary rabies vaccines in the Netherlands.

INTRODUCTION

In Western Europe several commercial rabies vaccines are available for pre- and post-exposure rabies prophylaxis in humans and pre-exposure prophylaxis in animals (27).

[1] Centre for Exotic Virus Infections, Department of Clinical Virology, Erasmus University Hospital Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, the Netherlands.

[2] National Institute of Public Health and Environmental Protection, P.O.Box 1, 3720 BA Bilthoven, the Netherlands.

[3] Veterinary Public Health Inspectorate (VHI), P.O.Box 5406, 2280 HK Rijswijk, the Netherlands.

[4] corresponding author: prof. dr. A.D.M.E. Osterhaus, Head, Institute of Virology, Erasmus University Rotterdam, P.O.Box 1738, 3000 DR Rotterdam, the Netherlands. Tel: +31 10 408 8066. Fax: +31 10 436 5145.

According to international requirements for the approval of inactivated rabies vaccines (18,32), manufacturers are obliged to guarantee the quality of vaccines before release. Part of the procedure of approval is efficacy control in the NIH potency test (26), as such recommended by the World Health Organization (32). For veterinary rabies vaccines, the European Pharmacopoeia adopted a modified protocol of the NIH potency test which requires a single inoculation of serially diluted vaccine in outbred mice, followed by the intracerebral inoculation of virulent CVS-26 rabies virus 14 days after the start of the test (18). After challenge, the mice are observed for the development of rabies symptoms during 2 weeks. The potency is calculated by comparison of the ED_{50} (median effective dose) of the sample and that of a simultaneously tested reference vaccine with an established potency (IU). Veterinary rabies vaccines should have a minimal potency value of 1.0 IU (18). In the Netherlands the Veterinary Public Health Inspectorate (VHI) is charged with the supervision of public health regulations in the veterinary field. In this context we collected six commercial veterinary rabies vaccines from stocks in veterinary practices for examination in the mouse potency test. They were also tested in recently developed enzyme-linked immunosorbent assays (ELISAs) for quantitation of their glycoprotein (GP) and nucleoprotein (NP) contents.

Since in recent years approximately 7 per cent of all the examined bats sent in for rabies diagnosis to the Institute for Animal Science and Health in the Netherlands were found to be rabies positive (20), the efficacy of these vaccines against infection with the Dutch bat rabies virus EBL1-12 was also tested.

MATERIALS AND METHODS

Veterinary rabies vaccines

Commercially released, inactivated rabies vaccines for veterinary use were collected from stocks in veterinary practices by the veterinary inspector in the district Arnhem (the Netherlands). The first panel ($n=6$) and second panel ($n=5$) of rabies vaccines were against the virus strains SAD, PV, PM, and Flury-LEP. The vaccines had been stored in a refrigerator until use, and were tested before the expiration date had been reached.

Reference vaccines

The fifth International Standard for rabies vaccine (17) and the in-house reference (R0/41A, PM dog kidney cell vaccine) were used for calibration of the mouse potency test and the competition ELISA systems. The potency is expressed in international units (IU) rabies vaccine, the antigenic value for glycoprotein and nucleoprotein in GP units and NP units, respectively.

Dutch bat rabies virus EBL1-12

A homogenate of the brain of a rabid Dutch bat (*Eptesicus serotinus*), kindly provided by Dr. J. Haagsma (Institute for Animal Science and Health, Lelystad, the Netherlands), was passaged *in vivo* in susceptible mice. Once the mice showed clinical signs of rabies, a brain suspension was prepared for *in vitro* passage in mouse neuroblastoma (NA) cells, using standard techniques. To prepare a sufficiently large stock of challenge virus, the NA adapted virus was passaged twice in the brains of adult outbred NIH mice. A 10 % mouse brain suspension containing $10^{3.3}$ LD_{50} per ml was used as challenge virus in vaccine potency tests.

This challenge stock was also used for typing in an immuno fluorescence assay (IFA) after passage in BHK-21 cells, with the general purpose rabies nucleocapsid specific FITC-conjugate (Sanofi Diagnostics Pasteur) and a panel of nucleoprotein specific MAbs for rabies virus identification (19), kindly provided by Dr. M. Lafon (Institut Pasteur, Paris, France).

In vivo potency testing

Mouse potency test

The mouse potency test was performed according to the specifications of the European Pharmacopoeia (18): SPF outbred NIH mice, strain Riv:NIH, at the age of 4 weeks, were distributed in a randomized manner into groups of 20 animals (one sex per group). Freshly prepared serial fivefold dilutions of rabies vaccines and the in-house reference vaccine were inoculated intraperitoneally (0.5 ml per mouse). After 14 days, the mice were challenged intracerebrally with rabies virus CVS-26 (30 µl per mouse). The challenge dose

(LD_{50} titre) was titrated in control mice and assessed at 275 LD_{50} rabies virus CVS-26 per mouse. The median effective dose (ED_{50}) for the reference and vaccine samples was assessed and the respective potency of the samples was assessed according to the formula:

$$\text{potency sample} = \frac{ED_{50} \text{ sample x potency reference}}{ED_{50} \text{ reference}}$$

Rabies virus EBL1-12 challenge test

The rabies virus EBL1-12 challenge test was carried out according to the European Pharmacopoeia mouse potency test (18) in groups of 10 mice with the challenge virus CVS-26 replaced by EBL1-12. The challenge dose was titrated and assessed at 37 LD_{50} rabies virus EBL1-12 per mouse.

Measurement of GP and NP contents by competition ELISA

Glycoprotein (GP) and nucleoprotein (NP) contents of the vaccines were quantitated in a competition ELISA system (25). Briefly, serial twofold dilutions of rabies vaccine were incubated with a titrated amount of monoclonal antibodies specific for GP (murine MAb 2-22) or NP (human MAb 56), in a microtitre plate with low binding capacity for protein. Subsequently, the mixture of vaccine and MAb was transferred to a microtitre plate precoated with a standard preparation of rabies virus antigen, to determine the amount of uncomplexed MAb. After a 1-hour incubation, the plate was washed and species-specific horseradish peroxidase (HRP) conjugated second antibody (anti-mouse Ig HRP, anti-human Ig HRP, respectively) was incubated in the plate. After the plate was washed, a tetra methyl benzidine (TMB) substrate buffer was added and the enzymatic reaction was stopped after 10 minutes (21). The optical density at wavelength 450 nm was measured for each well in a spectrophotometer. The procedure for calculating the antigenic values of GP and NP was based on the method for linear regression analysis described elsewhere (25).
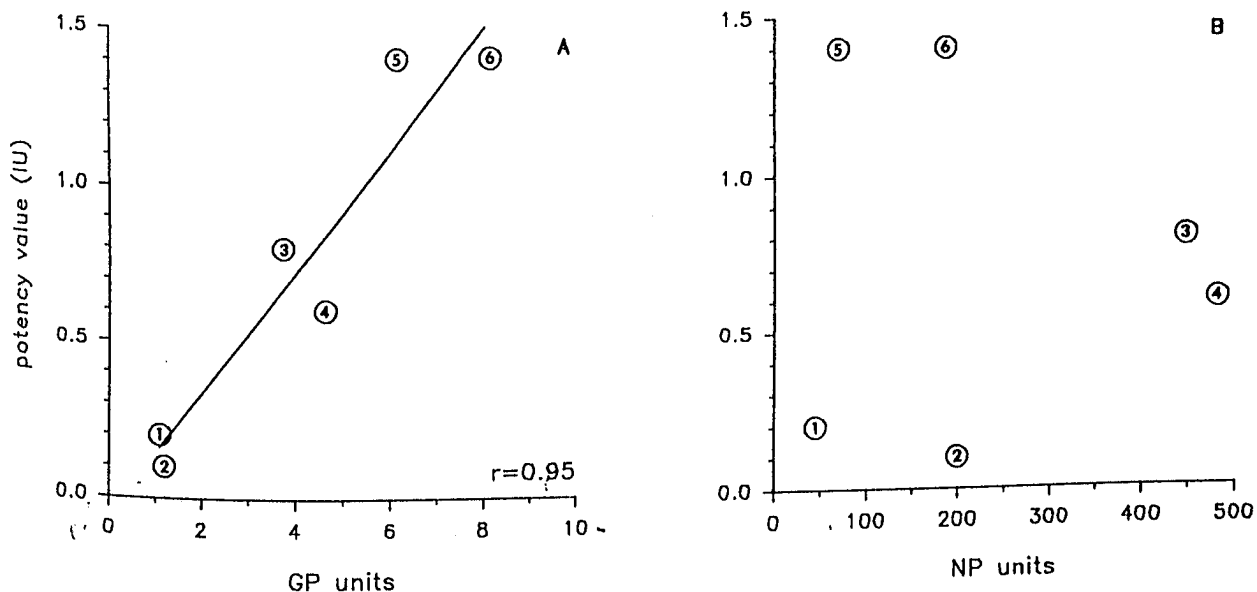

Figure 1. Comparison between potency (IU) and rabies virus glycoprotein (GP; A) and nucleoprotein (NP; B) content (measured by ELISA) for the first panel of six veterinary rabies vaccines (indicated by numbers 1-6). Correlation between potency and GP content proved to be significant (r=0.95, p<0.01).
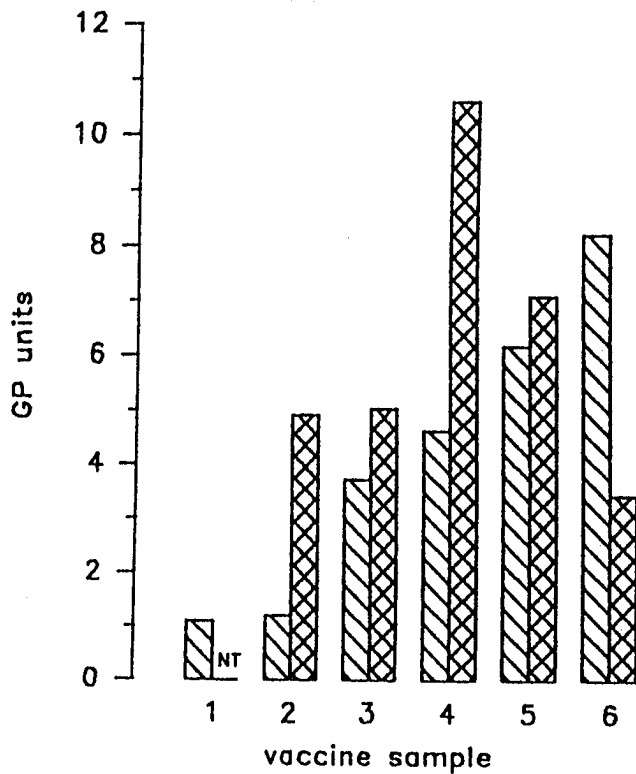
Figure 2. GP content of veterinary rabies vaccines assessed in GP ELISA. Comparison of GP values between subsequent batches of the same vaccine (first panel, diagonal hatch; second panel, cross hatch). NT, not tested.
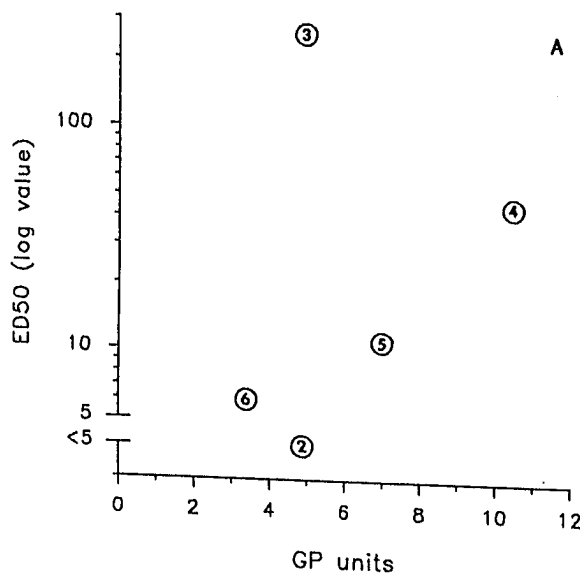
Table 1. Comparison of the antigen profiles characteristic for the rabies virus serotype 1 and the European Bat Lyssavirus 1 (EBL1) serogroup with the profiles found for the prototype rabies virus serotype 1 (CVS) and the Dutch bat rabies virus isolate (EBL1-12).

| | anti-nucleocapsid MAbs | | | | | | | |
	IP1	IP2	IP3	IP4	IP5	IP6	IP7	IP8
serotype 1	+	+	-	-	+	+	-	+
EBL1 serogroup	+	-	+	-	+	-	-	-
CVS	+	+	-	-	+	+	-	+
EBL1-12	+	-	+	-	+	-	-	-

Indirect IFA on fixed BHK cells: +, positive; -, negative

2. GP content follow up

As shown in figure 2, comparison of the GP content of the first and second panels showed considerable differences between subsequent batches, for three of the five vaccines tested. The most striking difference was observed between the first and second lots of vaccine no. 2, which showed a more than four-fold increase in GP content. A second lot of vaccine no. 1 was not available, since it had been withdrawn from the market.

3. Protection studies with rabies virus isolated from a Dutch bat
3.1 Characterization of the cell culture adapted rabies virus isolate (EBL1-12)

The antigen profile of the BHK cell adapted virus EBL1-12 proved to be characteristic for the European Bat Lyssavirus 1 (EBL1) serogroup, which is distinct from the prototype rabies virus serotype 1 (Table 1).

3.2 Vaccine induced protection against rabies virus EBL1-12 challenge

The ED_{50} values for the second panel of vaccines (n=5) in the EBL1-12 challenge test ranged from 5.9 to 257 for four vaccines, whereas for one vaccine the 50 % protection level was not achieved (ED_{50} value < 5), as shown in figure 3. Comparison of the ED_{50} values with the GP and NP contents assessed in the respective competition ELISAs showed there to be no significant correlation (r~0, p>0.05, n=5).

DISCUSSION

In the present paper we have shown that two out of six veterinary rabies vaccines collected from veterinary practitioners
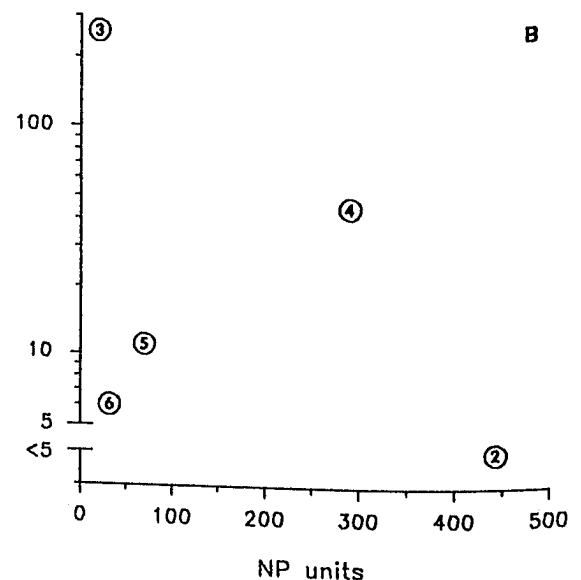
RESULTS

1. Assessment of potency values and comparison with GP and NP contents

The potency of six veterinary rabies vaccines (first panel) ranged from 0.1 to 1.4 IU, with two vaccines clearly below the requirement of 1.0 IU (nos. 1 and 2), two just below the required value (nos. 3 and 4), and two clearly above this value (nos. 5 and 6).

As shown in figure 1, comparison of potency values with antigenic values assessed in the ELISA showed a good correlation with the GP content (r=0.95, p<0.01, n=6), but not with the NP content (r~0, p>0.05, n=6).

Figure 3. Comparison between ED_{50} values in EBL1-12 challenge test and ELISA values for GP (A) and NP (B) for the second panel of five veterinary rabies vaccines tested (numbers 2-6).

in the Netherlands did not meet the minimal requirements for potency, as determined in the mouse potency test according to the European Pharmacopoeia (18). The other vaccines either met or were close to the minimal requirements for potency of 1.0 IU. Furthermore we have shown that one of the two vaccines which did not meet the requirements failed to provide acceptable levels of protection in a mouse potency test with the recently isolated rabies virus (EBL1-12) from a bat in the Netherlands. The other vaccine was not tested further because it was withdrawn from the market by the manufacturer after the first test results became available. Although the product specifications of the manufacturers indicated higher potency values than those found by us, it should be emphasized that several factors may influence the outcome of the potency tests in different laboratories. First, the vaccines that we tested had been collected directly from veterinary practices without a systematic control of cold chain conditions. This approach was chosen in order to achieve the most realistic evaluation of the potency of veterinary vaccines used in the field. Second, the use of reference vaccines of different origins and of different challenge doses, which is allowed within the specifications of the European Pharmacopoeia (18), may lead to considerable differences in test results between laboratories. In spite of these considerations we concluded that two of the vaccines tested clearly did not meet the minimal requirements.

Because of the variations observed in the outcome of mouse potency tests, but also for practical and ethical reasons, alternative methods for potency control should be sought. To date, limited information is available on possible alternatives for the actual replacement of the mouse potency test (2,3,10,11) that would be independent of laboratory animal use, which causes most of the aforementioned problems. The alternative in vitro methods that have been considered include the modified antibody binding test (4,5), the single radial immuno-diffusion test (12,13), and ELISAs (1,21,22,23, 29,31). These methods can generally not be used for adjuvanted vaccines and were used to measure the GP content. Since other viral proteins may also contribute to the induction of protective immunity (7,8,14,28,30), it may be desirable to measure the content of other viral proteins, including NP, in alternative in vitro potency tests. We tested the potential of an ELISA based on the principle of antigen competition in a monoclonal antibody inhibition system (25) to measure the GP and NP contents of the veterinary vaccines in this study. Both assays proved to be useful for potency testing of human PM vaccines produced on dog kidney cells (PM-DKC) (25) and proved not to be affected by the presence of alum-based adjuvants (unpublished results). Testing of LEP-, PM-, PV-, and SAD- based veterinary vaccines in the present study showed that a good correlation existed between the GP content and potency, whereas no such correlation was found with the NP content. This absence of correlation may be due to differences in NP content in the presence of the same GP content caused by differences in vaccine strains and manufacturing procedures. Therefore we conclude that the GP competition ELISA is a valuable tool to at least in part replace the mouse potency test in routine quality control procedures and in the screening of vaccines used in the field. Evaluation of the potential of the ELISA, with the ultimate purpose to replace the mouse potency test, should be carried out in relation to parameters of humoral and cellular protective immunity, and with special attention being paid to antigenic differences between vaccine strains.

Since the first cases of bats infected with rabies virus in the Netherlands were reported (24), limited protection studies have been carried out with rabies viruses of the European Bat Lyssavirus 1 (EBL1) serogroup (8,15,16), which proved to be antigenically different from the usually applied vaccine strains (6,9). Pre-exposure immunization studies with a rabies vaccine for human application, produced in primary dog kidney cells with the PM vaccine strain (PM-DKC) used at that time in the Netherlands, indicated that this type of vaccine induced protection in mice against intracerebral inoculation with the bat rabies virus strain EBL1-12 (unpublished results). The present data of the veterinary rabies vaccines of LEP, PM, PV, and SAD origin that we tested in the EBL1-12 challenge test confirm that protection against EBL1-12 virus infection is induced with these vaccines. The low level of protection found for one vaccine (no. 2) cannot directly be related to the type of virus strain used for the production of this vaccine, since acceptable levels of protection were induced by another vaccine produced with the same virus strain. The efficacy against EBL1-12 infection appeared not to be consistent with the GP and NP contents of the vaccines, which is probably explained by differences in antigenic composition between rabies vaccine strains and the EBL1-12 virus isolate. Antigenic differences between viruses may have implications for cross-protection against rabies-related viruses, as was reported for Duvenhage and Mokola virus previously (8). Our data show that concern about a possible inability of PM and LEP based rabies vaccines to induce protection against rabies viruses of bats, as was reported previously for the rabies-related virus Duvenhage (8,15), is less relevant for the EBL1 virus from Dutch bats.

In conclusion, the demonstrated presence of insufficient potency of veterinary rabies vaccines and of differences in their GP content, illustrates the need for veterinary authorities to maintain adequate quality control procedures for these vaccines.

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