**POTENCY AND EFFICACY for bovine vaccines containing bovine herpesvirus 1 (BoHV-1) causal agent of the Infectious Bovine Rhinotracheitis (IBR)**

REBUTAL LETTER to the queries from USA in CAMEVET

-Donna Gatewood, Section Leader, Virology, USDA/APHIS/VS/CVB PEL

-David Siev, Statistics Stetion Leader, USDA

Questions

1- **CVB allows in vitro potency tests using a parallel line assay and a validated reference vaccine.**
   The statement was included in the test. A proper literature reference given by APHIS is needed.

2- **Original data set to obtain the SPLIT POINTS values for vaccine classification**

In all cases the measure unit was consider the **potency** of the calibrating vaccines, expressed as the mean Ab titers of groups of 5 guinea pigs/bovines receiving two doses of each vaccine.

Calibrating vaccines were formulated to contain increasing concentrations of BoHV-1 antigen/dose, differing in 1log10.

*Guinea pig: calculation of vaccine classification split points*

**Table 1.** Guinea Pigs ELISA (n= 5 per group)

| Experimental group | log10 BoHV-1 conc./dose | Mean ELISA Ab titer | 90% prediction interval | |
|---|---|---|---|---|
| | | | lower | upper |
| 1 | 8 | 3,65 | 3,2 | 4,32 |
| 2 | 8 | 4,13 | 3,2 | 4,32 |
| 3 | 8 | 3,64 | 3,2 | 4,32 |
| 4 | 8 | 4,01 | 3,2 | 4,32 |
| 1 | 7 | 3,05 | **3,02** | 4,07 |
| 2 | 7 | 3,52 | **3,02** | 4,07 |
| 3 | 7 | 3,40 | **3,02** | 4,07 |
| 4 | 7 | 3,28 | **3,02** | 4,07 |
| 5 | 7 | 3,25 | **3,02** | 4,07 |
| 6 | 7 | 3,54 | **3,02** | 4,07 |
| 1 | 6 | 2,96 | 1,93 | 2,98 |
| 2 | 6 | 2,84 | 1,93 | 2,98 |
| 3 | 6 | 2,80 | 1,93 | 2,98 |
| 4 | 6 | 2,80 | 1,93 | 2,98 |
| 5 | 6 | 2,27 | 1,93 | 2,98 |
| 6 | 6 | 2,28 | 1,93 | 2,98 |
| 1 | 5 | 0,56 | -0,04 | 1,04 |
| 2 | 5 | 0,82 | -0,04 | 1,04 |
| 3 | 5 | 0,30 | -0,04 | 1,04 |
| 4 | 5 | 0,30 | -0,04 | 1,04 |
| 5 | 5 | 0,30 | -0,04 | 1,04 |
| 6 | 5 | 0,30 | -0,04 | 1,04 |

**Table 2.** Guinea Pigs VN (n= 5 per group)

| Experimental group | log10 BoHV-1 conc./dose | Mean VN Ab titer | 90% prediction interval | |
|---|---|---|---|---|
| | | | lower | upper |
| 1 | 8 | 2,92 | 2,03 | 3,25 |
| 2 | 8 | 3,00 | 2,03 | 3,25 |
| 3 | 8 | 2,6 | 2,03 | 3,25 |
| 4 | 8 | 2,56 | 2,03 | 3,25 |
| 1 | 7 | 2,32 | **2,05** | 3,21 |
| 2 | 7 | 2,84 | **2,05** | 3,21 |
| 3 | 7 | 2,46 | **2,05** | 3,21 |
| 4 | 7 | 2,00 | **2,05** | 3,21 |
| 5 | 7 | 2,44 | **2,05** | 3,21 |
| 6 | 7 | 2,15 | **2,05** | 3,21 |
| 1 | 6 | 2,38 | 1,31 | 2,46 |
| 2 | 6 | 2,52 | 1,31 | 2,46 |
| 3 | 6 | 1,72 | 1,31 | 2,46 |
| 4 | 6 | 2,28 | 1,31 | 2,46 |
| 5 | 6 | 1,98 | 1,31 | 2,46 |
| 6 | 6 | 1,99 | 1,31 | 2,46 |
| 1 | 5 | 0,36 | -0,18 | 1,01 |
| 2 | 5 | 0,42 | -0,18 | 1,01 |
| 3 | 5 | 0,30 | -0,18 | 1,01 |
| 4 | 5 | 0,30 | -0,18 | 1,01 |
| 5 | 5 | 0,30 | -0,18 | 1,01 |
| 6 | 5 | 0,30 | -0,18 | 1,01 |

Note: Split point were obtained as the lower limits of the 90% prediction interval (corresponding to the 95% of coverage) for vaccine containing 10^6 and 10^7 DICT50 of BoHV-1 per dose.

**Comment [MVL1]:** VN

*Regression analysis*

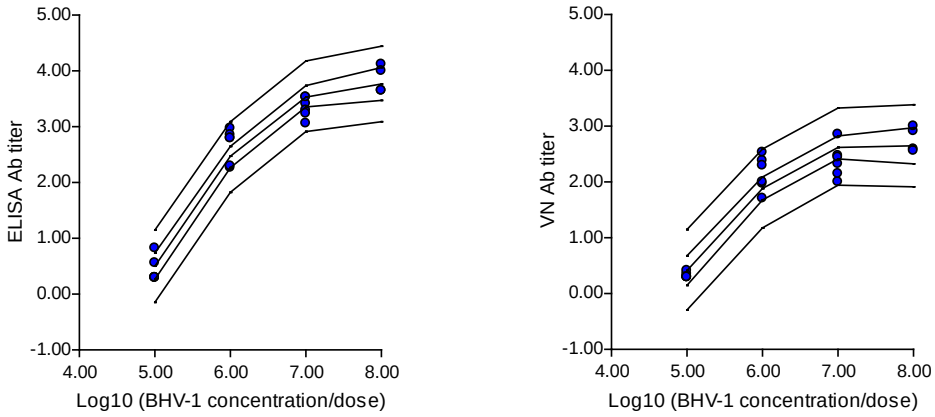
| Variable | N | $R^2$ | $R^2$ Aj |
|---|---|---|---|
| ELISA Ab titer | 22 | 0,96 | 0,95 |

| Coef | Est. | EE | LI(95%) | LS(95%) | T | p-value | CpMallows |
|---|---|---|---|---|---|---|---|
| constant | -22,38 | 2,56 | -27,75 | -17,01 | -8,73 | <0.0001 | |
| BHV-1 conc./dose | 6,76 | 0,81 | 5,05 | 8,46 | 8,31 | <0.0001 | 67,65 |
| BHV-1 conc./dose^2 | -0,44 | 0,06 | -0,57 | -0,3 | -6,92 | <0.0001 | 47,56 |

| Variable | N | $R^2$ | $R^2$ Aj |
|---|---|---|---|
| VN Ab titer | 22 | 0,9 | 0,89 |

| Coef | Est. | EE | LI(95%) | LS(95%) | T | p-value | CpMallows |
|---|---|---|---|---|---|---|---|
| constant | -17,86 | 2,82 | -23,75 | -11,96 | -6,34 | <0.0001 | |
| BoHV-1 conc./dose | 5,48 | 0,89 | 3,61 | 7,34 | 6,13 | <0.0001 | 37,78 |
| BoHV-1 conc./dose^2 | -0,36 | 0,07 | -0,51 | -0,22 | -5,26 | <0.0001 | 28,36 |

Figure 1. Regression analysis and estimation of split point for vaccine classification in guinea pig by ELISA and VN




| Guinea pig: Vaccine classification | | | |
|---|---|---|---|
| | Low <10^6 | Satisfactory [10^6 – 10^7] | Very satif. >10^7 |
| **ELISA** | X < 1.93 | 1,93≤ X ≤3,02 | 3,02 < X |
| **VN** | X < 1,31 | 1,31≤ X ≤ 2,05 | 2,05 < X |

*Bovines: calculation of vaccine classification split points*

**Table 3**

| Exp | conc BHV-1 | Mean VN Ab titer | Mean ELISA Ab titer | ELISA 90% Prediction interval | | VN 90% Prediction interval | |
|---|---|---|---|---|---|---|---|
| | | | | lower | upper | lower | upper |
| 1 | 8 | 2,62 | 3,4 | 2,53 | 4,25 | 1,89 | 2,86 |
| 2 | 8 | 2,72 | 3,28 | 2,53 | 4,25 | 1,89 | 2,86 |
| 3 | 8 | 1,96 | 2,8 | 2,53 | 4,25 | 1,89 | 2,86 |
| 4 | 8 | 2,32 | 4,16 | 2,53 | 4,25 | 1,89 | 2,86 |
| 1 | 7 | 2,05 | 2,95 | **2,72** | 4,34 | **1,96** | 2,88 |
| 2 | 7 | 2,42 | 3,16 | **2,72** | 4,34 | **1,96** | 2,88 |
| 4 | 7 | 2,02 | 3,77 | **2,72** | 4,34 | **1,96** | 2,88 |
| 5 | 7 | 2,62 | 4,13 | **2,72** | 4,34 | **1,96** | 2,88 |
| 6 | 7 | 2,6 | 3,4 | **2,72** | 4,34 | **1,96** | 2,88 |
| 1 | 6 | 1,96 | 2,68 | **1,69** | 3,32 | **1,27** | 2,19 |
| 2 | 6 | 2,04 | 2,65 | **1,69** | 3,32 | **1,27** | 2,19 |
| 4 | 6 | 1,82 | 3,28 | **1,69** | 3,32 | **1,27** | 2,19 |
| 5 | 6 | 1,72 | 1,96 | **1,69** | 3,32 | **1,27** | 2,19 |
| 6 | 6 | 1,5 | 2,2 | 1,69 | 3,32 | **1,27** | 2,19 |
| 1 | 5 | 0,3 | 0,3 | -0,54 | 1,18 | -0,15 | 0,82 |
| 2 | 5 | 0,3 | 0,3 | -0,54 | 1,18 | -0,15 | 0,82 |
| 5 | 5 | 0,3 | 0,3 | -0,54 | 1,18 | -0,15 | 0,82 |
| 6 | 5 | 0,3 | 0,3 | -0,54 | 1,18 | -0,15 | 0,82 |

Note: Split point were obtained as the lower limits of the 90% prediction interval (corresponding to the 95% of coverage) for vaccine containing 10^6 and 10^7 DICT50 of BoHV-1 per dose.

*Regression analysis*

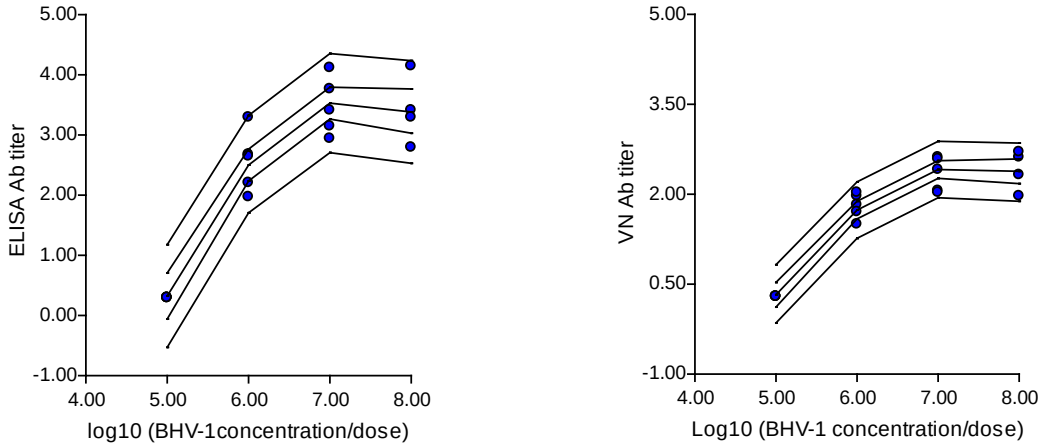
| Variable | N | $R^2$ | $R^2$ Aj |
|---|---|---|---|
| ELISA | 18 | 0,90 | 0,89 |

| Coef | Est. | EE | LI(95%) | LS(95%) | T | p-valor | CpMallows | |
|---|---|---|---|---|---|---|---|---|
| const | -28,06 | 4,34 | -37,3 | -18,81 | -6,47 | <0.0001 | | |
| conc BHV-1 | 8,58 | 1,36 | 5,68 | 11,48 | 6,31 | <0.0001 | 39,39 | |
| conc BHV-1^2 | -0,58 | 0,1 | -0,8 | -0,36 | -5,57 | 0,0001 | 31,17 | |

| Variable | N | $R^2$ | $R^2$ Aj |
|---|---|---|---|
| VN | 18 | 0,93 | 0,92 |

| Coef | Est. | EE | LI(95%) | LS(95%) | T | p-valor | CpMallows | |
|---|---|---|---|---|---|---|---|---|
| const | -17,53 | 2,46 | -22,76 | -12,29 | -7,14 | <0.0001 | | |
| conc BHV-1 | 5,38 | 0,77 | 3,74 | 7,02 | 6,98 | <0.0001 | 47,78 | |
| conc BHV-1^2 | -0,36 | 0,06 | -0,49 | -0,24 | -6,11 | <0.0001 | 37,11 | |

Figure 2. Regression analysis and estimation of split point for vaccine classification in guinea pig by ELISA and VN




| Bovine: Vaccine classification | | | |
|---|---|---|---|
| | Low <10^6 | Satisfactory [10^6 – 10^7] | Very satif. >10^7 |
| **ELISA** | X < 1.69 | 1.69≤ X ≤2.72 | 3,02 < X |
| **VN** | X < 1.27 | 1.27≤ X ≤ 1.96 | 1.96 < X |

3- **Guinea pig receives two doses of vaccine, 21 days apart. Why the 2 vaccination schedules?**

The guinea pigs were vaccinated with two doses of vaccines since this schedule allowed us to obtain detectable Ab responses in vaccines of low immunogenicity and also the shape of the curve of the kinetic of the Ab response using this vaccine regime better resemble the kinetic obtained in the target species, than the one obtained when administering 1 vaccine dose.

**4- Note that the cutoffs proposed by the authors are intended to discriminate only between differences of at least one common logarithm (i.e. tenfold).**

**Note also that observed VN specificity was 0.83 and observed ELISA specificity was 1.0. (Specificities were not reported in the paper but are more useful than the kappa statistic.)**

It is important to remark that serial 10-fold dilutions was considered to be the most appropriate scale to use in the dose response assay in order to homologate the scale and its associated error to that used for titration of virus batches selected in vaccine formulation and VN Ab testing. In general, in the Virology field it is recommended to carry out 10-fold dilutions for virus titration as well as for virus back titration within virus neutralization assays. In addition, at the initial stage of this study vaccines containing

5x10^6 and 5x10^7 TCID50 were formulated and tested. The results showed that the lab animal model and the host species could not discriminate among vaccines formulated with antigen doses within of the same order of magnitude (i.e. 5x10^6 and 1x10^6 TCID50 induce a detectable Ab response while 5x10^5 and 1x10^5 TCID50 induced responses in some guinea pigs but did not induce detectable response in bovines), then a 1log10 difference among Ag concentration was chosen to conduct the validation.

Regarding the **specificity** of the guinea pig model to properly classified vaccines, we agree this is a good parameter to validate the model in addition to the agreement with bovine. During the validation process the specificity of the model using both serologic techniques (VN and ELISA) was estimated using animals vaccinated with 22 gold standard vaccines of known antigen concentration.

| Guinea Pig ELISA | Gold Standard Vaccines (known Ag concentration) | | |
|---|---|---|---|
| | (vaccine with 10^5 TCID50/dose and placebos and non vaccinated controls | Vaccines formulated with $10^6$ TCID50/dose | Vaccines formulated with ≥$10^7$ TCID50/dose or higher |
| **Low** (<1.93) | 6 | 0 | 0 |
| **Satisfactory** (1.93 – 3.02) | 0 | 6 | 0 |
| **Very satisfactory** (>3.02) | 0 | 0 | 10 |

Weighted Kappa: 1.00 ASE= 0.00 LCI= 1.00 LCS= 1.00

| Guinea Pig VN | Gold Standard | | |
|---|---|---|---|
| | ≤$10^5$ (E y Control) | $10^6$ (D) | ≥$10^7$ (A, B, C) |
| **Low** (<1.31) | 6 | 0 | 0 |
| **Satisfactory** (1.31 – 2.05) | 0 | 3 | 1 |
| **Very satisfactory** (>2.05) | 0 | 3 | 9 |

Weighted Kappa= 0.80 ASE= 0.10 LCI= 0.61 LCS= 0.99

| Gold Standard Vaccines (known Ag concentration) | Guinea Pig ELISA | | |
|---|---|---|---|
| | **Low** (<1.93) | **Satisfactory** (1.93 – 3.02) | **Very satisfactory** (>3.02) |
| **(vaccine with 10^5 TCID50/dose and placebos and non vaccinated controls** | 6 | 0 | 0 |
| **Vaccines formulated with $10^6$ TCID50/dose** | 0 | 6 | 0 |
| **Vaccines formulated with ≥$10^7$ TCID50/dose or higher** | 0 | 0 | 10 |

Predictivity for 10^5 vaccines 100%
Predictivity for 10^6 vaccines 100%
Predictivity for 10^7 or higher 100%

Precision for 10^5 vaccines 100%
Precision for 10^6 vaccines 100%
Precision for 10^7 or higher 100%

Accuracy 100%

According to ECVAM (2002) the test performance is excellent

| Gold Standard Vaccines (known Ag concentration) | Guinea Pig VN | | |
|---|---|---|---|
| | **Low** (<1.31) | **Satisfactory** (1.31 – 2.05) | **Very satisfactory** (>2.05) |
| **(vaccine with 10^5 TCID50/dose and placebos and non vaccinated controls** | 6 | 0 | 0 |
| **Vaccines formulated with $10^6$ TCID50/dose** | 0 | 3 | 3 |
| **Vaccines formulated with ≥$10^7$ TCID50/dose or higher** | 0 | 1 | 9 |

Predictivity for 10^5 vaccines 100%
Predictivity for 10^6 vaccines 75%
Predictivity for 10^7 or higher 75%

Precision for 10^5 vaccines 100%
Precision for 10^6 vaccines 50%
Precision for 10^7 or higher 90%

Accuracy 82%

According to ECVAM (2002) the test performance is excellent for predictivity and precision of 10^5 vaccines and precision of 10^7 or higher vaccines, insufficient for precision of 10^6 vaccines and good for the rest of the criteria.

The definitions (*predictivity*, *precision*) used in the 3x3 contingency tables were adapted from the definitions (*specificity, sensitivity*) of 2x2 contingency tables (Brown, 2002; ECVAM, 2002).

Brown, N. (2002). Selection of test chemicals for the ECVAM international validation study on *in vitro* embryotoxicity tests. *ATLA* **30**, 177-198.

ECVAM. 2002. The Use of Scientifically-Validated *In Vitro* Tests for Embryotoxicity. Available at http://ecvam.jrc.ec.europa.eu/publication/Embryotoxicity_statements.PDF