Replacement of Foot-And-Mouth Disease Virus Cattle Tongue Titration by *In Vitro* Titration

Aldo Dekker [1], Froukje van Hemert-Kluitenberg [1], Anna H. Oosterbaan [2], Kimberly Moonen [2] and Laure Mouton [3]

[1]Wageningen Bioveterinary Research, Lelystad, The Netherlands; [2]Boehringer-Ingelheim, Lelystad, The Netherlands; [3]Boehringer-Ingelheim, Lyon, France

Abstract
Titration of foot-and-mouth disease cattle challenge virus in cattle tongue has been the standard for many years in many countries, although titration in animals has been replaced by *in vitro* methods for all other applications. The objective of the analysis was the replacement of *in vivo* titration of cattle challenge virus by *in vitro* titration. Using data from 32 *in vivo* titration experiments together with the *in vitro* titration results of the same samples obtained by plaque count on primary lamb or pig kidney cells, as well as data from the virus isolation control chart used in the laboratory, we show that the reproducibility of the *in vitro* titration is much higher than that of the *in vivo* titration. The titer on primary kidney cells was on average 1.4 $\log_{10}$ higher than the titer determined by titration in cattle tongue (PFU/ml compared to bovine ID_{50}/ml), but the difference varied among different strains. The study also shows that the probability of infection in cattle tongue is high even when a lower challenge dose is used, which makes the variability between strains less important. Based on these results, we propose to change the standard dose for cattle challenge from 10^4 bovine ID_{50} to $10^{5.4}$ PFU, and to replace the *in vivo* cattle tongue titration method with the *in vitro* titration method.

1 Introduction

Foot-and-mouth disease (FMD) is a contagious disease of cloven hoofed animals, for which vaccination is the best control option in densely populated livestock areas (Backer et al., 2009, 2012a,b). The vaccines must be of good quality. For quality control of the finished vaccine, both the World Organisation for Animal Health (OIE) manual for terrestrial animals (OIE, 2017) as well as the European Pharmacopeia (EDQM, 2017a) prescribe challenge experiments in FMD-vaccinated cattle. The OIE prescribes that 10,000 bovine ID_{50} (median bovine infectious dose; BID_{50}) is used for challenge; the European Pharmacopeia does not specify the method of titration of the virulent bovine virus. To determine the BID_{50}, the virus used for challenge is titrated in cattle.

Titration in cattle tongue was described in the early days of FMD virology (Henderson, 1949) when *in vitro* cell culture was not available. In the report of the 1972 OIE conference on standardization of potency tests, many authors considered 10,000 BID_{50} the standard challenge dose (Anon., 1972). However, it is not clear when and why 10,000 BID_{50} became the standard, especially as at the same conference it was reported that the potency of vaccines was similar for different challenge doses (10, 10,000 or 1,000,000 BID_{50}) (Terré et al., 1972).

Although *in vivo* titration of FMD virus (FMDV) has been replaced by *in vitro* titration for most applications, several FMD laboratories still use *in vivo* titration for cattle-passaged viruses intended for challenge. Until recently that was also the case in Wageningen Bioveterinary Research (WBVR, formerly known as Central Veterinary Institute) in Lelystad, The Netherlands. A quantitative relation between *in vitro* virus dose and infection by aerosol challenge has been reported before (French et al., 2002), but very limited data are available comparing the relation between *in vitro* dose and response to needle infection into the dermis of the tongue.

From a methodological point of view, it is necessary to standardize the dose used for challenge. For a standardized dose it is essential that the method used to determine the dose is robust and delivers reproducible results. From an ethical point of view, we should try to replace, reduce and refine animal experiments (EU, 2010). If animal experiments are performed, they should be as robust and reproducible as possible (https://www.nc3rs.org.uk).

In the present study, we analyze the reproducibility of cattle tongue titration from data collected over 30 years of cattle tongue titrations in comparison to the results obtained with the same samples using *in vitro* titration. The objective of the

analysis was to determine whether *in vivo* titration of cattle challenge virus could be replaced by *in vitro* titration.

2 Animals, materials and methods

Animals

Wageningen Bioveterinary Research is licensed to perform animal experiments. Permission from the animal ethical committee was obtained for all experiments prior to performing the experiment, according to the *Wet op de dierproeven* of 1977 and its amendments applicable at the times the respective experiments were performed (current license number AVD401002015265).

Conventionally reared, healthy cattle (common Dutch breeds, mainly Holstein Friesian, Friesian, MRY and Belgian blue or crossbreeds of those breeds, more than 90% female, 6-9 months old) were used. The cattle were bought by an animal provider from a Dutch farm. The cattle were individually uniquely identified.

The cattle were housed together in a tie-stall with rubber matting as bedding and were fed pelleted grass supplemented with pelleted oat husks with ad-lib water present. In the more recent experiments the cattle were provided with salt licks to fulfil licking needs, and a small amount of hay. The experiments were performed by a trained veterinarian.

Medium ingredients

The current supplier of minimal essential medium (MEM) and antibiotics is Gibco (USA), and DMSO, amido-black and fetal bovine serum (FBS) are supplied by Sigma-Aldrich (USA), but in older experiments details on producers were not recorded.

Viruses

In vivo titration data were available for the 27 strains (the names of the strains are proprietary knowledge) within serotype A (n = 10), O (n = 6), C (n = 3), Asia1 (n = 3), and SAT2 (n = 5). For 3 strains a challenge virus batch was produced twice, once for the same producer because the titer of the first batch was too low, and twice for two different producers; thus, a total of 30 different batches were tested. Cattle challenge viruses were obtained by passage of the original FMDV isolate (without cell passage, or only 1 or 2 cell passages) in cattle tongue. On approximately 20 sites 0.1 ml of the isolate, diluted between 10 and 1,000-fold in MEM supplemented with 2% FBS and antibiotics, was injected into the upper epidermal/dermal layer of the tongue of a cow under anesthesia. The cow used for passage was euthanized by captive bolt and exsanguinated when a vesicle presented (mostly after 26-28 hours). The vesicular material was collected and a suspension was made in MEM supplemented with 2% FBS and antibiotics using sterile sand and a pestle and mortar. The cattle challenge virus was stored at -70°C until use.

Cattle tongue titration

Data were available from 32 experiments performed to determine the *in vivo* titer of the challenge virus at Wageningen Bioveteri-

nary Research in Lelystad, The Netherlands, using 63 cattle (one titration was performed in only 1 cow) for the testing of 30 different batches of FMD cattle challenge virus belonging to 5 FMD serotypes (A, O, C, Asia1, and SAT2).

For each standard cattle tongue titration, two cattle were randomly assigned to receive either the low virus doses or the high virus doses. Two cattle were chosen to allow for a sufficient range of dilutions to determine the titer. The experiment was not blinded because the observer was the same person who did the inoculation. Both cattle were anesthetized in the morning in the stable by intravenous injection of Xylazine (0.2 mg/kg BW, currently Sedamun®, Dechra, The Netherlands). In both cattle, the part of the tongue between the lingual fossa and the tip was marked into three equal sections by intradermal lingual injection of ink (East Indian ink, Talens, The Netherlands) using a 0.7 x 32 mm hypodermic needle. In total, 4 dilutions of the challenge virus were tested (normally the dilutions 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} in MEM supplemented with 2% FBS and antibiotics were tested). The three highest dilutions were tested on the tongue of the first cow and the three lowest dilutions were tested on the tongue of the second cow. In each section of the tongue one dilution was tested by intradermal lingual injection into 7 spots of 0.1 ml of test dilution using a 0.7 x 32 mm hypodermic needle. In each cow the highest dilution was injected into the upper part of the tongue to avoid high concentrations of virus leaking out of the injection site onto the next injection site (see Fig. S1[1]). In the last 8 experiments, both cattle were treated with non-steroidal anti-inflammatory drugs, initially with injectable drug (Ketofen 10%, Boehringer Ingelheim, The Netherlands), but in the last experiment with Fynadyne transdermal® (MSD, The Netherlands) at the time of infection using the dose prescribed by the manufacturer.

After approximately 24 hours both cattle were anesthetized in the stable intravenously using 0.2 mg/kg bodyweight of Xylazine (currently Sedamun®, Dechra, The Netherlands) combined with 2 mg/kg of Ketamine (10%, Alfasan, The Netherlands). The tongues were inspected and the primary FMD lesions were noted. At the same time, it was evaluated whether the cattle fulfilled the humane endpoint for early euthanasia (unable to stand), which never occurred.

After approximately 48 hours, both cattle were euthanized using an overdose of pentobarbital (Euthasol 40%, AST Farma, The Netherlands) and the tongues were inspected at post-mortem. The number of injection sites that produced a vesicle were counted for each dilution and the titer, bovine ID_{50}, was calculated using the non-parametric Spearman method (Spearman, 1908). The virus dilutions used for titration in cattle tongue were frozen at -70°C and later tested by plaque count on primary porcine or ovine kidney cells, see below (Dekker, 1998; Bouma et al., 2004). For each injection site dilution, the *in vitro* titer in that specific dilution was calculated.

In vitro titration

For *in vitro* titration, the plaque count was determined in primary kidney cells derived from young, healthy (3-6 weeks of age)

lambs or pigs, specifically euthanized, using an overdose of pentobarbital (Euthasol 40%, AST Farma, The Netherlands), for the culture of the cells. The kidneys were brought to the laboratory and cells were separated by chopping the cortex into small pieces and treating them with 0.06% trypsin. When individual cells were separated, the cells were washed and suspended in MEM with Hank's Salts with 5% FBS and antibiotics. The cells were then cultured at 37°C in 24 closed culture flasks (300 cm^2, Falcon, USA). After approximately 1 week the cells were collected and suspended in medium containing 20% FBS and 10% DMSO and subsequently frozen in liquid nitrogen and stored until use.

Primary kidney cells were thawed quickly and suspended in MEM containing 5% FBS and antibiotics. After spinning down the cells, they were suspended in the same medium and seeded in 6-well collagen coated plates (Corning, USA); at least 240 plates can be cultured from 1 set of kidneys. Previous studies in our laboratory had shown that titers on lamb and pig kidney cells were very similar. In the plaque count, 200 μl of the dilutions of the sample was incubated for 1 hour at 37°C in a humidified atmosphere, before 2.5 ml of MEM containing 2% FBS and 1% methylcellulose and antibiotics was added to each well. Monolayers were stained after 1 or 2 days (depending on the plaque formation observed microscopically) using amido-black (0.1% amido-black in 1 M acetic acid, 0.09 M sodium acetate, 10% glycerol). In wells with 1 to 50 plaques, the plaques were counted and the number of plaque forming units in the original suspension was calculated. Information on variation in the plaque count was obtained from the samples included in titration studies, as well as from the control chart of the positive control (C$_1$Detmold challenge virus) used in virus isolation between 2004 to 2017 (n = 228) (Tab. S1[1]).

Statistics

A logistic regression model (EDQM, 2017b) using a binomial error distribution and a logit link function was used to determine the titer of the virus in each experiment (n = 32) and for each cow (n = 60, 2 cattle were excluded as they did not show any lesion, and 1 because lesions were seen on all injection sites). In the logistic regression model, the result variable was "lesions formation", and possible explanatory variables were the *in vitro* dose injected, the experiment and the animal. Normality of the data was assessed by evaluating the normal Q-Q plot. All statistical analyses were performed in R.[2]
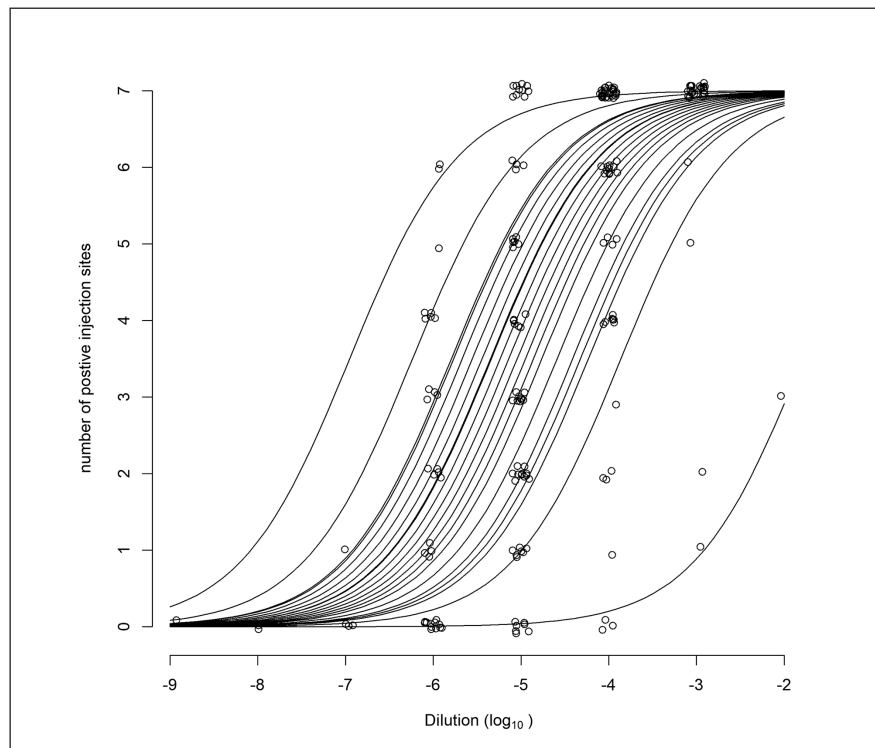
3 Results

All cattle were healthy at the start of the experiments. In the last 8 experiments the cattle were treated with NSAIDs at the time of virus application as a refinement. There were no adverse events and only the *in vitro* titer sometimes indicated that different dilutions would have been preferable. The log$_{10}$ dilutions -6, -5, -4, and -3 were tested in most experiments, but in some experiments other dilutions were also included.

All observations are plotted together with the estimated dose response curves for each experiment in Figure 1. The logistic re-

Fig. 1: Observations on *in vivo* response in relation to dilutions after injection of 0.1 ml into the tongue
The lines represent the estimated logistic regression lines for each experiment. Individual observations are slightly displaced randomly on the vertical axis to avoid overlap.
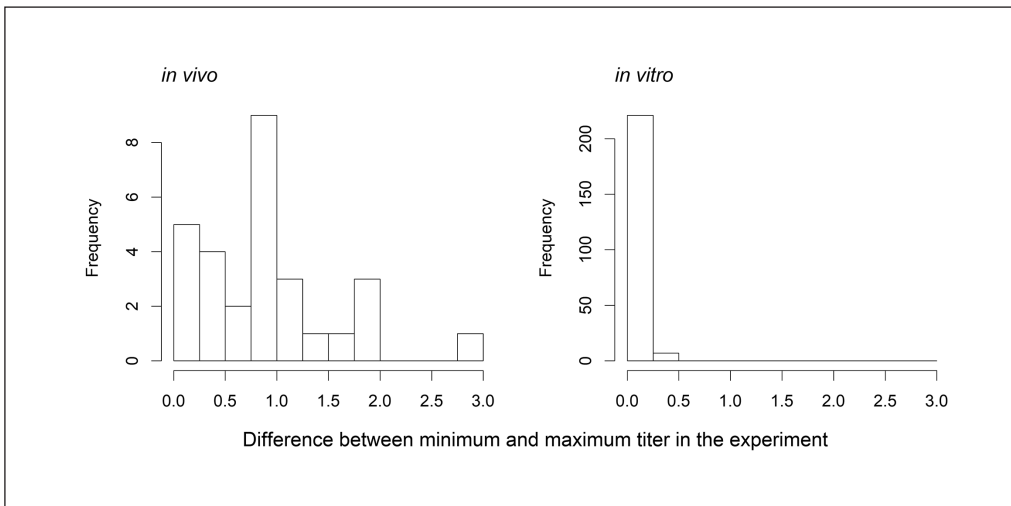
Fig. 2: Histograms of the difference between minimum and maximum titer observed in the same experiment, for both the *in vivo* titration and the results from duplicate control samples in *in vitro* titration

gression model with both *in vitro* dose and experiment as explanatory variables (the curves shown in Fig. 1) fitted significantly better than a model with only the *in vitro* dose as explanatory variable (p < 0.001, likelihood ratio test). The logistic regression model with both *in vitro* dose and experiment was used to calculate the *in vivo* titer (BID_{50}/ml) for each experiment. The logistic regression model with both *in vitro* dose and animal as explanatory variables fitted significantly better than a model with only the *in vitro* dose as explanatory variable (p < 0.001, likelihood ratio test). Therefore, the logistic regression model with both *in vitro* dose and animal was used to calculate the *in vivo* titer (BID_{50}/ml) for each cow (cattle with lesions at all sites or no lesions at all were excluded). The AIC (Akaike information criterion) for the logistic regression model using *in vitro* dose and experiment was 668, whereas the AIC for logistic regression

model using *in vitro* dose and animal was 515. The models using interaction were not further explored.

The between-experiment minimum, maximum, mean and standard deviation (SD) of the *in vivo* as well as *in vitro* titers were calculated (Tab. 1). The difference between *in vivo* and *in vitro* titration was on average 1.4 $\log_{10}$, but the difference varied for different strains (SD = 0.77 $\log_{10}$). The range in difference between *in vivo* and *in vitro* titration was 0.06-2.7. This shows that the analytical sensitivity of primary cells was always higher than the analytical sensitivity of a cattle tongue.

For analysis of the *in vivo* reproducibility (within test variation), the within-experiment differences of the 2 *in vivo* observations are shown in Figure 2. The within-experiment SD of the *in vivo* titration varied between 0 and 1.6, with a mean of 0.5. Table 2 shows the results of the experiment (O Panasia 2 strain) where the largest

Tab.1: Summary statistics of titers found in various experiments and individual animals

Titer	Minimum	Maximum	Mean	SD
In vivo ($\log_{10}$ BID_{50}/ml) per experiment (n = 32)	2.8	8.0	6.0	0.9
In vivo ($\log_{10}$ BID_{50}/ml) per animal (n = 60)	3.0	7.8	5.9	0.8
in vitro ($\log_{10}$ PFU/ml)	5.2	8.5	7.4	0.8

Tab. 2: Result of titration of FMD cattle challenge strain O Panasia 2
In this experiment the highest difference (2.9 $\log_{10}$ PFU) between the 50% point in both cattle was observed.

Dilution ($\log_{10}$)	Titer injected ($\log_{10}$ PFU per dose)	Positive sites (vesicular lesions)	
		Cow 7372	**Cow 7373**
-6	0.57	NA	2
-5	1.57	0	7
-4	2.57	2	7
-5	1.57	2	NA

difference observed between 2 individual cattle was observed. For repeatability and reproducibility (between test variation) of the *in vitro* titration only very limited data were available for the challenge viruses; several were only titrated once. Therefore, the data of the control chart 2004-2017 was analyzed (Tab. S1[1]). In total, 228 test data were available of duplicate titration of C_1Detmold challenge virus used as positive control in virus isolation in the laboratory. Figure 2 shows the observed differences between the 2 duplicates, the within test SD varied between 0 and 0.3 $\log_{10}$ PFU/ml with a mean of 0.1 $\log_{10}$ PFU/ml. Over a period of 13 years, the average titer of the positive control was 2.6 $\log_{10}$ PFU/ml with an SD of 0.3.

For 2 strains, A_5Westerwald and O/ITA/1993, the *in vivo* titration was repeated. For A_5Westerwald, the dilutions chosen were too high in the first experiment (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}). In the repetition, dilutions 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were tested. The estimated titers were $10^{6.3}$ and $10^{6.7}$ BID_{50}/ml, respectively for the first and the second experiment. In the case of O/ITA/1993, the experiment was excluded from the reproduction analysis as the titration was repeated because a mistake was suspected in the first experiment.

Based on the model, the probability that one injection site becomes positive when injecting 5,000 BID_{50} was 99.7%. So, the probability that a cow develops a lesion on at least one site when injected with 10,000 BID_{50} into two sites of the tongue (as recommended by the OIE manual), is 100%. Using the logistic model with the *in vitro* titer and experiment as explanatory variables, the probability that an injection with 5.1 $\log_{10}$ PFU results in a vesicular lesion is 97.7-100%, so also 100% probability of developing at least one vesicular lesion when injected at two sites (which is a total of 5.4 $\log_{10}$ PFU and based on the comparison of the mean *in vivo* titer and the mean *in vitro* titer similar to 10,000 BID_{50}). The use of 10,000 PFU (3.7 $\log_{10}$ per site) would result in a probability of infection of at least one of the 2 sites of 97-100%.

4 Discussion

The objective of the analysis was the replacement of *in vivo* titration of cattle challenge virus by *in vitro* titration.

In the analysis of the *in vivo* titration data we used logistic regression as recommended by the European Pharmacopoeia (EDQM, 2017b). By combining results of all tests into one logistic regression model without interaction we assume a common slope for all experiments (Fig. 1). The model with both *in vitro* dose and experiment did fit better than the model with only *in vitro* dose. This shows that the relation between *in vivo* and *in vitro* virus titer is different for each strain. This confirms a previous finding that the sensitivity of *in vivo* and *in vitro* virus titration is different for different strains (House and House, 1989). Analysis of the SD of *in vivo* and *in vitro* titration (Tab. 1), which reflects mainly the difference in virus concentration in different samples, shows that the overall variation between both methods is similar.

The fact that the logistic regression model with both *in vitro* dose and animal has a lower AIC (value = 515) than the logistic regression model with both *in vitro* dose and experiment (value = 668) shows that the variation in response is better explained by differences in both strain and animal (which are not independent) than with difference in strain alone. Therefore, we also analyzed the *in vivo* between-animal variation and compared that with the variation found in *in vitro* titration. The maximum and mean of the within test SD is much larger in *in vivo* titration (Fig. 2). This shows that the *in vitro* titration has a higher reproducibility (within test variation). For the *in vivo* titration only one valid result of a repeated test was available, so it is not possible to draw any conclusions on reproducibility (between test variation) of the *in vivo* titration. For the *in vitro* titration it was easy to find data on reproducibility; an SD of 0.3 $\log_{10}$ was found in the data from the virus isolation control chart. Based on these results, it can be concluded that the reproducibility (variation in results in the same test) of *in vivo* titration is lower than that of the *in vitro* titration.

Based on the low reproducibility of the *in vivo* titration as well as the methodological short-comings, *in vitro* titration of FMD cattle challenge virus is the preferred method. Although *in vivo* titration is probably not used a lot for other agents, it is advisable that a similar analysis is performed for these cases. It is likely that also in those instances an *in vitro* method would demonstrate a higher reproducibility.

The next question is the dose that should be used for a challenge test. Review of old literature did not reveal the reason for the choice of 10,000 BID_{50}; the analysis of the results, however, supports this choice as it shows that the probability that at least one vesicle is produced when injected at 2 sites is 100%. So, the probability of control cattle developing FMD generalization is extremely high under the assumption that production of one vesicle is enough to produce generalization in control cattle. This assumption is probably largely true because the potency of 10, 10,000 or 1,000,000 BID_{50} has been shown to be very similar (Terré et al., 1972).

In our studies we observed a difference in mean titer between *in vivo* and *in vitro* values of 1.4 $\log_{10}$. So, we propose that the best dose for infection would be 5.4 $\log_{10}$ PFU (5.1 $\log_{10}$ PFU per site), at which the overall probability of infection would also be 100% when injecting at 2 sites. Using a lower *in vitro* dose could lead to a slightly lower probability of infection of the control cattle, although still over 97% probability of infection of one of the 2 sites used for infection when using 10,000 PFU. Laboratories that cannot use primary cells should use a different dose as in some cells higher *in vitro* titers are seen than in primary cells, e.g., in LBFK-αVβ6 cells (LaRocco et al., 2013, 2015) or a lower *in vitro* titer than in primary kidney cells (House and House, 1989). Even though a high number of *in vitro* titrations can be performed with the kidneys of one animal, it would be better to use a continuous cell line for which the relation with the titer in primary cells is known.

The current study has a few limitations: There was only one valid repetition of the *in vivo* virus titration, which limits the analysis of *in vivo* reproducibility. Furthermore, the analysis of the titration in cattle tongue assumes that observations at different sites of the tongue are independent. However, as they are

observed in the same animal, this assumption is not valid. It also assumes that all parts of the tongue are equally sensitive to FMD infection and lesions can be as easily formed at the back of the tongue as on the tip. This assumption is most likely also not valid. Dependence on the site of injection probably could explain the huge difference found between cattle. So, from a methodological point of view, *in vivo* titration is not a valid method, which supports the change to *in vitro* titration of challenge virus.

The analysis was performed to support changes to legislation and to replace titration in cattle tongue by *in vitro* titration. Based on the analysis, *in vivo* titration of FMD cattle challenge virus can be replaced by *in vitro* titration in primary kidney cells using a total of 5.4 $\log_{10}$ PFU for intradermal injection into two sites of the tongue (0.1 ml per site).

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Conflict of interest