Research Article

Replacement of foot-and-mouth disease virus cattle tongue titration by in vitro titration

Aldo Dekker1, Froukje van Hemert-Kluitenberg1, Anna H. Oosterbaar2, Kimberly Moonen2, Laure Mouton3

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 for all other applications titration in animals has become replaced by in vitro methods. 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, obtained by plaque count on primary lamb or pig kidney cells, of the same samples, as well as data from the virus isolation control chart used in the laboratory, we show that the repeatability of the in vitro titration is much higher than the repeatability of the in vivo titration. The titer on primary kidney cells was on average 1.4 log10 higher than the titer determined by titration in cattle tongue (PFU/ml compared to bovine ID50/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 ID50 to 10^5.4 PFU, and therefore replace the in vivo cattle tongue titration method with the in vitro titration method.

Keywords: FMDV, potency test, challenge, vaccine, animal study

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; Backer et al., 2012a; Backer et al., 2012b). The vaccines should, however, be of good quality. For quality control of the finished vaccine, both the World Organisation for Animal Health (OIE) terrestrial Manual (OIE, 2017) as well as the European Pharmacopeia (Anonymous, 2017a) describe challenge experiments in FMD-vaccinated cattle. The OIE prescribes that 10 000 bovine ID50 (Median bovine infectious dose; BID50) is used for challenge, the European Pharmacopoeia does not specify the method of titration of the virulent bovine virus. To determine the BID50, the virus for challenge is titrated in cattle.

Titration in cattle tongue was developed in the early days of FMD virology (Henderson, 1949), but was primarily developed when in vitro cell culture was not available. In the report of the 1972 OIE conference on standardization of potency tests, it was shown that many authors consider 10 000 BID50 as standard challenge dose (Anonymous, 1972). It is, however, not clear when and why the use of 10 000 BID50 has become a standard, considering that at the same conference it was reported that the potency of vaccines was similar when using different challenge doses (10, 10 000 or 1 000 000 BID50) (Terré et al., 1972).

As 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 used 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 have been reported before (French et al., 2002), but very limited data are available on comparison between the relation between in vitro dose and response to needle infection into dermis of the tongue.

From a methodological point of view, it is essential to standardize the dose used for challenge. For a standardized dose it is essential that the method used to determine the dose is repeatable and reproducible. 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 possible1.

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1 https://www.nc3rs.org.uk
In the present study, we analyze the repeatability and reproducibility of cattle tongue titration from data collected over 30 years of both cattle tongue titrations and the results obtained by in vitro titration. The objective of the analysis was the replacement of in vivo titration of cattle challenge virus by in vitro titration.

2 Materials and methods

Animals
The institute Wageningen Bioveterinary Research is licensed to perform animal experiments, and for all experiments permission from the animal ethical committee was obtained prior to performing the experiment, according to the Wet op de diertproeven, 1977 and its amendments applicable at the time the experiments were performed (current license number AVD401002015265).

Conventionally reared, healthy cattle (common Dutch breeds, mainly Holstein Friesian, Friesian, MRY and Belgian blue or crosses 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 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 and antibiotics is Gibco (USA), and DMSO, amido-black and fetal bovine serum is supplied by Sigma-Aldrich (USA), but in older experiments details on producers were not recorded.

Viruses
In vitro 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, one time for the same producer because the titer of the first batch was too low, and twice for 2 different producers, therefore 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 1000 fold in minimal essential medium supplemented with 2% fetal bovine serum and antibiotics, was injected in 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 minimal essential medium supplemented with 2% fetal bovine serum 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 Bioveterinary 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 chose 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 Sedamus®, Dechra, the Netherlands). In both cattle, the part of the tongue between the lingual fossa and the tip was divided into three equal sections by intradermal lingual injection of ink (East Indian ink, Talens, the Netherlands) using a 0.7x32 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 were made in minimal essential medium supplemented with 2% fetal bovine serum and antibiotics). 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 at 7 spots of 0.1 ml of test dilution using a 0.7x32 mm hypodermic needle. In each cow the highest dilution was injected in the upper part, to avoid high concentrations of virus leaking out of the injection site onto the next injection site (see Fig. S1). 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 injection using the dose prescribed by the producer.

After approximately 24 hours both cattle were anesthetized in the stable intravenously using 0.2 mg/kg BW of Xylazine (currently Sedamus®, 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 the cattle were evaluated whether they fulfilled the humane endpoint (unable to stand) for early euthanasia, which never occurred.

After approximately 48 hours both cattle were euthanized using an overdose of pento-barbital (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 ID50, 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.

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on primary porcine or ovine kidney cells, see below (Dekker, 1998; Bouma et al., 2004). For each injection site dilution, the 

**In vitro titration**

For **in vitro** titration, 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 pento-barbital (Euthasol 40%, AST Farma, the Netherlands), for the culture of the cells. After euthanasia the kidneys for cell culture 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 are washed and suspended in minimal essential medium, with Hank’s Salts, with 5% fetal bovine serum and antibiotics. The cells were cultured at 37°C in 24 closed culture flasks (300 cm², Falcon, USA). After approximately 1 week the cells were collected and suspended in medium containing 20% fetal bovine serum and 10% DMSO and subsequently frozen for storage in liquid nitrogen until use. Primary kidney cells from the liquid nitrogen are thawed quickly and suspended in minimal essential medium containing 5% fetal bovine serum and antibiotics. After spinning down the cells, the cells are 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 an humidified atmosphere, before 2.5 ml of minimal essential medium containing 2% fetal bovine serum and 2% 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 (Ci:Detmold challenge virus) used in virus isolation between 2004 to 2017 (n = 228) (Tab. S1).

**Statistics**

A logistic regression model (Anonymous, 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 as possible explanatory variables: the **in vitro** dose injected, experiment and animal. Normality of the data was assessed by evaluating the normal Q-Q plot. All statistical analyses were performed in R.3

### 3 Results

In all experiments the cattle were healthy. 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 was sometimes an indication to use different dilutions. In most experiments the log<sub>10</sub> dilutions -6, -5, -4 and -3 were tested, but in some experiments other dilutions were also included.

In Figure 1 all observations are plotted together with the estimated dose response curves for each experiment. The logistic regression model with both **in vitro** dose and experiment as explanatory variables (the curves shown in Figure 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<sub>50</sub>/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, logistic regression model with both **in vitro** dose and animal was used to calculate the **in vivo** titer (BID<sub>50</sub>/ml) for each cow (cattle with lesions on 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<sub>10</sub>, but the difference varied for different strains (SD = 0.77 log<sub>10</sub>). 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** repeatability (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 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 were analyzed (Tab. S13). In total, 228 test data were available of duplicate titration of Ci:Detmold 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 as a measure of repeatability varied between 0 and 0.3 log<sub>10</sub> PFU/ml with a mean of 0.1 log<sub>10</sub> PFU/ml. Over a period of 13 years the average titer of the positive control was 2.6 log<sub>10</sub> PFU/ml with a SD of 0.3, which is a measure of reproducibility.

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Fig. 1: Observations on in vivo response in relation to dilutions injected after injection of 0.1 ml in 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 in the same experiment, for both the in vivo titration and the results from duplicate control samples in in-vitro titration

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

<table>
<thead>
<tr>
<th>Titer</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo (log_{10} BID_{50}/ml) per experiment (n = 32)</td>
<td>2.8</td>
<td>8.0</td>
<td>6.0</td>
<td>0.9</td>
</tr>
<tr>
<td>In vivo (log_{10} BID_{50}/ml) per animal (n = 60)</td>
<td>3.0</td>
<td>7.8</td>
<td>5.9</td>
<td>0.8</td>
</tr>
<tr>
<td>In vitro (log_{10} PFU/ml)</td>
<td>5.2</td>
<td>8.5</td>
<td>7.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Dilution (log_{10})</th>
<th>Titer injected (log_{10} PFU per dose)</th>
<th>Positive sites (vesicular lesions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-6</td>
<td>0.57</td>
<td>Cow 7372</td>
</tr>
<tr>
<td>-5</td>
<td>1.57</td>
<td>Cow 7373</td>
</tr>
<tr>
<td>-4</td>
<td>2.57</td>
<td></td>
</tr>
<tr>
<td>-3</td>
<td>1.57</td>
<td></td>
</tr>
</tbody>
</table>

For 2 strains, A5Westerwald and O/ITA/1993, the in vivo titration was repeated. For A5Westerwald, the first experiment the dilutions chosen were too high (10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}). In the repetition 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions 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 in the first experiment was suspected.

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 have used logistic regression which is recommended by the European Pharmacopoeia (Anonymous, 2017b). By combining results of all tests into one logistic regression model without interaction we assume a common slope for all experiments (Figure 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 (Table 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 (Figure 2). This shows that the in vitro titration has a higher repeatability (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; a 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 repeatability (variation in results in the same test) with in vivo titration is lower than the repeatability 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. 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 get generalization in control cattle. This assumption is probably largely true because in potency tests using 10, 10 000 or 1,000,000 BID$_{50}$ the potency was 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 for infection. 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 titration 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 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).

References


**Conflict of interest**
The authors declare the following competing financial interest(s): Laure Mouton, Anna H. Oosterbaan and Kimberly Moonen are employed by one of the vaccine producers that own the cattle challenge strains. The opinions expressed in this article are not informed by this affiliation.