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# POTENCY TEST FOR BOVINE VACCINES CONTAINING BOVINE PARAINLFUENZA TYPE 3 VIRUS




**Guideline n° 2 - G.B.**

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**POTENCY test for bovine vaccines containing**

**Bovine Parainlfuenza type 3 virus**

## 1. INTRODUCTION

Bovine Parainfluenza type 3 virus (PI-3) is a member of the genus Respirovirus (Murphy et al., 1995) of the subfamily *Paramixovirinae*, order Mononegavirales, family Paramixovirindae. Viral particles are spherical to pleomorphic, 150-to 200 -nm in diameter and consist of a nucleocapsid surrounded by a lipid envelope that derives from the plasma membrane of the cell from which it buds. In this envelope two viral glycoproteins are present: the hemagglunitin-neuraminidase (HN) and the fusion (F) glycoprotein, which mediates attachment to, and penetration of, the host cell, respectively. These glycoproteins represent the main viral antigens and induce protective antibody responses in the infected animals (Robert M. Chanock, 2001). Hemagglutination, hemadsorption, hemolysis and fusion are biologic activities associated to these viral glyproteins.

PI-3 virus has been recognized as an endemic agent in the cattle population worldwide. Currently PI-3 is included within the bovine respiratory disease complex (BRC) but its role in the pathogenesis is considered of less importance than the bovine respiratory sincytial virus (BRSV). Clinical disease due to PI-3 infection is highly variable from asymptomatic infections to severe respiratory disease and pneumonia characterized by cough, pyrexia and nasal discharge (Morein and Dinter, 1975). Clinical disease generally occurs in naive calves with low level of maternal passive antibodies or in animals under stress conditions. Lung lesions and immunosuppression after PI-3 infection contribute to the establishment of secondary bacterial infections (*Mannheimia haemolytica* and

mycoplasma spp) that are common feature of enzootic pneumonia in calves and the bovine respiratory disease complex in feedlot cattle, leading to severe bronchopneumonia (Haanes et al., 1997). The virus was first isolated in the United States from the nasal discharge of cattle with shipping fever (Ellis, 2010). In South American contries, for example Argentina PI-3 infection was first detected by serology in the 80' (Lager, 1983). Serologic surveys conducted in 2000 in non-vaccinated herds from Jujuy and Neuquén provinces gave 100% prevalence in adult cattle, suggesting its broad distribution in the country (Marcoppido et al., 2010 ; Robles, 2008).

Regarding genetic and antigenic characterization, bovine PI-3 are classified in three genotypes: genotype A mainly distributed in United States and Europe, genotype B circulating in Australia and genotype C only reported in China (Zhu et al., 2011). In Argentina the virus was detected from cases of respiratory disease in bovines and buffaloes. The strains found in bovines were classified as genotype A and C, while the strains detected in buffaloes were typed as genotype B, being so far, the first country reporting the circulation of the three genotypes (Maidana et al., 2012).

Specific antibodies (Ab) induced in the infected animals possess the property to block viral Hemagglutinin (HA) function. These antibodies target specific HA antigens involved in the binding to red blood cells that can be measured by hemaggutination inhibition test (HI), a rapid and economic technique, which does not require complicated infrastructure and that can be easily implemented in veterinary laboratories to evaluate the protective antibody responses to PI-3. This technique is a useful tool to conduct serologic surveys in the field and to evaluate vaccine potency in the target species as well as in a laboratory animal model (Annex I). Animals exposed to PI-3 (after infection/vaccination) significantly increase their HI Ab titers. For the viral agents within the orthomixoviridae and paramixoviridae families, the HI Ab titer in serum is associated to protection against infection (Beyer et al., 2004; de Jong et al., 2003; Lee et al., 2001).

There are numerous multivalent vaccines to prevent the BRC in the market, containing PI-3. Vaccines are fomulated with attenuated or inactivated virus. Vaccines containing inactivated PI-3 are formulated in aqueous or oil adjuvant together with other

viral (BoHV-1, BVDV and BRSV) and bacterial antigens. It was postulated that a 1/32 titer of HI of passive maternal Abs in calves is the threshold of protective immunity against PI-3 infection (Ellis, 2010). In our method this Ab titer expressed as hemagglutination inhibition units (HIU) is 32 * 8= 256 HIU; $\log_{10}$ transformed= 2.4.

To our knowledge, a unified criterion to evaluate the potency of PI-3 vaccines in the region was not yet established.

## 2. POTENCY CONTROL IN GUINEA PIGS: AIMS and Background

This guide describes an *in vivo* method conducted in laboratory animals (guinea pigs) to evaluate the potency (immunogenicity) of vaccines used in the prevention of the BRC against PI-3.

For the validation of the model the recommendations given by international animal health agencies were followed (EMEA/P038/97, 1998; Taffs, 2001). Experimental and commercial vaccines were tested in parallel in guinea pigs and bovines. Vaccines included aqueous and oil immunogens containing PI-3 combined with variable concentration of other viral (IBR, BVDV, VRSV) and bacterial (*Pasteurella multocida, Mannheimia haemolytica and Histophilus sommi)* antigens. Vaccine immunogenicity measured, in both species, as the HIU Ab againt PI-3 showed high levels of agreement between the model and the target species (Parreño, 2010; Parreño, 2008). The technical and statistical details of the validation are described in Annex II. The guinea pig model can be used to test the batch to batch quality of PI-3 vaccines to be released in the market and represent a practical tool for both, the vaccines companies as well as the animal health authorities, to warrant optimal products in the market.

Regarding animal health, an *in vivo* test is still considered inevitable to assess potency of multivalent inactivated vaccines. The developed guinea pig model is aligned with the 3R principle of animal welfare (reducing, refinement and replacement), since the test included a reduced number of animals (n=6 for the tested vaccine and 4 non-vaccinated

controls/placebos) and does not involve viral challenge, just vaccination and serum sampling (Akkermans and Hendriksen, 1999). In addition, the same serum sample can be used to evaluate the vaccine potency against each one of the different viruses included in multivalent vaccines.

## 2.1 Guinea pig model: design of the test

### *2.1.1 Guinea pigs*

Groups of guinea pigs, 400 ± 50 grams in weight are included in the test. Males and females can be used, but each group should contain animals of the same sex. At entry, a period of 7 days should be taken for animal adaptation to the new environment. After this adaptation period and prior to immunization, serum sampling is recommended to check the presence of antibodies to PI-3 in the guinea pigs. Seropositive reactors should be excluded of the assay. Animals are kept under study during a minimum of 30 days.

### *2.1.2 Procedure*

The trial assay for viral vaccine testing in guinea pig is based on the immunization of 6 guinea pigs with two doses of vaccine (21 days appart), applied subcutaneously, of a volume equal to 1/5 the bovine dose. Together with the assessment of unknown vaccine(s) (n=6), two groups of guinea pigs are included, one vaccinated with the reference vaccine of known potency (n=6) and the unvaccinated control group (n=3). Serum samples taken prior to vaccination and 9 days post-revaccination (30 days post-vaccination) are tested by HI to determine the Ab titer to PI-3, the technical details are described in ANNEX 2.

### *2.1.3 Interpretation*

Validation of the guinea pig model for PI-3, based on a linear regression analysis of the Ab titers determined by HI, indicated a dose-response relationship between the HI Ab responses induced by vaccination and the PI-3 concentration in the vaccine, in bovines and guinea pigs immunized with calibrating vaccines (dose-response assay, ANNEX 1). The guinea pig model was able to significantly discriminate among vaccines containing 1 $\log_{10}$

difference in its Ag concentration. Based on the results obtained in the dose-response curve, splits points or ranges of Ab titers anti-PI-3 were estimated. These splits points allows vaccines to be differentiated by the immunogenicity induced in guinea pigs and bovines. Two split points and three categories were established (Table 1), see details in ANNEX 1.

| ESPECIE | VACCINE POTENCY AGAINST PI-3 | | |
|---|---|---|---|
| | **No Satisfactory** | **Satisfactory** | **Very Satisfactory** |
| **GUINEA PIG** | $\bar{y} < 1.50$ | $1.50 \leq \bar{y} \leq 2.4$ | $2.4 < \bar{y}$ |
| **BOVINE** | $\bar{Y} < 2.80$ | $2.80 \leq \bar{Y} \leq 3.1$ | $3.1 < \bar{Y}$ |

**Table 1.** Cut offs represent the Ab titer to PI-3 determined by HI, expresseded as the $\log_{10}$ of the hemagglutination units (HIU) obtained in the serum of the vaccinated animales. Arithmentic mean Ab titer of groups of 5 guinea pigs, evaluated 30 days post vaccination (dpv) and groups of 5 bovines evaluated 60 dpv. Bovines receive two doses of vaccine with a 30-day interval, following vaccine manufacter´s recommendations, and are sampled at 0 and 60 dpv. This latter point corresponded to the peak or plateau of Ab titers reached by aqueous or oil vaccines, respectively. Guinea pigs receive two doses of vaccine (1/5 the volume of the bovine dose) with a 21-days interval and are sampled at 0 and 30 dpv. The two dose regimen chosen in the laboratory animal model allow detecting the immune response induced by vaccines of low potency. The 21 interval between doses was adopted in order to obtain a curve of Ab kinetic response similar to that obtained in bovines, but in a shorter period of time providing a faster alternative method for vaccine potency testing than the one conducted in bovines.

Vaccines of satisfatory immunogenecity (potency) for PI-3 may induce HI antibody titers equal or higher than 1.5 in guinea pig and 2.8 in bovines, while vaccines inducing Ab titer equal or higher than 2.4 in guinea pig and 3.1 in bovines are considered of very satisfactory potency. Finally vaccines inducing HI Ab titer lower than 1.5 in guinea pigs and 2.8 in bovines are considered of low immunogenecity (no satisfactory) (ANNEX I).

*2.1.4 Validation criterion for guinea pig testing*

Potency testing in guinea pigs is considered valid when the mean Ab titer obtained from animals vaccinated with a reference vaccine of satisfactory potency results to be the expected value (higher than 1.50 in immunized guinea pigs and higher than 2.80 in bovines), and unvaccinated control animals remain seronegative for Ab against PI-3 throughout the experience.

*2.1.5 Calculation*

All serums of animals immunized with the vaccine under control will be evaluated. FIVE (5) sera taken at 30 dpv with the highest Ab titers to PI-3 (expressed as the $\log_{10}$ transformed of HIU) will be selected and an average will be calculated on that basis.

*2.1.6 Vaccine approval criterion by potency testing in guinea pigs*

For the **APPROVAL** of the vaccine submitted to control, mean Ab titers obtained must be higher or the same as **1.50**

## 3. Harmonization of assays for the region

A panel of positive and negative control sera and reference vaccines should be elaborated and made available for regional users to harmonize the results obtained for each assay laboratory adopting the control method. The reference vaccine will allow defining the conformity of each immunization assay, while the panel of reference sera will be used to validate the results of the serologic assays (HI test) and for the standardization of alternative assays (ELISA, VN).

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# ANNEX I

**PI-3 Hemagglutination inhibition test**

This assay detemines the presence of antibodies (Abs) directed to the bovine PI-3 viral hemagglutinin, in the serum of infected and/or vaccinated animals. Prior to the assay, serum is treated with kaolin to adsorb unespecific inhibitors of the hemagglutination. The sample is also treated with red blood cells to absorb inespecific hemagglutinatin substances present in the serum. Treated serum will end in a 1/5 dilution. Serial 2-fold dilutions (5, 10, 20, 40, etc.) of the serum are mixed with a fix concentration of PI-3 virus established as 8 HA units/25 µl. The reaction is developed adding guinea pig red blood cells. In positive sera for Abs directed to the viral hemagglutinin, the formation of Ag-Ab complex will inhibit PI-3 red blood cell hemagglutination, and red blood cells will agregates forming a red button in the botton of the well. The end point of the hemagglutination inhibition activity of a serum sample determines it HI Ab titer to PI-3 and is expressed as the reciprocal of the highest dilution of serum in which complete hemagglutination did not occur. This value mutiplied by the constant 8 (representing virus concentration) define the PI-3 hemagglutination inhibition units (HIU) of the sample.

MATERIALS

- U-bottom 96 wells plates
- Cuvettes
- 200µl and 1000 µl Tips
- 1.5 ml, 15 and 50 ml plastic tubes
- Pasteur pipettes
- Needle “25/8
- 5 ml syringe

Equipment

- Mono-channel Micropipettes: up to 50µl, 200µl, 1000 µl
- 8-12 Multi-channel Micropipettes 5-50µl.
- Microcentrifuge (up to 14.000 rpm)
- Refrigerate Centrifuge (up to 5.000 rpm)

REAGENTS

- Virus: PI-3 viral suspension produced in MDBK cells, containing 8 HAU/25 ul (16 HAU/ 50 ul)
- Phosphate buffer, pH: 7.2-7.4 (PBS)
- Kaolin solution:

| | |
|---|---|
| Kaolin | 0,04g |
| PBS | 5 ml |

- Guinea pig Red Blood cell suspension
- Alsever Anticoagulant
- **Positive control serum:** pool of sera from guinea pigs immunized with two doses (21 days appart) of a reference vaccine containing a 10^7 DICT50/dose of PI-3 in oil adjuvant, sampled at 30 dpv. Selection of serum with 320-640 HIU are recommended.
- **Negative control serum:** pool of sera from non immunized guinea pigs

**Guinea pig Red Blood cell (RBC) suspension**

1. Take a simple of blood with anticoagulant (1 ml anticoagulant + 4 ml of blood) from a guinea pig by cardiac puncture. Blood extraction must be conducted under anesthesia, following ECVAM recommendations for animal welfare.
2. Discard the needle and poor the blood in a 15 ml tube.
3. Centrifuge the blood at 1500 ± 200 rpm, for 5 ± 1 min, at 4-8º C.
4. Discard supernatant and washed twice with PBS in the same manner.
5. Prepare a 0.8% RBC working suspension:
    a. suspension1/4= 1 ml of RBC + 3 ml PBS,
    b. suspension 1/120= 1 ml of ¼ suspension + 29 ml PBS
    Count RBC in the Neubauer chamber and adjust the final suspension to contain 5 X $10^7$ cells/ml

IMPORTANT: the RBC suspension should be prepare fresh at the moment of running the HI assay. After each wash the supernatant should remain clear. The presence of a redish color is an indication of hemolisis and the RBC are not suitable for the assay.

**PI-3 virus titration by Hemaggutination assay (HA)**

1. Thaw the PI-3 virus.
2. Add 50ul PBS in a U bottom 96 well plate
3. Add 50 ul of virus in four wells (4 replicates) 1-A, B, C , D
4. Perform serial 2-fold dilution transferring 50 ul from 1 to 12
5. Add to the plate 50 ul of the 0.8% RBC suspension
6. Incubate at room temperature (20-27 ºC) for 1 hour
7. Once the red buttons are present in the control wells, the assay is ready to read.
8. The virus for the assay should have 16 HA/50ul (8 HA/25ul). If the obtained titer is lower than that, the viral suspension is not suitable for the assay. If the viral suspension has a higher titer, perform a proper dilution in PBS and repeat the titration.

**Treatment of serum samples prior to HI testing**

1. Serum heating at 56ºC for 30 min.
2. In a 1.5 ml test tube, mix 50 ul of serum with 50 ul of Kaolin, vortex and incubate for 10 ± 2 min at room temperature (20-27º C).
3. Centrifuge for 15 ± 2 min at 1500 rpm.
4. Take 50ul of the supernatant and transfer to a new tube 75ul of PBS. (Final serum dilution: 1/2 * 2/5=1/5)
5. Add 10 ul of RBC package, incubate in gently agitation at 37ºC for 30 min, centrifuge for 15 ± 2 min at 1500 rpm. Transfer the supernatant to a new tube. Run the HI assay thereafter or save the treated sample at -20ºC until used, within one week.
6. Positive and negative control sera are treated in the same manner.

**HI assay**

1. Add 25ul PBS in U bottom 96-well-plates, all wells except G row
2. Add 25ul of the treated serum in wells 1 F, G, H (sample 1), 2 F,G,H (sample 2) an so on.
   Vertical design: 12 samples are tested from row 1 to 12 and 7 serial dilutions (G-A)
   Horizontal design: 8 samples are tested from row A to H and 11 dilutions (2-12).
3. Perform serial 2-fold dilutions transferring 25 ul from F to A.
4. Run in the assay positive and negative standard sera of known HI titer, randomly mixed among the samples and plates.
5. In the last plate of the assay include a positive and negative control serum and PBS as blank of reaction (RBC control). Confirm the titration of the virus.
6. Add to the plates 25 ul of the working dilution of the PI-3 virus (8 HA/25 ul) in all the rows, except H. This well will serve as control of serum sample + RBC. The absence of a red button in this well indicates that the serum sample still possesses unspecific hemagglutination activity, and its HI titer will not be determined.
7. Incubate the plates with the serum-virus mix for 1 hour at room temperature (24-27º C).
8. Add 50 ul of the 0.8% RBC suspension in all the plates. Incubate at room temperature. Once the red buttons are present in the control wells, the assay is ready to read by direct inspection.

**ASSAY APPROVAL**

A HI assay is approved if:
The back titration of the working virus suspension gives a HA titer of 16 HA/50ul (8 HA/25ul)
The RBC buttom in the control wells (RBC+PBS) should be solid
The standard and control sera give the expected HI titers (± one 2-fold dilution)

**INTERPRETATION**

The HI titer is considered the reciprocal of the highest dilution of serum in which complete agglutination did not occur. The titer is expressed in hemagglutination units (HIU), multiplying the reciprocal of the final dilution by the number of HA units of the virus (8 HA). See table below

Table 2. Ab titer against PI-3

| Well (vertical design) | Reciprocal of the serum dilution in the well | serum HI units (reciprocal dil*8) | Ab HI titer to PI-3 ($log_{10}$ HIU) |
|---|---|---|---|
| G | 5 | 40 | 1.6 |
| F | 10 | 80 | 1.9 |
| E | 20 | 160 | 2.2 |
| D | 40 | 320 | 2.5 |
| C | 80 | 640 | 2.8 |
| B | 160 | 1280 | 3.1 |
| A | 320 | 2560 | 3.4 |