

## CONCLUSIONS AND RECOMMENDATIONS

XXI Seminar on Harmonization of Registration and Control of  
Veterinary Medicines  
Americas Committee for Veterinary Medicines (CAMEVET)  
Antigua, Guatemala  
November 9 – 13, 2015

### *Opening speeches*

Mr. Leonel Rodas Serrano, Vice-president of the Organizing Committee, welcomed all the participants, together with Dr Elisabeth Erlacher, Deputy Head of the Scientific and Technical Department of the OIE, Dr Martín Minassian, Technical Assistant of the OIE Regional Representation for the Americas, Dr Enrique Argento, Secretary of CAMEVET, and Dr María Eugenia Paz, President of the Committee and OIE Focal Point for Veterinary Products.

Dr Minassian highlighted the importance of veterinary products, and the priority afforded by the OIE to the topic of antimicrobials and its resistance.

Mr DrRodas Serrano thanked the collaboration received from the Executive Board and the sponsor companies.

Dr María Eugenia Paz expressed a warm welcome for the participants, and noted the importance of this event for her country and the entire region.

Dr Enrique Argento thanked the efforts made throughout the year by the Organization Committee, and noted that it had been a difficult year for the host country.

### *Taking office by the President*

Dr María Eugenia Paz took office as President of the Seminar.

### *Session I – CAMEVET Relations*

#### *Implementation of the documents harmonized by the Committee*

#### *Procedures for the participation of CAMEVET in the proposals for the creation and modification of OIE standards.*

#### *Standards currently under review.*

Dr Martín Minassian, Technical Assistant of the OIE Regional Representation for the Americas, made a presentation that included the description of the structure of the OIE, including positions renewed during 2015.

He also gave a description of the Codes of the OIE, describing the functions of the Specialized Committees and their annual work plan, and listing the instances in which OIE Member Countries can issue comments and propose modifications.

Dr Minassian included in his presentation a selection of the Resolutions adopted during the 83rd. General Session of the OIE having an impact on CAMEVET activities, including Resolution N° 26, directed at reducing the resistance to antimicrobials, as well as the modifications to Codes and Manuals, and the approval of a diagnostic kit.

Lastly, he made reference to the Chapters of the Terrestrial Animal Health Code currently under review and open for the submission of comments until 8 January 2016, requesting the Focal Points to work on-line with their Delegates and jointly with the private sector in

this process.

### ***Implementation status of harmonized documents in member countries.***

Dr Enrique Argento presented the results of a survey sent to the countries of the Americas, which reflects the implementation status of harmonized documents. He reminded of the need for each country to send its answers, since this survey will shed light on the impact of the work of the Committee.

It was highlighted that, although in many cases harmonized documents are not incorporated into national regulations, they are used as technical support, or applied without being incorporated to the regulations, and therefore it is important to have this information available. In this regard, it was reminded that the previous Seminar proposed the criterion of using harmonized documents as a reference, setting aside the concept of internalization.

It was noted that the presentation will be distributed to each of those present to serve as information on the status of each country. It was proposed that the survey be completed, or updated – where applicable, by the countries that have not yet done so.

### ***Participation of CAMEVET in the VICH Outreach Forum***

Dr Enrique Argento made a presentation regarding his participation in representation of the Committee, with a brief review of previous meetings held by the Forum, and informed on developments at the meetings held in February 2015 in Washington, and in October 2015 in Tokyo.

He highlighted the importance of the participation at the meetings of representatives from countries of the American continent, as well as the participation of CAMEVET in the Working Group for the Modification of the VICH Stability Guideline for Veterinary Active Ingredients and Pharmaceutical Products relative to climate zones 3 and 4.

The participation of industry in the Forum was also highlighted, and it was noted that CAMEVET and the official and private delegations of Argentina took part in the Workshops relating to bioequivalence and training.

It was mentioned that the topics considered a priority by the majority of the participants of the Forum, and which resulted from the workshops carried out at the Tokyo meeting, were: pharmacovigilance; and, at the proposal of Argentina, resistance to antiparasitics, as a new topic to be developed.

The presentations made at both events were expounded, most notably:

From the meeting in Washington:

The survey carried out among CAMAVET member countries. In this regard, it was noted that very few responses had been received and that this reduced the value of conclusions. The comparison between some VICH Guidelines with their CAMEVET counterparts, making reference to their great similarity.

From the meeting in Tokyo:

The use of VICH Guidelines by member countries and members of the Forum; this presentation will be distributed to participants to inform of these uses.

It is important to highlight the advisability of insisting on the possibility of defining an equivalence criterion for counterpart guidelines. The aim is to show that, even though documents are not identical, they are equivalent in terms of their scientific basis.

Comments were received from the Brazilian official sector to the effect that the VICH Guidelines are being used, and are considered important for international trade involving

veterinary products.

The official sector expressed the convenience of expanding the participation of the countries of the region in the VICH Outreach Forum. It was noted that this decision does not depend on CAMEVET, but on the VICH Steering Committee.

It was reminded that the conditions for participating in VICH include bearing all costs relating to participation and undertaking to maintain ongoing participation and continuity of representatives.

Dr Silvia Piñeiro (FDA – USA) explained the form of work of the VICH.

She highlighted the different ways of participating in the generation and updating of Guidelines.

She expressed that guidelines are used by way of suggestion for the proper drafting of protocols for product registration.

She also informed that the FDA is translating VICH Guidelines, and has already translated 5 of them, and a further 5 translations are in draft form. She noted that final translations are available on the FDA Web page, and offered to share the working drafts.

Dr Argento informed that the United States of America reiterated the invitation to hold the meeting of the Steering Committee and the Outreach Forum in Buenos Aires in February 2017. He commented that the Executive Committee approved this proposal as it is considered very important for the region. Finally, he noted that the Steering Committee formalized the proposal.

### ***Conclusions of the Plenary Meeting of the Official Sector***

Dr Gloria Alarcón, Focal Point of the OIE for Veterinary Products for Paraguay, presented the conclusions of this meeting.

The final document of the meeting is included as an Annex.

The representative of CLAMEVET proposed the inclusion in the corresponding harmonized document the inclusion in product labels on suitable disposal of containers/packaging.

This task will be taken up by the working group currently reviewing the Labeling Guideline under review.

### ***Conclusions from the Plenary Meeting of the Private Sector.***

Topics discussed during the meeting of the private sector were presented.

The final document of the meeting is included as an Annex.

### ***CAMEVET Priority Topics - Resistance to Antimicrobials***

A round table was organized, with the participation of Dr Minassian, Dr César Díaz, Coordinator of Veterinary Product Registration at the National Agrofood Health and Quality Service (SENASA) of Argentina, and Dr Horrys Friaça, Agricultural Health and Food Safety Specialist of the Inter-American Institute for Cooperation on Agriculture (IICA).

Dr Minassian described the first work draft of the strategic plan for the implementation of the OIE standards on resistance to antimicrobials for the Americas. The plan comprises three stages, which include obtaining information from the countries of the region, definition of gaps or obstacles, and drafting of an action plan to cover the gaps identified. For the first stage, an electronic survey will be conducted, and the data gathered will be used to continue with the subsequent stages, with a view to presenting the results at the next Conference of the OIE Regional Commission for the Americas, in November 2016.

Dr César Díaz informed on the development of the national plan to control resistance to antimicrobials, with work carried out jointly by the Ministry of Agriculture and the Ministry of Health on the topic, and commented that SENASA will conduct sampling and isolation at bovine, swine and avian packing plants.

Dr Horrys Friaça described a Training Plan on resistance and good use of veterinary medicines carried out by the IICA.

The representative of CAPROVE supported these proposals, and commented that responsible use of medicines rather than their prohibition must be sought, and that any measures taken in this regard should be science-based. He also reminded the availability of the guide on Good Practices for the Use of veterinary products adopted in 2005.

## **SESSION II Work Documents**

### ***Guide for registration of homeopathic veterinary medicines***

The representation of SINDAN, as coordinator of the working group, made a presentation through Dr Mario Real, an expert on the topic.

At the indication of the Brazilian official sector of differences in criteria regarding the topic, it was decided that the topic would be returned to the work group for consideration via electronic communication, remaining in Step II status.

### ***Guide for the registration of nutraceutical/dietary supplement veterinary products***

Dr Carlos Rufrano, coordinator of the Work Group, presented the final version of the Document, which was unanimously approved. The Document is included as an **Annex**.

### ***Guide for Potency tests for inactivated vaccines containing the PI3 virus, Guinea pig model.***

Dr Viviana Parreño, Coordinator of the Working Group, presented the final document which is currently in Step IV status. Having fulfilled all the requirements, the approval of the document was proposed.

Comments were received from Dr Carlos Francia (CAPROVE), who noted that the Guides are reference material and not for mandatory implementation. Dr Ricardo Rego Pamplona (MAPA Brazil) informed that alternate techniques aside from the Guides can be applied, provided they have been agreed with the registration agency.

There being no objection, the document was approved unanimously, and is attached as an **Annex**.

### ***Guide for Good Storage, Distribution and Transport Practices for Veterinary Products***

Dr Carlos Rufrano, Coordinator of the Work Group, presented a progress report on the document, and highlighted that some comments had been received and already included in the Spanish version, and await inclusion in the English and Portuguese translations. Having done this, the document will be circulated through the Secretariat to all the members of CAMEVET and, if no objections are made within a term of 60 days, the Secretariat will be informed, and will request the official sector for their approval. The official sector expressed its agreement with the approval through this procedure, and undertook to send an e-mail confirming each decision.

### ***Considerations for regulation of aquaculture vaccines in the Americas***

Dr Glen Gifford, Coordinator of the working group, presented its first work paper, which is pending for its translation into Portuguese.

Dr Carlos Francia highlighted the innovation proposed by Dr Gifford regarding the recognition and consultation by regulatory agencies to other agencies having greater experience in the topic.

The working group will continue to work on the draft already underway, currently in Step II status. Such draft shall be distributed for comments as soon as the working group finalizes the review of the translations, and shall be presented during next Seminar.

### ***Safety of bovine vaccines – Guide for studies with inactivated vaccines.***

Dr María Marta Vena, Coordinator of the Working Group, presented the final document, currently in Step IV status. Having fulfilled all the requirements, the approval of the document was proposed.

The document was approved unanimously and is attached as an **Annex**.

### ***Guide for Potency Tests for inactivated vaccines containing bovine rotavirus***

Dr Viviana Parreño, Coordinator of the Work Group, presented the final document, currently in Step IV status. Having fulfilled all the requirements, the approval of the document was proposed.

Dr Ricardo Rego Pamplona (MAPA – Brazil) informed that Brazil cannot take on the responsibility for supplying standard serua.

The document was approved unanimously and is attached as an **Annex**.

### ***Bioequivalence Guide***

Dr Carlos Francia, Coordinator of the Work Group, presented the progress made in the document referred to.

He highlighted the excellent work carried out by the Group and its high technical standard.

Dr Francia expressed that a meeting was held in person during the Seminar, at which agreement was reached on certain points in conflict. As a result, it was suggested to set aside room in the Agenda for the next Seminar for this type of meetings.

Comments were received from the Brazilian official sector regarding the possibility of extrapolating results from different animal species. The answer was that this Guide does not envisage this as a possibility.

Ricardo Hoigjelle, representative of the Industrial Chamber of Nicaragua, asked about the dates for the distribution of documents, and the institution he represents was subsequently invited to form part of the Work Group.

The Work Group will continue to work on the available draft, which is currently in Step II status, in order to circulate a version to the countries during the following year.

Finally, Dr Argento highlighted the high technical standard of the documents presented, and the efficacy of the Work Groups in complying with pre-established deadlines, and reminded that the main objective of the CAMEVET is to generate and harmonize recommendations based on recognized scientific principles.

***Instructions for completing CAMEVET forms for registration of Pharmacological and Biological products.***

Dr César Díaz presented the document generated by the Working Group. He explained that two trends were evidenced, one that sought to add the highest level of detail, and another seeking more general submissions. These diverging opinions were solved at a meeting in person of the members of the working group. The group will continue to work on the completion of the proposed guide, which is currently in Step II status.

***Labeling of Veterinary Products***

Dr Niels Scherling, of CAPROVE, Argentina, presented the results of the survey carried out relating to the terminology used in the different countries. The possibility of concordance in wording in phrases of mandatory inclusion was analyzed. It was found that labeling could be simplified by using phrases which are accepted in most countries of the region. A decision was made to continue working on the matter, which will require a review of the CAMEVET Rot 001 Guide on Labeling of Veterinary Products, also including Rot 002, Synonymity Agreement. This Group will also include the recommendations relating to the indication on labels of proper disposal of containers/packaging.

***Policy on Generic products – Criteria for applying definitions for registration of innovative, generic, similar and new products.***

Dr Ofelia Flores, Focal Point for Mexico, made a presentation that included the definitions of each type of product. The registration procedure in Latin American countries was put forward. In these countries products for registration are classified as innovative, new generics or interchangeable generics, as opposed to the procedure that allows registration of an innovative product, with the subsequent registration of generic products referencing the innovative product with bioequivalence studies. Dr Flores highlighted that it must be considered that two possibilities exist. One would involve modifying the registration procedure, whereby all generics should be interchangeable, accepting the use of bioequivalence. The other option is to maintain the current system. To evaluate the situation, Dr Flores proposed conducting a survey to allow each country to issue its opinion and send the legislation currently in force regarding the matter. Dr Fernando Zambrano, from the Chilean official sector, proposed the use of international Guides. Dr Javier Carracedo, from ALANAC, Brazil, proposed contributing the Brazilian legislation on generics. It was decided that a survey would be conducted, whose content would be coordinated by Dr Ofelia Flores. For that, it was agreed to merge both Groups currently working on the topic, and to include the already operative Working Group on Bioequivalence. Work on the topic, which is currently in Step I of the process, will continue.

***Growth Promotors***

Dr Laura Lorenzetti Jorge (SINDAN – Brazil) presented the document on Growth Promoters and Food Additives. She introduced Dr Richard Coulter, expert on growth promoters (Phibro Animal Health), who made a presentation on antimicrobials and their use as Growth Promoters.

Based on the discussion that followed, a decision was made to continue drafting the work document, which is currently in Step II of the process.

Based on a suggestion from Dr Milson Da Silva Pereyra, Dr César Díaz, representative from SENASA Argentina, informed that this agency has been working on these products with a traceability system since 2013.

Dr G. Ardiles (ANVET – Chile) proposed including other active ingredients with growth promotor activity, such as vegetable extracts, and the proposal was accepted.

### *Piracy and Forgery of Veterinary Products*

Dr Javier Carracedo presented the program for controlling veterinary product piracy and forgery currently implemented in Brazil, with the collaboration of various Government agencies.

### *Round Table and Discussion – Present and Future of CAMEVET*

#### **CAMEVET Strategic Plan**

Dr Carlos Francia, acting for Dr Federico Luna, Focal Point for Argentina, presented the Strategic Plan for CAMEVET 2015 – 2020. He commented on the work modality of the group that drew up the plan based on proposals received from the countries.

The new plan includes the continuity of some objectives from the previous plan, such as training and communication, and new objectives such as pharmacovigilance and good use of veterinary medicines. After the lack of comments received, Secretariat shall be in charge of circulating the document, providing a 60 days deadline for receiving comments, plus a new final circulation.

#### **CAMEVET Training Plan**

Dr Liliana Revolledo presented the progress made in the Training Plan. She informed that a full program has been drafted with the collaboration of Drs. Adela Encinosa and Emilio Gimeno. The next step consists in drafting six pilot modules, which will be available in July 2016. Following the launch of the modules, comments and suggestions will be received and work will continue to reach final versions, to be launched in 2017.

#### **Communications**

In response to requests made by industry representatives from Central America for further information on CAMEVET, Dr Ofelia Flores proposed that a brief induction be offered at the commencement of each Seminar, in a similar manner as the OIE does. The reading of CAMEVET documents currently available on the web is suggested. The motion made by Dr Flores was approved.

Miss. Ana Sgammini, assistant to the Secretariat of CAMEVET, presented the new web page: <http://www.rr-americas.oie.int>

The design and content of the new web page was opened to suggestions from Committee members. Proposals will be received by the Secretariat.

Dr Suzan McLennon, representative for Jamaica, suggested the inclusion in the web page

of informative messages in the form of flashes or banners. The possibility was also mentioned of including a section in each document open for comments, as done by the FDA. The Executive Committee will evaluate whether this is possible in line with the provisions of the OIE.

***GS1 Progress in standardization of coding systems and their implications for the Veterinary Product Industry.***

Dr Mario Chávez Bocanegra, Information Technology director at GS1 Guatemala, made the presentation, and explained what GS1 is about, its organization and activities. He described the current application of the traceability system in Argentina for veterinary pharmaceuticals and agrochemicals, by way of example of the possibilities for its use. Dr César Díaz, from SENASA Argentina, added a description of the scope of the implemented system, which includes products containing Ketamine, Growth Promoters and Estradiol. He suggested that a specific presentation should be made at the next Seminar.

Dr Carlos Francia, from CAPROVE Argentina, considered that the unitary traceability system is somewhat complicated and only justified for special cases, such as Ketamine, in view of the deviations in its use. The batch traceability system is simple and easy to implement.

Dr Carlos Rufrano, from CLAMEVET Argentina, added that traceability has been in use through commercial documentation since the implementation of the GMP regulation in Argentina, and that the GS1 system would be supplementary to this.

Dr Martín Minassian indicated that the OIE has signed a memorandum of understanding with GS1 to share standards.

***Guide for Registration of Diagnostic Kits for diseases.***

Since Dr Byron Rippke was unable to attend the Seminar, Dr Emigdio Lemes made a presentation on the progress made on this work document.

Dr Lemes added that the English draft was received, and a Spanish translation was made in Cuba and sent to the Secretariat. It was suggested to have the document translated into Portuguese for subsequent circulation. For that, SINDAN offered the translation services for the draft.

Dr Martín Minassian indicated that the document must be in line with OIE standards, as well as the OIE Standard Operating Procedure for the registration of diagnostic kits. This document shall remain in Step II status.

***New Topics Proposed***

***Pharmacovigilance***

The Working Group will be coordinated by ANVET (Chile), with the participation of the Official Sector from Argentina, Canada, Chile, Costa Rica, Guatemala, Mexico and Uruguay, as well as CAPROVE and CLAMEVET (Argentina), ALANAC and SINDAN (Brazil), ASIFAN and Cámara de Insumos Agropecuarios (Costa Rica), INFARVET-CANIFARMA (Mexico), CADIN (Nicaragua), ADIPRAVE and CEV (Uruguay).

***Resistance to Antiparasitics:***



The Working Group will be coordinated by the official sector of Uruguay with the collaboration of ADIPRAVE and CEV (Uruguay) and made up by the official sectors of Argentina, Brazil, Costa Rica, Cuba, Guatemala and Mexico, as well as CAPROVE and CLAMEVET (Argentina), SINDAN (Brazil), ASIFAN and Cámara de Insumos Agropecuarios (Costa Rica), ASOVET (Guatemala) and INFARVET-CANIFARMA (Mexico).

***Criteria for the authorization of Mixed Plants***

The Working Group will be coordinated by CLAMEVET, and made up by the official sectors from Argentina and Guatemala, with the participation of CAPROVE (Argentina), ALANAC and SINDAN (Brazil), ALANAC and SINDAN (Brazil), and ALFA (El Salvador), .

***Criteria for registration exemption for veterinary products***

The Working Group will be coordinated by ALANAC and made up by the official sectors of Argentina, Chile, Canada and Guatemala, as well as CAPROVE and CLAMEVET (Argentina), SINDAN (Brazil), ANVET (Chile), ASIFAN and Cámara de Insumos Agropecuarios (Costa Rica), and ASOVET (Guatemala).

***Members of the Executive Committee***

Due to failure by the representatives of the Industry Associations present to reach a consensus for electing a position representing the Industry in the Executive Board, it was resolved that this post shall remain vacant until the New Executive Board is formed in 2016. This proposal was unanimously adopted by all the representatives from the official sector.

***Approval of the proposal of new host countries for the next Seminars***

The proposal of Mexico to hold the XXII Seminar was accepted, and the proposal by the delegation from Paraguay to hold the XXIII Seminar in their country in 2017 was also accepted.

***Budget and resources of CAMEVET***

Dr Martin Minassian presented the balance sheet of CAMEVET for the current budget year until 31 October.

A sum of up to 50.000 US Dollars was signed for supporting the participation of National Focal Points. Accounting balance was approved and is included as Annex.

***Conclusions and recommendations. Reading and approval of the final document.***

The document containing the conclusions and recommendations was read and approved.

**List Annexes:**

**Annexes I: Minute plenary meeting of the official sector**

**Annexes II: Minute plenary meeting of the private sector.**

**Annexes III: Guide for the registration of nutraceutical/dietary supplement veterinary products**

**Annexes IV: Guide for Potency tests for inactivated vaccines containing the PI3 virus, Guinea pig model.**

**Annexes V: Safety of bovine vaccines – Guide for studies with inactivated vaccines.**

**Annexes VI: Guide for Potency Tests for inactivated vaccines containing bovine rotavirus**

**Annexes VII: Balance 2015-2016**

*List of acronyms used in the document*

<b>ADIPRAVE:</b>	ASOCIACIÓN DE LAS INDUSTRIAS DE PRODUCTOS AGROQUÍMICOS Y VETERINARIOS (URUGUAY)
<b>ALANAC:</b>	ASOCIACIÓN DE LABORATORIOS FARMACÉUTICOS NACIONALES (BRASIL) (ASSOCIAÇÃO DOS LABORATÓRIOS FARMACÊUTICOS NACIONAIS)
<b>ANVET:</b>	ASOCIACIÓN NACIONAL DE LABORATORIOS VETERINARIOS (CHILE)
<b>CAMEVET:</b>	COMITÉ DE LAS AMÉRICAS DE MEDICAMENTOS VETERINARIOS
<b>CAPROVE:</b>	CÁMARA ARGENTINA DE LA INDUSTRIA DE PRODUCTOS VETERINARIOS
<b>CEV:</b>	CÁMARA DE ESPECIALIDADES VETERINARIAS (URUGUAY)
<b>CLAMEVET:</b>	CÁMARA DE LABORATORIOS ARGENTINOS MEDICINALES VETERINARIOS
<b>FDA:</b>	U S FOOD AND DRUG ADMINISTRATION - ADMINISTRACIÓN DE MEDICAMENTOS Y ALIMENTOS
<b>FIVETCA:</b>	FEDERACIÓN DE INDUSTRIA VETERINARIA CENTROAMERICANA
<b>INFARVET / CANIFARMA:</b>	INDUSTRIA FARMACÉUTICA VETERINARIA – CANIFARMA (MÉXICO)
<b>OIE:</b>	ORGANIZACIÓN MUNDIAL DE SANIDAD ANIMAL
<b>OIRSA:</b>	ORGANISMO INTERNACIONAL REGIONAL DE SANIDAD AGROPECUARIA
<b>SENASA:</b>	SERVICIO NACIONAL DE SANIDAD Y CALIDAD AGROALIMENTARIA (ARGENTINA)
<b>SINDAN:</b>	SINDICATO NACIONAL DA INDUSTRIA DE PRODUTOS PARA SAÚDE ANIMAL
<b>VICH:</b>	INTERNATIONAL COOPERATION ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF VETERINARY MEDICINAL PRODUCTS
<b>CAPALVE</b>	CÁMARA DE LABORATORIOS PARAGUAYOS DE PRODUCTOS VETERINARIOS
<b>CADIN</b>	CAMARA DE INDUSTRIAS DE NICARAGUA
<b>ASOVET</b>	
<b>LABIOFAM</b>	GRUPO EMPRESARIAL LABIOFAM
<b>CIA</b>	CÁMARA DE INSUMOS AGROPECUARIOS
<b>ASIFAN</b>	ASOCIACIÓN FARMACÉUTICA DE LA INDUSTRIA NACIONAL
<b>ALFA</b>	ASOCIACIÓN DE LABORATORIOS FARMACÉUTICOS DE EL SALVADOR

## ANNEX I

### Plenary Session of the Official Sector

The session commenced at 18:00 hours in the Hall of the hotel “Casa Santo Domingo” in Antigua, Guatemala; which is the venue of CAMEVET 2015. Dr Maria Eugenia Paz Diaz, National Focal Point was nominated as the coordinator of the plenary session by the Executive Committee of CAMEVET. The topics discussed were as follows:

1. Pharmacovigilance

Given the importance of this topic there was a need for it to be discussed at the CAMEVET level. The development of a guideline for notifications and the subsequent establishment of a network for notification among CAMEVET member countries are of vital importance.

2. Labelling

Jamaica indicated that the reusing of empty veterinary product vials and medicated feed bags is a problem. Mexico agreed. Dominican Republic suggested that the Guide for the reusing of packages and vials should be included in a document of Good Agricultural Practices. It was recommended that the guide for Labelling be revised and “disposal of bottles” be included. Argentina indicated that waste disposal was the purview of another ministry, the Ministry of Environment; however, the support of other entities was vital. One health approach can be taken to provide guidelines to this Ministry for the proper disposal of veterinary product vials.

3. Biotechnology

Guatemala is interested in obtaining guidelines for the registration of vaccines based on biotechnology and request relevant training. Cuba informed that the proposal for these guidelines have been in place for the past 2 years.

4. Participation of Focal Points in CAMEVET

Mexico emphasized that due to administrative problems in our respective countries the official sectors participation was becoming difficult. They proposed that the private sector financed their participation.

Adjournment at 19:30 hours.

## **ANNEX II**

### INDUSTRY MEETING

The session began at 5:20 PM in the Atrio saloon of Hotel Santo Domingo, in Antigua Guatemala, headquarters of CAMEVET 2015, where Mr. Leonel Rodas began informing the themes proposed by the CAMEVET's directive board.

- 1) Change of the date for the next CAMEVET. The new date would be in August.
- 2) Pharmacovigilance
- 3) Resistance to antiparasitic drugs. It is necessary to present a proposal.
- 4) Recognition of Dr. Milson, from SINDAN, Brasil, who proposes Laura Lorenzetti Jorge to take his place in the directive board.

Meeting:

1.- In the general industry meeting the last ítem 4) was discussed and it was proposed to vote following the regulations.

2.- The Central America's Industry poses the lack of staff in the veterinary services, and expresses that it has not been established the homologation of the records of the veterinary products in some countries of the región.

3.- Bruno Forti, from Argentina (CLAMEVET) asks to be considered the fact that in those counter products where no registration is required in Europe and in the USA, in some countries of our región the registration is required as medicines.

4.- It is requested to analyze the fact that the veterinarian drugs manufactured in plants that also produce human medicines, as in some countries of our region they must be manufactured separately ,so it is impossible to import them.

5.- It should be very clear that the adopted guidelines are just guidelines, not rules to be applied mandatorily. Carlos Francia clarified that the guidelines are reference papers, emphasizing that.

6.- It was expressed by some participants that there is a lack of official registration staff in some countries, which worries the industry and was point of emphasis of ALANAC ,through its representative Javier Carracedo, considering the importance of the forum, he asks to make a communication in this regard. Dr. Argento consideres it should be submitted for discussion at the directive board, the kind of communication related to this issue.

7.- Lack of commitment to the review of technical documents, which are circulated, and participation and opinion of all involved is needed.

**ANNEX III**

**CAMEVET**  
**Cod:**  
**STEP I**  
**October 2012**

## DIETARY COMPLEMENTS

## DIETARY COMPLEMENTS REGISTRATION

### Introduction

Several oral administration products for animals exist in various countries, and, due to their nature, they are difficult to be defined as medicine or as food. This makes their treatment ambiguous from a regulatory point of view. To correct this, a proposal to create, within the area of registration of veterinary products, a specific Registration Form for these products identified as "Dietary Complements", including among others: nutraceuticals, dietary supplements, probiotics, prebiotics and plant extracts.

### Key Points

- Dietary Complements play an increasingly important role as an aid in the treatment and prevention of diseases in humans and animals.
- In many cases it is difficult to specify if they are food or medicine. Therefore they are not covered by registration authorities and are not subject to a pre-market evaluation.
- The lack of specific regulations which give information on safety and effectiveness for dietary complements, does not justify that the potential therapeutic benefit of some of these products should be ignored.
- No one should assume they are safer or better than medicine simply because they are "natural" because many dietary complements may be synthetic. Natural is not synonymous of safe.

### Purpose and Scope

**Objective:** To outline a specific Application for Registration of Dietary Complements.

**Scope:** Dietary Complements

### Terms and Definitions

Dietary complements are substances or a mixture of substances, which are obtained naturally or synthetically, administered only by mouth, in different pharmaceutical dosage forms: liquid, solid or semisolids, such as solutions,

suspensions, emulsions, syrups, drops, granulated, powders, tablets, capsules, pastes and gels, in order to improve the health and welfare of animals.

## Procedure

1. Adopt the Application for Registration of Dietary Complements, included as part of the present document, for the registration of these products with the official authority for the registration of veterinary products.
2. This Application for Registration shall be considered an affidavit, committing the responsible firm to ensure security, innocuousness and absence of hazardous residues in quantities greater than those permitted for any substance in the products register.
3. Dietary complements products covered by this regulation will be manufactured in veterinary drug manufacturing plants duly authorized by the local health authority and must comply with the GMP standards applicable to this type of product.

## References

Argentine Food Code, Section 1381 et seq.

Ekta K Kaira, Nutraceutical - Definition and Introduction. PharmaSci AAPS 2003, 5 (3) Article 25 (<http://www.pharmasci.org>)

Dawn Merton Boothe, DVM, PhD, ACVIM, ACVCP. FUNCTIONAL FOODS IN VETERINARY MEDICINE Part I - Definitions and Regulations. Department of Veterinary Physiology and Pharmacology, Veterinary College and Texas A & M University College Station, Texas.

Dawn Merton Boothe, DVM, PhD, ACVIM, ACVCP. FUNCTIONAL FOODS IN VETERINARY MEDICINE Part II - Safety and Efficacy. Department of Veterinary Physiology and Pharmacology, Veterinary College and Texas A & M University College Station, Texas.

## Authors

Argentine Chamber of Veterinary Drug Laboratories (CLAMEVET)

## Level of approval

– XV Seminar on Harmonization of Standards for Registration and Control of Veterinary Drugs - Guadalajara - Mexico 10 to 15 August 2009 (Step I)

## Effective Date

## Frequency of review

10 years



# REGISTRATION FORM FOR VETERINARY DIETARY COMPLEMENTS

## DATE

### 1. - PRODUCT NAME

### 2. - CLASSIFICATION: Dietary Complement

### 3. – APPLYING ESTABLISHMENT: OWNER / LEGAL REPRESENTATIVE

3.1 .- Name:

3.2 .- Address (Street - City - Country):

3.3 .- Official Authorization N°:

3.4 .- Technical Manager:

3.4.1 .- Profession:

3.4.2 .- Professional Identification Number (License or Registration):

### 4. – MANUFACTURING FACILITY (for products manufactured in the country)

4.1 .- Name:

4.2 .- Address (Street - City - Country):

4.3 .- Official Authorization N°

4.4 .- Technical Manager:

4.4.1 .- Profession:

4.4.2 .- Professional Identification N° (License or Registration):

### 5. – FRACTIONATION FACILITY (for products manufactured in the country)

5.1 .- Name:

5.2 .- Address (Street - City - Country):

5.3 .- Official Authorization N°

5.4 .- Technical Manager:

5.4.1 .- Profession:

5.4.2 .- Professional Identification N° (License or Registration):

### 6. - MANUFACTURING FACILITY IN ORIGIN (for imported products)

6.1 .- Name:

6.2 .- Address (Street - City - Country):

6.3 .- Official Authorization N°

6.4 .- Technical Manager:

6.4.1 .- Profession:

6.4.2 .- Professional Identification Number ° (License or Registration):

**7 .- LEGAL DOCUMENTS** (when applicable)

7.1 .- Manufacturing agreements.

7.2 .- Representation Agreement of Manufacturer in origin.

7.3 .- Official Authorization of the Manufacturing Facility.

7.4 .- For imported products: Certificate of Registration and Non-regulated Trade or equivalent. If the product does not require registration in the country of origin: a disclaimer.

**8. – PHARMACEUTICAL FORM**

**9. - QUALITATIVE–QUANTITATIVE FORMULA**

Regular names recommended by recognized international organizations, if these exist, will be used, on the contrary, the usual regular name or chemical description will be used.

**10. - FORMULA COMPONENT SPECIFICATIONS**

**11. - PRODUCT MANUFACTURING METHODOLOGY**

Briefly describe the manufacturing process.

**12. FINISHED PRODUCT CONTROL**

Finished product specifications.

Control Process

**13. – PHARMACEUTICAL FORM AND CONTENT**

**14. - PACKAGING SPECIFICATION AND CONTROL**

14.1 - Packaging characteristics

14.2 - Inviolability System

14.3 - Packaging Quality Control

**15. - INDICATIONS FOR USE**

16.1. - Main instructions and/or complementary ones, if any.

**16. - APPLICATION MEANS and FORM OF ADMINISTRATION or USE OF PRODUCT**

The route of administration must be oral only.

**17. - PREPARATION OF THE PRODUCT AND INSTRUCTIONS FOR CORRECT USE AND CONSERVATION.**

**18. – ADMINISTRATION SCHEME**

- 18.1 - Indicate the amount of intake of the product by species or age
- 18.2 - Duration of treatment.

**19. - POSSIBLE SIDE EFFECTS AND PHARMACOLOGICAL ANTAGONISMS AND INCOMPATIBILITIES**

- 19.1. - Contraindications and limitations of use (when administration may lead to adverse effects).
- 19.2. - Precautions to be taken before, during or after administration.

**20. - SPECIAL PRECAUTIONS**

Describe adequate product storage, transportation and destruction instructions.

**21. – POSSIBLE CAUSES OF VARIATIONS IN PRODUCT QUALITY**

Precipitation, dissociation, decrease or loss in activity of the active ingredients, cold, heat, light, humidity, compression in storage or pallets.

**22. - CORRECT PRODUCT STORAGE**

**23. – PERIOD OF VALIDITY. (Shelf life)**

**24. - LABELS AND HANDOUTS**

Draft printed material must be attached.  
The label should include the words "dietary complement – Non-Prescription Drugs for sale in Professional Veterinary Businesses “

**25. - BIBLIOGRAPHIC AND/OR TESTING REFERENCES.**

**THIS IS AN AFFIDAVIT**

Name and signature of the  
Technical Director

Name and signature of the  
Legal Representative of the Company

THE COMPANY GUARANTEES THE EFFECTIVENESS AND SAFETY OF THE PRODUCT AND THE LACK OF RESIDUES ABOVE PERMITTED AMOUNTS

FOR ANY ACTIVE PRINCIPLE IN THE PRODUCT TO BE REGISTERED IF SUPPLIED TO ANIMALS INTENDED FOR HUMAN CONSUMPTION

ONCE THE CERTIFICATE AUTHORIZING USE AND MARKETING IS ISSUED, THE ESTABLISHMENT REQUESTING THE REGISTRATION MUST INFORM THE DATE OF THE FIRST BATCH PRODUCED OR THE FIRST BATCH TO BE IMPORTED TO THE COMPETENT AUTHORITY (REGISTRANT).

**CONDITIONS OF THE PRODUCT TO BE CONSIDERED DIETARY COMPLEMENT:**

Dietary complements are substances or a mix of substances, which are obtained naturally or synthetically, which are administered by mouth only, in order to improve the health and welfare of the animals.

Dietary complements must not include therapeutic indications.

If an internationally accepted therapeutic dosage exists, the dosage indicated for the dietary complement must not exceed 50% of it.

REGISTRATION FORM FOR  
DIETARY COMPLEMENTS  
VETERINARY USE

DATE

1. - PRODUCT NAME

2. - Classification: Dietary (Supplement) Complement

3. – Company requesting application: :-OWNER / LEGAL REPRESENTATIVE

3.1 .- Name:

3.2 .- Address (Street - City - Country):

3.3 .- Official Qualification No.:

3.4 .- Technical Manager:

3.4.1 .- Profession:

3.4.2 .- Professional Identification Number (registered or recorded):

4. – Manufacturing company (for products manufactured in the country)

4.1 .- Name:

4.2 .- Address (Street - City - Country):

4.3 .- Official Qualification No.:

4.4 .- Technical Manager:

4.4.1 .- Profession:

4.4.2 .- Professional Identification Number (registered or recorded):

5. – Conditioning company (for products manufactured in the country)

5.1 .- Name:

5.2 .- Address (Street - City - Country):

5.3 .- Official Qualification No.:

5.4 .- Technical Manager:

5.4.1 .- Profession:

5.4.2 .- Professional Identification Number (registered or recorded):

6. - Manufacturing facility in origin (for imported products)

6.1 .- Name:

6.2 .- Address (Street - City - Country):

6.3 .- Official Qualification No.:

6.4 .- Technical Manager:

6.4.1 .- Profession:

6.4.2 .- Professional Identification Number (registered or recorded):

7 .- LEGAL DOCUMENTS (as applicable)

7.1 .- Manufacture Agreement.

7.2 .- Agreement of representation with the producing company

7.3 .- certificate of approval of the manufacturing facility.

7.4 .- For imported products: Certificate of Registration and Free Sale or equivalent documentation. If the product does not require registration in the country of origin disclaimer.

## 8. - PHARMACEUTICAL FORM

### 9. – QUALI – QUANTITATIVE FORMULA

Common names recommended by recognized international organizations must be used where they exist, otherwise the usual common name or chemical description.

## 10 .- SPECIFICATIONS OF COMPONENTS OF THE FORMULA

### 11. - PRODUCT MANUFACTURE METHODOLOGY

Describe briefly the manufacturing process.

### 12. FINAL PRODUCT-CONTROL

Finished product specifications.

Process Control

## 13. – PHARMACEUTICAL FORM AND CONTENT

### 14. - PACKAGING SPECIFICATION AND CONTROL

14.1 Characteristics of the packaging

14.2 Inviolability System

14.3 Quality control of packaging

### 15. - INSTRUCTIONS FOR USE

15.1 .- Main use and / or complementary, if any.

16. - Route of application and method of administration ~~or use of proceeds~~

Only oral use is admitted.

### 17. - PREPARATION OF THE PRODUCT AND CORRECT INSTRUCTIONS FOR USE AND CONSERVATION.

### 18. – ADMINISTRATION SCHEDULE

18.1 Indicate the amount of intake of the product by species or age

18.2 Duration of treatment.

### 19. - POSSIBLE SIDE EFFECTS, AND PHARMACOLOGICAL ANTAGONISTS and INCOMPATIBILITIES

19.1 .- Possible side effects, incompatibilities and antagonisms.

19.2 .- Contraindications and limitations of use (where its administration may lead to adverse effects).

19.3 .- Precautions to be taken before, during or after administration.

## 20. - SPECIAL PRECAUTIONS

Describe the proper storage, transportation and destruction of the product.

## 21. - CAUSES THAT MAY PRODUCE A VARIATION IN THE PRODUCT QUALITY

Precipitation, dissociation, decreased or loss of activity of the active ingredients, cold, heat, light, moisture, compression or storage pallets.

## 22 .- CORRECT PRODUCT STORE

## 23 -. SHELF LIFE PERIOD

## 24. - LABELS AND BOOKLETS

Annexed to this paper projects of:

The label should include the words "dietary complement - Free sale only in premises with Veterinarian Advice"

## 25 .- REFERENCES AND / OR TESTING.

THIS IS AN AFFIDAVIT

Signature and clarification of  
Technical Director

Signature and clarification of  
Attorney of the Company

THE COMPANY GUARANTEES THE SECURITY AND SAFETY AND THE LACK OF RESIDUES IN EDIBLE TISSUE ABOVE LEVELS ADMITED FOR ANY ACTIVE INGREDIENT PRESENT IN THIS PRODUCT, IF USED IN ANIMALS FOR HUMAN CONSUMPTION.

Once the Free Sale Certificate is provided, the Company submitting this registration agrees to inform the Registration Office the date and series number of the first batch produced or to be imported.

CONDITIONS THAT MUST MEET A PRODUCT TO BE CONSIDERED DIETARY COMPLEMENT:

Dietary complements are pure or mixed non pharmaceutical substances, natural or synthetic, administered orally in order to improve the physiology and animal welfare.

Dietary complements may not include therapeutic indications.

If there is an internationally accepted ~~as a~~ therapeutic dose, the indicated dose for the dietary complement. must not exceed the 50%.



**ANNEX IV**

September 28th, 2012

C A M E V E T

Cod: 000

TRÁMITE I

DATE: October 4th, 2012

**POTENCY test for bovine vaccines containing  
Bovine Parainfluenza type 3 virus**

OIE Regional representation for The AMERICAS.

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Guide n° 2 - G.V.

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## Prologue

### ***PROSAIA: Food safety and the production of pharmaceutical veterinary products***

***“healthy animals, healthy food, healthy people”.***

Argentina as a quality food producer faces, among other challenges, the threat of emerging and re-emerging infectious diseases that, due to cultural changes occurred worldwide in the last years, are in continuous expansion (BSE, Avian influenza, Nipah, West Nile Fever, Rift Valley Fever, to name some). Many of these are zoonotic diseases, and that fact has caused deep changes in the assurance systems established by the public health authorities, for which food safety is an indispensable requirement. In order to achieve food safety, besides meeting other conditions, it is necessary to count with biological and veterinary pharmaceutical products of proven safety and purity that guarantee, together with its correct application, that products and sub-products derived from animals will not become disease-causing food, neither by the unintended presence of contaminants or pathogen agents –innocuousness–, nor by its deliberated presence –bioterrorism– thus contributing to preserve consumers health and protection.

For that sake, there are fundamental principles that must be born in mind in the formulation of supplies for food producing animals, including food and pharmacological products. These principles include the control of the source, the manipulation of materials employed and the design of an adequate elaboration system that considers:

#### ***Regulations, recommendations and national and international standards.***

This is a main aspect that must be fulfilled by all veterinary pharmaceutical products, since otherwise, products and sub-products derived from treated animals run the risk of being left out of the markets.

***Good Manufacturing Practices.***

“Good Manufacturing Practices is the part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by their marketing authorization.” WHO Good Manufacturing Practices for Pharmaceutical Products.

Therefore, keeping and developing a competitive business as food suppliers in this context implies complying with the implicit and explicit requirements requested by consumers. Among those requirements, innocuousness involves the application of quality assurance systems such as Good Agricultural Practices, Good Manufacturing Practices, HACCP, determination of levels or absence of residues, pesticides, antibiotics, assurance that pharmacological products used in the control of animal diseases comply with international regulations.

In this framework and in compliance with the objectives of its foundation, PROSAIA summoned the main representatives in the subject from the regulating organism SENASA, the academy and the representative committees of veterinary products to form an ad hoc group for the writing and update of guidelines, protocols and regulations for the correct development of veterinary products, as a contribution for the adequacy to the times we are living.



Dr. Carlos Van Gelderen



Dr. Alejandro Schudel

## **POTENCY test for bovine vaccines containing**

### **Bovine Parainfluenza 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 *Paramixoviridae*. The viral genome is simple-stranded non segmented negative-sense RNA. 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 hemagglutinin-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 syncytial 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 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 hemagglutination 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. 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 against 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 I. 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,  $400 \pm 50$  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 apart), 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, expressed as the  $\log_{10}$  of the hemagglutination units (HIU) obtained in the serum of the vaccinated animales. Arithmetic 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 manufacturer'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 satisfactory immunogenicity (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 immunogenicity (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 2

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### PI-3 Hemagglutination inhibition test

This assay determines 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 adsorb inespecific hemagglutinin 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  $\mu$ l. 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
- 200 $\mu$ l and 1000  $\mu$ l Tips
- 1.5 ml, 15 and 50 ml plastic tubes
- Pasteur pipettes
- Needle "25/8
- 5 ml syringe

### Equipment

- Mono-channel Micropipettes: up to 50 $\mu$ l, 200 $\mu$ l, 1000  $\mu$ l
- 8-12 Multi-channel Micropipettes 5-50 $\mu$ l.
- 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 apart) 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  $1500 \pm 200$  rpm, for  $5 \pm 1$  min, at  $4-8^\circ$  C.
4. Discard supernatant and washed twice with PBS in the same manner.
5. Prepare a 0.8% RBC working suspension:
  - a. suspension  $1/4 = 1$  ml of RBC + 3 ml PBS,
  - b. suspension  $1/120 = 1$  ml of  $1/4$  suspension + 29 ml PBSCount RBC in the Neubauer chamber and adjust the final suspension to contain  $5 \times 10^7$  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 hemolysis and the RBC are not suitable for the assay.

#### **PI-3 virus titration by Hemagglutination 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 ( $20-27^\circ$ C) 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 56°C 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 (20-27° 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 37°C 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 -20°C 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) and 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 (24-27° 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 button 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 <sub>10</sub> 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

# ANEXO I

VALIDACIÓN ESTADÍSTICA

MODELO COBAYO INTA

POTENCIA DE VACUNAS PARA

EL VIRUS DE PARAINFLUENZA-3 BOVINO

## **Lista de abreviaturas**

PI-3: virus parainfluenza tipo 3 bovino

Mín-Máx: Valores mínimo y máximo, respectivamente.

SD: Desvío estándar.

LCI: Límite de confianza inferior bilateral al 95 %.

LCS: Límite de confianza superior bilateral al 95 %.

Título de Ac. : Título de Anticuerpos transformados a logaritmos

IHA: Inhibición de hemoaglutinación

UIHA: unidades inhibitorias de la hemoaglutinación

ANOVA: Análisis de varianza

Ag: Antígeno

dpv: días post vacunación

## EL ESTUDIO

- 
- Objetivos**
- 1. Ensayo dosis respuesta de vacunas contra Parainfluenza 3 bovino (PI-3)**
    - Capacidad discriminadora del modelo cobayo y la especie de destino (bovinos) para diferenciar vacunas oleosas formuladas con concentraciones crecientes de Ag de PI-3.
    - Estimar el tiempo óptimo de muestreo en bovinos y cobayos para realizar la comparación de parámetros serológicos entre el modelo y la especie de destino.
  - 2. Establecer un criterio de clasificación de vacunas comerciales en el modelo cobayo y los bovinos.**
  - 3. Análisis de Concordancia entre el bovino y el modelo cobayo para clasificar vacunas gold std de concentración conocida y vacunas comerciales, según los puntos de corte establecidos.**
- 

**1. Diseño** Estudio experimental. La selección aleatoria de los animales ensayados estuvo a cargo del grupo de investigación.

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**2. Variables en estudio** Se evaluó el título de Ac por IHA para PI-3, a los 0, 30, 60 y 90 días dpv en bovinos y a los 0, 30 y 60 dpv en cobayos.

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**2. Grupo de Investigación** Laboratorio VD, Instituto de Virología, CICV y A, INTA

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**3. Período.** Los datos provienen de ensayos realizados durante 2004 – 2005- 2006 en el marco del CVT 1664 INTA-Biogénesis Bagó.  
Análisis estadístico basado en un informe previo realizado en 2006.  
Fecha de informe: Septiembre 2012.

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**5. Número de animales** **Dosis respuesta:** 80 bovinos mayores de seis meses de edad y 90 cobayos de 450 a 550 gr de peso en grupos de 5.

**Concordancia:** 500 bovinos y 428 cobayos en grupos de 5 a 10, correspondientes a 74 vacunas comerciales y experimentales.

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## Diseño Experimental, materiales y métodos

### Vacunas:

Se formularon dos sets vacunas oleosas, polivalentes conteniendo las valencias virales IBR, BVDV y dosis crecientes de antígeno de virus PI-3 bovino cepa de referencia SF4 (genotipo A). El rango de concentración viral utilizada en cada vacuna, varió en 1 o medio log<sub>10</sub> de diferencia entre cada vacuna. Cada set de 4 vacunas cada uno se evaluó en paralelo en bovinos y cobayos, en dos experimentos independientes según se detalla en la tabla 1.

**Tabla 1. Experimentos dosis respuesta en Bovinos y cobayos**

Set de vacunas	Set 1	Set 2
Ensayo a campo bovinos/ Ensayo en cobayos	1.1 – 1.2	2.1 -2.2
año	2004	2005
<b>PI-3</b>	A Oleosa (10 <sup>8</sup> )	C Oleosa (10 <sup>7</sup> )*
Vacunas: concentración del antígeno expresada en DICT50/dosis	B Oleosa (5x10 <sup>7</sup> )	C Oleosa (10 <sup>7</sup> )*
	C Oleosa (10 <sup>7</sup> )	D Oleosa (10 <sup>6</sup> )
	D Oleosa (10 <sup>6</sup> )	E Oleosa (10 <sup>5</sup> )
	Placebo/testigos	Placebo/testigos

\*En el año 2005, set de vacunas 2, se probaron dos vacunas con 10<sup>7</sup> DICT<sub>50</sub>/dosis de PI-3

### Bovinos:

Es importante destacar que la infección por el virus de parainfluenza tipo 3 bovino es endémica en el ganado bovino a nivel mundial (Ellis, 2010) y que todos los terneros reciben anticuerpos calostrales anti-PI-3 que pueden persistir hasta los 6 meses de edad. Se estima que cuando los títulos de Ac calostrales disminuyen a valores inferiores a 1/32 (256 UIHA) los terneros se encuentran altamente susceptibles a sufrir la infección por PI-3, que pueden ser sintomática o no y rápidamente desarrollan anticuerpos frente al agente.

En función de lo expuesto, para realizar el ensayo dosis-respuesta propuesto se utilizaron terneros de cría de las razas angus, heresford y sus cruza, mayores de 6 meses de edad. Las experiencias se realizaron en las temporadas 2004 y 2005, (set 1.1: Exp 301.04-1, grupos de 5 animales, un evento; set 1.2: Exp 301.04-bis, grupos de 5 animales, un evento; set 2.1 y 2.2: Exp 546-05, 2 grupos de 5 animales para cada grupo, en dos eventos independientes;). En todos los casos se determinó el título de Ac basales contra PI-3 por IHA para armar los grupos con medias de Ac basales homogéneas. Los animales incluidos en el ensayo debían ser primo-vacunados con las vacunas bajo estudio. Los animales fueron vacunados con dos dosis de 3 ml de vacuna con un intervalo de 30 días y se tomaron muestras de suero a los 0, 30, 60 y 90 días post vacunación (dpv) (Figura 1a). Se incluyó un grupo placebo y un grupo de animales no vacunados como testigos. El estudio completo involucró 80 bovinos de cría.

### Cobayos

Por su parte, los cobayos de 400-500 gramos de peso fueron controlados para determinar que fueran seronegativos para Ac IHA contra PI-3 y luego fueron vacunados con dos dosis de vacuna de un volumen de dosis correspondiente a 1/5 de la dosis bovina, por vía parenteral, con un intervalo de 21 días, los animales se muestrearon a los 0, 30 y 60 dpv (Figura 1a y b). El estudio completo involucró 90 cobayos (Exp 500.2-05; 500.3-05; 255.1-04; 255.2-04).

### Análisis de muestras:

La respuesta inmune humoral frente a la vacunación con PI-3 se evaluó en el sueros de los animales vacunados mediante la técnica de inhibición de la hemoaglutinación de GR de cobayos según la metodología que se detalla en el Anexo II de esta guía.

Figura 1.a

Figura 1.b

**Análisis de datos:**

Con los resultados obtenidos se construyó una base de datos del **ensayo dosis-respuesta**. Todos los ensayos realizados en cobayos y bovinos generaron resultados procesables. De esta manera se obtuvieron 17 valores promedio de título de anticuerpos IHA para cada especie, correspondiente a grupos conformados por 5 cobayos y 5 bovinos, vacunados con las vacunas calibradoras formuladas con concentraciones crecientes de PI-3 que permitieron hacer el estudio de la cinética y magnitud de la respuesta de anticuerpos en cada especie, la capacidad discriminante del bovino y el modelo cobayo de diferenciar entre vacunas de diferente concentración antigénica de PI-3.

**Vacunas probadas para evaluar la concordancia entre el modelo y la especie destino:**

Para estudiar el grado de concordancia entre el modelo cobayo y los bovinos se analizaron un total de 71 casos que incluyeron las 17 vacunas oleosas calibradoras, elaboradas para realizar el ensayo dosis-respuesta, 16 grupos controles negativos que recibieron placebos o no fueron vacunados (testigos), 6 vacunas experimentales de potencia conocida (gold estándar), de las cuales una fue oleosa y cinco acuosas. Finalmente se incluyeron 32 vacunas comerciales, 18 acuosas y 14 oleosas (apéndice I, vacunas análisis de concordancia).

**Métodos Estadísticos:**

El análisis estadístico abordó básicamente la comparación de promedios del título de Ac. de distintos tiempos de muestreo en vacunas experimentales. Los datos de títulos de Ac IHA en cobayos y bovinos determinados a los 0, 30 y 60/90 dpv, respectivamente, fueron analizados mediante un modelo mixto de medidas repetidas (Littell, 1998). Las diferencias entre medias se probaron empleando el criterio de Bonferroni. Se empleó el criterio de Akaike para la selección de la matriz de covarianzas (Wolfinger & Chang, 1998). En los casos en que la interacción Vacuna\*Momento resultó significativa la comparación de medias se efectuó dentro de cada momento.

Para determinar los puntos de corte de clasificación de vacunas en ambas especies se empleó análisis de regresión y árbol de clasificación (Breiman, 1984). Brevemente para los títulos de Ac IHA anti-PI-3 inducido por las 17 vacunas calibradoras utilizadas en los ensayos dosis respuesta en cada especie (Cobayo y Bovino), se estimó el modelo lineal polinómico (de segundo grado), que mejor ajustara los datos. Para cada caso se informan los coeficientes de cada modelo ajustado, la significación del modelo, el coeficiente de determinación ( $r^2$ ), los títulos estimados para las concentraciones de interés ( $10^5$  y  $10^7$  DICT<sub>50</sub>/dosis) y la estimación de los Límites Inferiores de los Intervalos de Predicción (LIP) para el título de Ac. esperado para dichas concentraciones. En cada caso se adjuntan también los gráficos de las curvas estimadas, con las correspondientes bandas de confianza y de predicción (del

Finalmente, para establecer el grado de concordancia entre el modelo cobayo INTA y bovino para clasificar vacunas de PI-3, según los puntos de corte establecidos, se analizaron las vacunas detalladas en el Apéndice 1 mediante el índice Kappa ponderado. Para cada especie (Bovino y Cobayo), se efectuó un análisis descriptivo, para evaluar promedios, medianas y medidas de variación, a través de las categorías propuestas. Se incluyen los Box & Whisker para visualizar el nivel de discriminación entre categorías. A partir de la aplicación simultánea del criterio de categorización en Bovinos y Cobayos, se generaron tablas de contingencia, para evaluar la concordancia entre ambos criterios. El nivel de concordancia fue evaluado mediante el índice KAPPA ponderado, acompañado de su SE asintótico y el intervalo de confianza del 95 % (Altman, 1991). En primer lugar, dicho índice fue estimado para el conjunto de vacunas de concentraciones conocida cuya información puede considerarse un Gold Standard, en este caso se evaluó la posible discrepancia con las predicciones efectuadas por cada modelo por separado. Luego se comparó la concordancia entre el modelo y la especie de destino y luego se completó en análisis incluyendo las vacunas comerciales. Valores de índice Kappa ponderado entre 0.41-0.60 indica concordancia moderada, valores de 0.61 a 0.80 indican concordancia sustancial o aceptable, mientras que valores mayores a 0.80 se considera concordancia casi perfecta (Viera and Garrett 2005).



## RESULTADOS

### 1-Ensayo Dosis respuesta Bovinos

- Evaluación de la inmunogenicidad y cinética de respuesta de anticuerpos IHA anti-PI-3 en bovinos vacunados**

Inicialmente se realizó un ensayo dosis respuesta que abarcó vacunas de concentración de Ag de PI-3 de  $10^8$  a  $10^6$  DICT<sub>50</sub>/dosis de PI-3. En este primer ensayo se evaluaron vacunas que diferían entre 1 y  $\frac{1}{2}$  logaritmo en su concentración antigénica. Todos los grupos de bovinos iniciaron la prueba con títulos basales de Ac contra PI-3 estadísticamente similares. El análisis de la cinética de respuesta de Ac IHA anti PI-3 en los bovinos vacunados con este 1º set de vacunas, probado en dos ensayos de campo en dos rodeos diferentes, indicó que las vacunas así formuladas fueron muy inmunogénicas. Los grupos vacunados seroconvieron para Ac IHA anti-PI-3 luego de una dosis a los 30 dpv y alcanzaron un título de Ac plateau que se mantuvo hasta los 90 dpv, diferenciándose estadísticamente del grupo control no vacunado o vacunado con placebo (Tabla 2; Figura 2).

Los bovinos no lograron discriminar entre vacunas que diferían en  $\frac{1}{2}$  o 1 log en su concentración antigénica en el rango de virus utilizado (Tabla 2), por lo que se decidió realizar un segundo experimento con una concentración menor de antígeno.

**Tabla 2. Set 1.1 + 1.2 (Exp 301-04/301bis-04).**

Vacuna	n	Momento de sangrado (días)			
		0	30	60	90
A Oleosa $10^8$	10	2.41 A	3.32 A*	3.38 A*	3.26 A*
B Oleosa $5 \times 10^7$	10	2.41 A	3.41 A*	3.41 A*	3.43 A*
C Oleosa $10^7$	10	2.51 A	3.23 A*	3.26 A*	3.28 A*
D Oleosa $10^6$	10	2.51 A	3.10 A*	2.98 A	3.26 A*
Testigos/Placebo	10	2.19 A	2.29 B	2.23 B	2.23 B

Nota: Los valores están expresados como medias aritméticas de los títulos de Ac.

Medias en **una misma columna** con diferente letra difieren significativamente.

Medias en la misma fila con asterisco indican diferencia significativa (seroconversión respecto del nivel de Ac basales).

La interacción Vacuna\*Momento fue significativa ( $p < 0.0001$ )

En el segundo experimentos se preparó un nuevo set de vacunas que fue aplicada a dos grupos de bovinos, en dos ensayos independientes, en el mismo rodeo. En este caso, se prepararon dos vacunas con  $10^7$ , una vacuna con  $10^6$  y otra con  $10^5$  DICT<sub>50</sub> de PI-3/dosis. Nuevamente los bovinos iniciaron la experiencia con títulos de Ac basales estadísticamente similares y seroconvitieron para Ac IHA anti-PI-3 luego de una dosis (vacunas  $10^7$  y  $10^6$ ), alcanzando un plateau que se mantuvo hasta los 90 dpv. OPor su parte, la vacuna formulada con  $10^5$  de PI-3/dosis elevó levemente el titulo de Ac luego de 1 y 2 dosis de vacuna pero a niveles que no se consideran seroconversión. A los 60 días post vacunación los títulos de Ac promedio inducidos por la vacuna con concentración de Ag de  $10^5$  fue significativamente inferior a los de las vacunas con concentraciones superiores y a los 90 dpv los bovinos vacunados con esta vacuna retornaron su nivel basal de Ac contra PI-3.

Tal lo observado en el experimento anterior, a los 60 dpv, los bovinos no fueron capaces de discriminar entre vacunas que poseen  $10^6$  y  $10^7$  DICT<sub>50</sub> de Ag de PI-3, pero si diferenciaron entre estas dosis y vacunas conteniendo  $10^5$ .

La capacidad de discriminar significativamente entre vacunadas que difieren en 1 log10 recién se logra a los 90 días. Este segundo experimento permitió determinar que vacunas con concentraciones de Ag de  $10^5$  ya no serian inmunogénicas para indicar niveles de Ac detectables por la técnica de IHA (representando el limite de detección de la técnica) (Tabla 3; Figura 3).

**Tabla 3. Set 2.1 + 2.2 (Exp 546-05)**

Vacuna	n	Anticuerpos IHA anti-PI3 Tiempo (dpv)			
		0	30	60	90
C Oleosa $10^7$	10	1.87 A	3.23 A*	3.23 A*	3.14 AB*
C Oleosa $10^7$	10	1.99 A	3.31 A*	3.38 A*	3.28 A*
D Oleosa $10^6$	10	1.93 A	3.11 A*	3.28 A*	2.81 B
E Oleosa $10^5$	10	2.02 A	2.37 B	2.54 B	2.09 C
Placebo	10	1.87 A	2.02 B	1.98 C	1.87 C

Nota: Los valores están expresados como medias aritméticas de los títulos de Ac.

Medias en **una misma columna** con diferente letra difieren significativamente.

Medias en una misma fila con asterisco difieren significativamente del titulode Ac basal (seroconversión)

La interacción Vacuna\*Momento fue significativa ( $p < 0.0001$ )

**Figura 3. Cinética de la Respuesta de Ac IHA anti-PI-3 en Bovinos (EnsayoDosis Respuesta Set 2 ensayos 2.1 + 2.2)**

**Ensayo dosis respuesta en cobayos:**

**Estudio de la inmunogenicidad, cinética de respuesta de Ac y capacidad discriminante del modelo cobayo como prueba alternativa de potencia de vacunas de PI-3**

En la tabla 4 se detalla en análisis estadístico del total de grupos de cobayos inmunizados con cada vacuna, agrupadas por dosis de Ag. Los cobayos iniciaron la experiencia seronegativos para Ac IHA contra PI-3. Luego de dos dosis de vacunas (0 y 21 ds), todos los cobayos de los grupos vacunados con vacunas conteniendo  $10^8$ ,  $5 \times 10^7$ ;  $10^7$  y  $10^6$  DICT<sub>50</sub> de PI-3/dosis seroconvierten a los 30 dpv. Las vacunas que contiene  $10^7$  de virus o más presentan títulos significativamente mayores a los 30 dpv que las vacunas formuladas con  $10^6$ , que a su vez se diferencian de las vacunas  $10^5$  de virus, que prácticamente no inducen respuesta detectable por IHA. Sólo se detecta un aumento significativo del título de Ac. promedio al día 60 en la vacuna con concentración  $10^6$ . En esta vacuna, la diferencia media verdadera apenas supera, como máximo, medio logaritmo. La vacuna con  $10^5$  de virus, al igual que lo observado en bovinos, no logra generar seroconversión biológicamente relevante en los 4 lotes de cobayos vacunados (Tabla 4, Figura 4).

Del resultado obtenido en estos ensayos en cobayos, se concluye que la administración de dos dosis de vacuna con un intervalo de 21 ds entre dosis permite obtener una cinética y magnitud de respuesta de Ac anti PI-3 similar a la observada en los bovinos. Asimismo, el cobayo, probablemente por su condición de seronegativo, posee mayor poder discriminante que el bovino para evaluar la inmunogenicidad de vacunas para PI-3 siendo los 30 dpv el momento óptimo de toma de muestra para realizar la evaluación. El modelo cobayo, al igual que los bovinos, no seroconvierte para Ac IHA al recibir vacunas con  $10^5$  de Ag de PI-3 (límite de detección).

**Tabla 4. Ensayo dosis respuesta Cobayos:  
(Set 2.1 + 2.2 + Set 1.1 + 1.2)**

Vacuna	n	Momento de sangrado (días)			SD*
		0	30	60	
A Oleosa $10^8$	25	1.00	3.15 A	3.07 A	0.34
B Oleosa $5 \times 10^7$	9	1.00	3.28 A	3.08 A	0.30
C Oleosa $10^7$	30	1.00	2.97 A	3.08 A	0.31
D Oleosa $10^6$	19	1.00	2.00 B	2.77 A	0.71
E Oleosa $10^5$	10	1.00	1.00 C	1.45 B	0.68

\* Desvío estándar de la diferencia.

Figura 4. Título de Ac. promedio y Error Estándar de vacunas oleosas con distinta concentración antigénica. IHA para PI-3 en cobayos

Figura 5. Cinética de respuesta de Ac anti-PI-3 y estudio de la capacidad discriminante del modelo cobayo para distinguir entre vacunas con concentración viral creciente. (cada set de vacunas elaborado en dos años consecutivos, probados en dos lotes de cobayos de 5 animales cada uno).

**Tabla 5. Experimento 1.1 + 1.2 PI-3 IHA**

Vacuna	Momento de toma de muestra (dpv)		
	0	30	60
C Oleosa $10^7$	1.00	3.26 A	3.45 A
C Oleosa $10^7$	1.00	3.56 A	3.71 A
D Oleosa $10^6$	1.00	2.77 B	3.06 B
E Oleosa $10^5$	1.00	1.00 C	1.45 C
Placebo/ testigos	1.00	1.00	1.00

Nota: Los valores están expresados como medias aritméticas de los títulos de Ac. Medias en una misma columna con diferente letra difieren significativamente. La interacción Vacuna\*Momento no fue significativa ( $p=0.475$ ).

**Tabla 6. Experimento 2.1 + 2.2 - PI-3 IHA**

Vacuna	Momento de toma de muestra (dpv)		
	0	30	60
C Oleosa $10^7$	1.00	3.47 A	3.63 A
C Oleosa $10^7$	1.00	3.65 A	3.77 A
D Oleosa $10^6$	1.00	2.88 B	3.10 B

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E Oleosa 10 <sup>5</sup>	1.00	1.00 C	1.38 C
Placebo/testigos	1.00	1.00	1.00

Nota: Los valores están expresados como medias aritméticas de los títulos de Ac.  
Medias en una misma columna con diferente letra difieren significativamente.  
La interacción Vacuna\*Momento no fue significativa (p= 0.734).

### Conclusiones de los ensayos dosis respuesta en bovinos y cobayos

- El patrón de respuesta fue similar en ambas especies animales tanto para la Cinética de la respuesta de Ac como para la Magnitud de la respuesta de Ac IHA anti-PI-3. Las respuestas promedio del título de Ac. tendieron a aumentar con las concentraciones crecientes de Ag en bovinos y cobayos. Se observó una relación directamente proporcional entre la Respuesta de Ac y la concentración de Ag en ambas especies, evidenciando un claro efecto dosis respuesta. Sin embargo la capacidad de discriminación de los bovinos entre vacunas de diferente concentración antigénica solo permite diferenciar vacunas de menos de 10<sup>6</sup> de Ag de PI-3 por dosis de vacunas con concentraciones de Ag de 10<sup>6</sup> o mayores.
- **La prueba en cobayo demostró mayor capacidad discriminante que el bovino, dado que discriminó significativamente entre vacunas oleosas formuladas con títulos de Ag de 10<sup>5</sup>; 10<sup>6</sup> y títulos de 10<sup>7</sup> o mayores.**
- El título de Ac IHA a los 30 días resulta más discriminante en la detección de vacunas de baja concentración de antígenos (D y E), ya que a los 60 días, el título de Ac. se elevó significativamente, en estas vacunas. Esto indicaría que **la medición a los 30 días se puede considerar como el punto óptimo para finalizar la prueba en cobayos**, resultando una prueba económica y rápida para evaluar la potencia de vacunas para PI-3.

### 2- **Análisis de regresión y estimación de puntos de corte para la clasificación de vacunas para PI-3 en bovinos y cobayos**

El análisis de regresión de los datos obtenidos a partir de los ensayos dosis respuesta en bovinos y cobayos en la Para establecer los puntos de corte para discriminar vacunas de diferente potencia se ilustra en la figura 6. Para ambas especies el modelo matemático que mejor ajustó a los datos fue un modelo lineal polinómico de 2º grado. El modelo permitió predecir los títulos de Ac IHA promedios inducidos por las vacunas con un 85% de ajuste para cobayos y un 81% de ajuste en bovinos. La F reportada y su valor p corresponden a la significación del modelo (Figura 6).

Figura 6. Análisis de Regresión a partir del cual se estiman los puntos de corte para clasificar vacunas de diferente calidad inmunogenica en bovinos y cobayos. Cada punto representa el promedio del log10 de los títulos de Ac inhibidores de la hemoaglutinación anti-PI-3, obtenido en grupos de 5 cobayos/bovinos vacunados con vacunas oleosas con concentraciones crecientes de Ag de PI-3. Se ilustra la curva estimada por el modelo acompañada las bandas correspondientes a los intervalos de confianza y predicción del 90%.

En la tabla 7 se detallan los límites de predicción obtenidos a partir del modelo matemático en bovinos y cobayos. El límite inferior del intervalo de predicción del 90% representa el valor mínimo de Ac IHA promedio que debe inducir una vacuna para cada concentración de Ag en la formulación cuando es administrada a un grupo de un mínimo de 5 cobayos o 5 bovinos, con un 95% de confianza (coverage). Dado que tanto el modelo como la especie de destino fueron capaces de discriminar entre vacunas formuladas con una concentración de  $10^6$  de Ag de PI-3 o mayor de vacunas con concentraciones inferiores, el punto de corte para declarar una vacuna como de calidad **mínima aceptable** fue el correspondiente al límite de predicción del 90% para dicha concentración antigénica estimado en un título mínimo de 2.8 para bovinos y 1.5 para cobayos. Adicionalmente, aquellas vacunas con concentraciones de antígeno  $\geq 10^7$  DICT<sub>50</sub>/dosis o mayores deben arrojar títulos promedios de Ac no menor a 3.1 en bovinos y 2.4 en cobayos.

**Tabla 6. Límites de predicción del 90% estimados a partir del análisis de regresión**

Set. Exp	VACUNA	Conc. PI-3 (log10 DICT50/dosis)	Bov T0	Bov T60	Cobayo T30	IPRE(LI) Bov T60	IPRE(LS) Bov T60	IPRE(LI) CobT30	IPRE(LS) Cob T30
1,1	A	8	2,69	3,41	3,59	3,05	3,67	2,67	4,46
1,1	B	7,7	2,32	3,41	3,41	3,09	3,67	2,67	4,34
1,1	C	7	2,38	3,29	3,18	3,05	3,62	2,39	4,02
1,1	C	7	2,26	3,35	3,18	3,05	3,62	2,39	4,02
1,1	D	6	2,44	3,11	1,48	2,8	3,37	1,52	3,17
1,2	A	8	2,14	3,35	3,71	3,05	3,67	2,67	4,46
1,2	B	7,7	2,51	3,41	3,17	3,09	3,67	2,67	4,34

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1,2	C	7	2,63	3,23	2,93	3,05	3,62	2,39	4,02
1,2	D	6	2,57	2,87	2,02	2,8	3,37	1,52	3,17
2,1	C	7	2,08	3,17	3,47	3,05	3,62	2,39	4,02
2,2	C	7	1,66	3,29	3,05	3,05	3,62	2,39	4,02
2,1	C	7	2,26	3,41	3,65	3,05	3,62	2,39	4,02
2,2	C	7	1,72	3,35	3,47	3,05	3,62	2,39	4,02
2,1	D	6	2,14	3,41	2,88	2,8	3,37	1,52	3,17
2,2	D	6	1,72	3,17	2,69	2,8	3,37	1,52	3,17
2,1	E	5	2,2	2,63	1	2,28	2,93	0,02	1,89
2,2	E	5	1,84	2,51	1	2,28	2,93	0,02	1,89

Tabla 7. Criterio de clasificación del Título de Ac. estimado a partir del modelo Cobayo y la especie de destino

Especie	Criterio de Clasificación Título de Ac IHA antiPI-3		
	Potencia Baja Menor a $10^6$	Media Entre $10^6$ y $10^7$	Alta $10^7$ o mayor
<b>BOVINO</b>	< 2.8	[2.8 a 3.1)	$\geq 3.1$
<b>cobayo</b>	< 1.5	[1.5 a 2.4)	$\geq 2.4$

### Conclusiones del análisis de regresión y estimación de puntos de corte para clasificar vacunas

- Como criterio de clasificación se pueden establecer límites del título de Ac. en cobayos para identificar la concentración antigénica de las vacunas comerciales. Para el caso de los títulos de Ac. para PI-3 medidos por IHA, el criterio sería calificar como de alta inmunogenicidad una vacuna con títulos superiores a 2.4 en cobayos (3.1 en bovinos) y vacunas de baja inmunogenicidad si el título alcanzado en promedio no supera 1.5. Valores intermedios indicarían que la vacuna posee bajos títulos de antígeno de PI-3 pero que podría ser inmunogénica en animales ya primados, considerándose de calidad inmunogénica intermedia.
- Es importante destacar que títulos de Ac IHA de 1/32 (en nuestro método con un título en UIHA =  $32 * 8 = 256$ ; expresado en  $\log_{10} = 2.4$ ) se considera el umbral mínimo (threshold) de anticuerpos colostrales que deben poseer los terneros para estar protegidos frente a la infección por PI-3 (Ellis, 2010).
- El licenciamiento de vacunas para PI-3 en Norteamérica requiere que la vacuna induzca en los bovinos al menos un incremento del título de Ac neutralizantes en suero de una dilución 1:4 postvacunación y una reducción significativa de títulos viral excretado en secreciones nasales en comparación con controles no vacunados. No se requiere reducción de enfermedad (CFR).

### 3- Estudio de concordancia entre los bovinos y el modelo cobayo para clasificar vacunas para PI-3

El estudio de concordancia se realizó inicialmente con un único punto de corte que diferencia vacunas satisfactorias de no satisfactorias (1.5 cobayos; 2.8 bovinos). El grado de concordancia del modelo cobayo y la especie de destino utilizando 39 líneas de comparación (17 vacunas formuladas para el ensayo dosis-rta, 6 vacunas gold standard de concentración de Ag conocida y 16 controles negativos) fue casi perfecto (Kappa ponderado = 0.898). Solo dos vacunas fueron clasificadas como no satisfactorias por el bovino y satisfactorias por el modelo cobayo. Ambas vacunas corresponden a formulaciones acuosas con  $10^7$  DICT50/dosis que fueron correctamente clasificadas por los cobayos.

Tabla 8. Concordancia entre el bovino y el cobayo. Vacunas Gold Standard

Cobayos (1.5)	Bovinos (2.8)		
	No satisfactoria	Satisfactoria	
No satisfactoria	19	0	19 (48,7%)
Satisfactoria	2	18	20 (51,3%)
	21 -53,80%	18 -46,20%	39
Weighted Kappa <sup>a</sup>	0,898		
Standard error	0,07		
95% CI	0,760 to 1,000		

Si al análisis se incorporan 32 vacunas comerciales de concentración de PI-3 desconocida se obtiene un grado de concordancia óptimo o sustancial (Kappa ponderado = 0.777). Registrándose sólo 8 discordancias sobre un total de 71 comparaciones.

Tabla 9. Concordancia vacunas comerciales y gold std

Cobayo (1.5)	Bovino (2.8)		
	No satisfactoria	Satisfactoria	
No satisfactoria	33	0	33 (46,5%)
Satisfactoria	8	30	38 (53,5%)
	41 (57,7%)	30 (42,3%)	71
Weighted Kappa <sup>a</sup>			0,777
Standard error			0,072
95% CI			0,635 to 0,919

Finalmente si se consideran los dos puntos de corte par discriminar entre vacunas de calidad intermedia de muy buenas, se obtiene un grado de concordancia también muy bueno

Tabla 10. Concordancia considerando dos puntos de corte

Cobayo	Bovino			
	Baja	Intermedia	Satisfactoria	
Baja	34	0	0	34 (47,9%)
Intermedia	4	7	2	13 (18,3%)
Muy satisfactoria	3	4	17	24 (33,8%)
	41 (57,7%)	11 (15,5%)	19 (26,8%)	71
Weighted Kappa <sup>a</sup>				0,757
Standard error				0,064
95% CI				0,632 to 0,882





**Apéndice I. Vacunas utilizadas en el análisis de concordancia**

Nº línea	Tipo de Vacuna	Aplicación VACUNA	SERIE	FORM	Composición (Ag virales)	Ag PI-3	PI3IHABov 0	PI3IHABov 60	PI3IHACob 30
1	DOSISRTA	Calibracion experimental	A	OLEOSA	IBR- BVDV -PI-3	8,00	2,08	3,17	3,47
2	DOSISRTA	Calibracion experimental	A	OLEOSA	IBR- BVDV -PI-3	8,00	1,66	3,29	3,05
3	DOSISRTA	Calibracion experimental	C	OLEOSA	IBR- BVDV -PI-3	7,00	2,26	3,41	3,65
4	DOSISRTA	Calibracion experimental	C	OLEOSA	IBR- BVDV -PI-3	7,00	1,72	3,35	3,47
5	DOSISRTA	Calibracion experimental	D	OLEOSA	IBR- BVDV -PI-3	6,00	2,14	3,41	2,88
6	DOSISRTA	Calibracion experimental	D	OLEOSA	IBR- BVDV -PI-3	6,00	1,72	3,17	2,69
7	DOSISRTA	Calibracion experimental	E	OLEOSA	IBR- BVDV -PI-3	5,00	2,20	2,63	1,00
8	DOSISRTA	Calibracion experimental	E	OLEOSA	IBR- BVDV -PI-3	5,00	1,84	2,51	1,00
42	DOSISRTA	Calibracion experimental	A	OLEOSA	IBR- BVDV -PI-3	8,00	2,14	3,35	3,71
43	DOSISRTA	Calibracion experimental	B	OLEOSA	IBR- BVDV -PI-3	7,70	2,51	3,41	3,17
44	DOSISRTA	Calibracion experimental	C	OLEOSA	IBR- BVDV -PI-3	7,00	2,63	3,23	2,93
45	DOSISRTA	Calibracion experimental	D	OLEOSA	IBR- BVDV -PI-3	6,00	2,57	2,87	2,02
47	DOSISRTA	Calibracion experimental	A	OLEOSA	IBR- BVDV -PI-3	8,00	2,69	3,41	3,59
49	DOSISRTA	Calibracion experimental	B	OLEOSA	IBR- BVDV -PI-3	7,70	2,32	3,41	3,41
50	DOSISRTA	Calibracion experimental	C	OLEOSA	IBR- BVDV -PI-3	7,00	2,38	3,29	3,18
51	DOSISRTA	Calibracion experimental	C	OLEOSA	IBR- BVDV -PI-3	7,00	2,26	3,35	3,18
54	DOSISRTA	Calibracion experimental	D	OLEOSA	IBR- BVDV -PI-3	6,00	2,44	3,11	1,50
20	GOLD STANDARD	Concentracion PI-3 conocida		ACUOSA	IBR- BVDV -PI-3	7,00	2,26	3,28	2,44
27	GOLD STANDARD	Concentracion PI-3 conocida		OLEOSA	IBR- BVDV -PI-3	7,00	2,62	2,56	1,42
48	GOLD STANDARD	Concentracion PI-3 conocida	A	ACUOSA	IBR- BVDV -PI-3	8,00	2,32	3,35	2,93
52	GOLD STANDARD	Concentracion PI-3 conocida	C	ACUOSA	IBR- BVDV -PI-3	7,00	2,32	2,81	1,56
53	GOLD STANDARD	Concentracion PI-3 conocida	C	ACPLUS	IBR- BVDV -PI-3	7,00	2,51	2,75	2,40
63	GOLD STANDARD	Concentracion PI-3 conocida		ACUOSA	IBR- BVDV -PI-3	7,00	1,54	2,26	2,44
9	CONTROL	Testigos/placebos		OLEOSA		0,00	1,96	2,02	1,00
10	CONTROL	Testigos/placebos		OLEOSA		0,00	1,70	1,90	1,00
46	CONTROL	Testigos/placebos		OLEOSA		0,00	2,31	2,20	1,00
55	CONTROL	Testigos/placebos		OLEOSA		0,00	2,08	2,26	1,00
19	CONTROL	Testigos no vacunados					2,38	2,50	1,00
24	CONTROL	Testigos no vacunados					2,10	2,20	1,00

*Validación Estadística del Modelo Cobayo INTA para evaluar la potencia de vacunas contra la infección por el virus de Parainfluenza tipo 3 bovino*

Nº línea	Tipo de Vacuna	Aplicación VACUNA	SERIE	FORM	Composición (Ag virales)	Ag PI-3	PI3IHABov 0	PI3IHABov 60	PI3IHACob 30
29	CONTROL	Testigos no vacunados					2,68	2,38	1,00
35	CONTROL	Testigos no vacunados					2,75	2,75	1,00
41	CONTROL	Testigos no vacunados					2,75	2,75	1,00
59	CONTROL	Testigos no vacunados					2,14	1,96	1,00
62	CONTROL	Testigos no vacunados					1,75	1,65	1,00
64	CONTROL	Testigos no vacunados					1,69	1,74	1,00
69	CONTROL	Testigos no vacunados					1,42	1,12	1,00
72	CONTROL	Testigos no vacunados					1,72	1,45	1,00
77	CONTROL	Testigos no vacunados					2,57	2,38	1,00
78	CONTROL	Testigos no vacunados					2,38	2,2	1,00
21	COMERCIAL	Síndrome respiratorio	33	ACUOSA	IBR- BVDV -PI-3		2,17	3,10	1,84
22	COMERCIAL	Multi proposito	21	OLEOSA	IBR- BVDV -PI-3 - RV		2,07	2,83	2,26
23	COMERCIAL	Pre-servicio	9	OLEDE	IBR- BVDV -PI-3		2,11	2,53	1,36
25	COMERCIAL	Multi proposito	20	OLEOSA	IBR- BVDV -PI-3 - RV		2,20	2,80	2,32
26	COMERCIAL	Queratoconjuntivitis	26	OLEOSA	IBR		2,38	2,26	1,00
28	COMERCIAL	Pre-servicio	9	OLEDE	IBR- BVDV -PI-3		1,96	2,20	1,00
30	COMERCIAL	Multi proposito	19	OLEOSA	IBR- BVDV -PI-3 - RV		2,38	3,17	2,75
31	COMERCIAL	Síndrome respiratorio	4	OLEDE	IBR- BVDV -PI-3		2,32	2,62	1,90
32	COMERCIAL	Síndrome respiratorio	5	OLEOSA	IBR- BVDV -PI-3		2,38	2,99	2,99
33	COMERCIAL	Síndrome respiratorio	1	ACUOSA	IBR - BVDV - PI-3 - BRSV		2,44	2,05	2,38
34	COMERCIAL	Pneumonia	6	OLEOSA	PI-3		2,38	2,99	2,93
36	COMERCIAL	Multi proposito	19	OLEOSA	IBR- BVDV -PI-3 - RV		2,38	3,17	2,87
37	COMERCIAL	Síndrome respiratorio	4	OLEDE	IBR- BVDV -PI-3		2,32	2,62	2,43
38	COMERCIAL	Síndrome respiratorio	5	OLEOSA	IBR - BVDV - PI-3 - BRSV		2,38	2,99	2,99
39	COMERCIAL	Síndrome respiratorio	1	ACUOSA	IBR - BVDV - PI-3 - BRSV		2,44	2,05	2,38
40	COMERCIAL	Pneumonia	6	OLEOSA	IBR- BVDV -PI-3		2,38	2,99	3,25
56	COMERCIAL	Síndrome respiratorio	32	ACUOSA	IBR- BVDV -PI-3		1,90	2,57	2,38
60	COMERCIAL	Síndrome respiratorio	45	ACUOSA	IBR- BVDV -PI-3			2,03	1,00
61	COMERCIAL	Síndrome respiratorio	31	ACUOSA	IBR- BVDV -PI-3		1,95	2,12	1,45
67	COMERCIAL	Síndrome respiratorio	236-152	ACUOSA	IBR- BVDV -PI-3		1,45	1,21	1,00
68	COMERCIAL	Síndrome respiratorio	2	OLEDE	IBR- BVDV -PI-3		1,21	1,12	1,00
70	COMERCIAL	Síndrome respiratorio	18	ACUOSA	IBR- BVDV -PI-3		1,69	2,14	2,51

*Validación Estadística del Modelo Cobayo INTA para evaluar la potencia de vacunas contra la infección por el virus de Parainfluenza tipo 3 bovino*

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Nº línea	Tipo de Vacuna	Aplicación VACUNA	SERIE	FORM	Composición (Ag virales)	Ag PI-3	PI3IHABov 0	PI3IHABov 60	PI3IHACob 30
71	COMERCIAL	Síndrome respiratorio	32	ACUOSA	IBR- BVDV -PI-3			1,60	1,00
73	COMERCIAL	Síndrome respiratorio	58	ACUOSA	IBR- BVDV -PI-3		2,07	2,39	1,30
74	COMERCIAL	Síndrome respiratorio	58	ACUOSA	IBR- BVDV -PI-3		2,07	2,39	1,00
75	COMERCIAL	Síndrome respiratorio	2	ACUOSA	IBR- BVDV -PI-3		1,90	2,43	1,00
76	COMERCIAL	Síndrome respiratorio	2	ACUOSA	IBR- BVDV -PI-3		2,13	2,38	1,34
79	COMERCIAL	Síndrome respiratorio		ACUOSA	IBR - BVDV - PI-3 - BRSV		2,51	2,88	1,53
80	COMERCIAL	Síndrome reproductivo		ACUOSA	IBR- BVDV		2,66	2,66	1,24
81	COMERCIAL	Síndrome respiratorio	1	ACUOSA	IBR - BVDV - PI-3 - BRSV		2,73	2,88	1,54
82	COMERCIAL	Síndrome respiratorio	2	ACUOSA	IBR - BVDV - PI-3 - BRSV		2,51	2,81	1,72
83	COMERCIAL	Síndrome respiratorio	3	ACUOSA	IBR - BVDV - PI-3 - BRSV		2,51	2,61	1,48

**SAFETY OF BOVINE VACCINES**  
**Guideline for studies with inactivated vaccines**

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**REPRESENTACIÓN REGIONAL DE LA OIE PARA LAS AMÉRICAS.**

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## 1. INTRODUCCIÓN

Inactivated bovine vaccines are basically composed of an antigenic element: either whole viruses or bacteria, or fractions of either, or toxoids, presented as one way or multivalent way and an adjuvant component (diverse principles in water solution or emulsion). Unlike pharmaceutical medicinal products, the formula for vaccines is not entirely definable, due to the complex composition of viruses and bacteria. Also, normalized potency trials for various bacterial vaccines (Leptospirosis, Clostridial diseases, Pasteurellosis, etc.) are challenged in laboratory animals; therefore, it is probable that those vaccines have not been tried in bovines during their development. For all the above mentioned, a well documented safety test in bovines is an ineludible step for every new vaccine applying for a license.

Continuous progress in the harmonization of international standards allows diminishing repetition of safety studies at the target species, avoiding the conduct of the same study in various countries. Thus, not only research costs but also the number of animals needed is reduced, taking into consideration the principles of animal welfare.

This guideline has been elaborated based on norms currently in force both in the American countries and the European Union, with the purpose of providing unified concepts that allow the acceptance of safety data by the regulatory authorities.

## 2. OBJETIVE

The aim of this guide es to provide recommendations for the conduct of studies that evaluate the safety of final formulations of inactivated vaccines for bovines. The document applies to batches at laboratory scale (experimental vaccines), at pilot scale and vaccines for registry, grouped under the acronym LPR.

## 3. SCOPE

The scope of application of this guideline is limited to evaluations of the health condition and welfare of bovines, who are subjected to a protocol of vaccination in accordance with the conservation and administration indications specified by the manufacturer. Recommendations at this guideline are addressed at the laboratories that manufacture inactivated vaccines for bovines, at the steps of development and pilot batches prior to the product license application and also at the evaluations of the finished product that may be requested. The guidance is not intended to cover safety studies conducted as part of post-approval batch release requirements, since these are conducted in laboratory animals (9. Cf., 113.33 and 113.38) or through documentary demonstration of manufacturing consistency parallel to studies in bovines (VICH. GL 50).

## 4. TERMS Y DEFINITIONS

- ❖ **Adverse Effect:** Any unfavorable observation suspected to be related to the LPR under investigation.
- ❖ **Adverse Event:** Any unfavorable and unintended observation which occurs after the use of a LPR, whether considered or not to be product related.
- ❖ **Class:** Subset of bovines that present certain characteristics in common such as age, reproductive status and/or use (calf, heifer, dairy cow, bulls, etc.).

- ❖ **Dosage:** Volume o (ml), frequency and interval between doses.
- ❖ **Field Safety Study:** Clinical study conducted using the vaccine under actual marketing conditions and following manufacturer's indications to assess safety in the target animal.
- ❖ **Good Clinical Practices (GCP).** - Application of standardized procedures for the design, conduct, data recording, analysis and reporting of clinical studies. Adherence to the standardized procedures provides assurance that the data and reported results are complete, correct and accurate.
- ❖ **Good Laboratory Practices (GLP).** - Application of standardized procedures for the design, conduct, data recording, analysis and reporting of non-clinical studies. Adherence to the standardized procedures provides assurance that the data and reported results are complete, correct and accurate.
- ❖ **Laboratory Safety Study:** Clinical study conducted with the LPR in controlled conditions and following manufacturer's indications, to assess safety in bovines. They are conducted before Field Safety Studies.
- ❖ **Masking/ Blinding:** A procedure to reduce potential study bias in which designated study personnel are kept uninformed of the treatment assignment.
- ❖ **Negative Control:** Healthy animals that are untreated or which receive placebo.
- ❖ **Pilot Batch:** A batch of a vaccine manufactured by a procedure fully representative of the one to be applied at commercial scale. Manufacturing methods should be identical, except for the scale of production.
- ❖ **Positive Control:** Healthy animals that are given a similar vaccine, which is normally registered in the country where the study is conducted. The product is chosen by the manufacturer and it is indicated for the same disease and target species claimed for the LPR under evaluation.
- ❖ **Production Batch:** A batch of a vaccine manufactured in the production facility where the vaccine will actually be manufactured by the method described in the application for registration.
- ❖ **Protocol:** A document that fully describes the objective(s), design, methodology, statistical considerations and organization of a study. The document is signed and dated by all the responsible persons taking part in the assay. The protocol may also include the background and rationale for the study, but that information may also appear in other study protocol-referenced documents. The term includes all protocol amendments.
- ❖ **Safety.-** In this guidance, the term safety (in Spanish *seguridad*) is used as a synonym of **innocuity**, term defined by the CAMEVET glossary as: "*Property of a veterinary product that indicates that its correct administration at the target species will not determine the presence of adverse effects in a statistically significant proportion*". In some documents from Spanish-speaking countries, the term **tolerancia** is also used with the same

meaning (MAPA Brazil, 2008 and SENASA Argentina, 2006).

## **5. GENERAL RECOMMENDATIONS**

In order to plan a safety study using vaccines in bovines, the available information on several aspects, such as the type of vaccine, the nature of adjuvants, excipients, dose and proposed usage regimen, claims, previous usage history of similar products, target species class, and breed, need to be borne in mind; as well as all other data on safety that has been reported during the steps of development of the product. The mentioned data are important to support the design of the safety studies, defining the critical parameters that need to be examined.

The data from safety studies on combined vaccines may be used to demonstrate safety of vaccine(s) from the same manufacturer(s) containing less amount and/or a lower concentration of antigenic fractions, provided the remaining components are identical in each case and it is only the number of antigens what decreases in future formulations. Adverse events must be described and included in the final report, and determination of causality for the adverse event attempted.

## **6. GUIDELINE FOR THE CONDUCT OF STUDIES**

The following recommended procedures refer to Laboratory Safety Studies, Field Safety Studies and Reproductive Safety Studies.

### **6.a) STANDARD PROCEDURES**

Safety studies in bovines should be performed and managed in accordance with Good Laboratory Practices (GLP), Good Clinical Practices (GCP) and internationally accepted animal welfare regulations.

### **6.b) LABORATORY SAFETY STUDIES**

Laboratory safety studies are the first step in the evaluation of safety and provide basic information for the second stage, field studies.

The animals should be in the age, sex and class proposed by the vaccine label. Treated and control animals should be managed similarly and their environmental conditions should be the same. Every animal should be unequivocally and individually identified.

Facilities should be adequate for the purpose of the study and conform to local animal welfare regulations. Animals should enter study facilities one week before the initiation of the study, to acclimatize. Any sanitary treatment should be completed and reported before initiation of the study. Reduction or elimination of suffering during the study is essential. Euthanasia and necropsy of moribund animals is recommended.

Essential parameters to be evaluated for safety are: clinical observation of the animals, local and systemic reactions related to the vaccine and its resolution, as well as the effects of the vaccines on reproduction, when applicable. Study



protocol should be detailed and include the appropriate sheets for data collection.

Complementary tests, such as hematology, blood chemistry, necropsy or histological examination may be required. Where these tests are conducted in a subset of animals, these animals should be randomly selected with an adequate sampling rate before study initiation to avoid bias, unless otherwise justified. Samples should be properly selected, so that, in case of unexpected reactions or results, the cause of the problem observed may be identified.

The personnel participating in the studies should be masked (blinded) to treatment identification to minimize bias.

Working protocol may vary if applicable.

- **One Dose and Repeat Dose Test**

One Dose or Repeat Dose Studies should be conducted with the LPR containing the maximum declared antigen concentration, or in case this is not specified, with a multiple of the minimum antigen concentration.

For vaccines that require primary vaccination series followed by booster vaccination (2<sup>nd</sup> dose), the interval applied should be the shortest of the vaccination regime recommended by the manufacturer. For practical reasons, the interval between administrations may be reduced up to 14 days.

In general, 8 animals per group should be used, unless properly justified. The most sensitive class, age and sex proposed on the label should be used. If multiple routes and methods of administration are specified for the product concerned, administration by all routes is recommended. If one route of administration has been shown to cause the most severe effects, this single route may be selected as the only one for use in the study.

- **Overdose Test**

When considered appropriate, an overdose of vaccine, for each of the indicated routes and for the subset of animals indicated by the manufacturer should be administered. For inactivated vaccines, the overdose study is established as an administration of the double of the indicated dose, in only one application. (E.P. 7.0, 2013)

- **Data Collection**

General clinical observations should be made every day for 14 days after each administration, trying to maintain the same observation time. In addition, other relevant criteria such as rectal temperature or performance measurement (weight gain, dairy production, etc.) are to be recorded within this observation period with appropriate frequency. Injection sites should be examined daily or at other justified intervals by inspection and palpation and measurement for a minimum of 14 days after each administration of the LPR being tested. When injection site adverse reactions are present at the end of the 14 days observation, the observation period should be extended until clinically acceptable resolution of the

lesion has occurred or, if appropriate, until the animal is euthanized and histopathological examination is performed.

When previous information on similar vaccines is available, it is convenient to set acceptance criteria (hyperthermia up to certain time, acceptable size of local reactions, etc.). When designing recording sheets, it is useful to anticipate the annotation of the length of eventual systemic and local reactions, as well as their way of resolution

- **Statistical Analysis**

In laboratory studies the safety implications are best addressed by applying descriptive statistical methods to the data. Tables and descriptive text are common methods of data summarization; however, it may also be valuable to make use of graphical presentations in which patterns of adverse events are displayed both within treatments and within individual animals. In field studies, if applicable, selection of the general form for a statistical model and the factors to be included in the model will depend on the nature of the response variable being analyzed and the study design. Regardless of the methods chosen, the process and steps used to conduct any statistical evaluations should be described. The outcomes of the data analysis should be clearly presented to facilitate evaluation of potential safety concerns. The terminology and methods of presentation should be chosen to clarify the results and expedite interpretation.

Although there may be interest in the null hypothesis of no difference between treatments, study design constraints limit the statistical power and discriminatory ability of these studies. Under these conditions, statistical analysis alone may not detect potential adverse effects and provide assurance of safety. A statistically significant test does not necessarily indicate the presence of a safety concern. Similarly, a non-significant test does not necessarily indicate the absence of a safety concern.

Therefore, results should be evaluated based on statistical principles but interpretation should be subject to veterinary medical considerations.

## 6.c) REPRODUCTIVE SAFETY STUDIES

Examinations of reproductive performance of animals in breeding animals must be considered when data suggest that the starting material from which the product is derived may be a risk factor. Laboratory Studies together with Field Safety Studies are required to support use in breeding animals. If Reproductive Safety Studies are not performed, an exclusion statement must be included on the label, unless a scientific justification for absence of risk for use of the LPR in breeding animals is provided.

For examination of reproductive safety, animals appropriate for the purpose of the study will be vaccinated with at least the recommended dose according to the vaccination scheme indicated. If multiple routes and methods of administration are specified for the product concerned, administration by all routes is recommended. If one route of administration has been shown to cause the most severe effects, this single route may be selected as the only one for use in the study. Generally, 8 animals per group should be used unless

otherwise justified. The animals should be observed for a period appropriate to determine reproductive safety, including daily safety observations. Exceptions should be justified. A control group should be included.

Vaccines recommended for use in pregnant animals must be tested as described above in each of the specific periods of gestation recommended for use by the manufacturer. An exclusion statement will be required for those gestation periods not tested. The observation period must be extended to parturition, to examine any effects during gestation, parturition or on progeny. Exceptions should be justified.

When scientifically warranted, additional studies may be required to determine the effect(s) of LPR on semen. The observation period should be appropriate for the purpose of the study.

#### 6.d) FIELD SAFETY STUDIES

Where epidemiology of the disease to be prevented and husbandry practices are similar between regions or countries, international data from field studies may be used, as long as it is accepted by the regulatory authorities. The manufacturer is responsible for ensuring that field studies are conducted under animal husbandry conditions representative of those regions and countries in which authorization is sought. Appropriate health authorizations must be obtained and consultation with regulatory authorities regarding study design prior to conduct of the studies is recommended.

If a label indicates use in breeding animals, appropriate field safety studies need to be performed to show the safety of the LPR in field conditions.

- **Animals and study sites**

Bovines should be in the age, range and class intended for treatment as indicated in the proposed labeling. Previous serological status may be considered, although it is not a restrictive condition. A control group should be included.

Two or more different geographical sites are recommended to conduct safety studies. The recommended dosage(s) and route(s) for vaccination should be used. The studies should be conducted using representative production batch(es) of the vaccine.

### 7. FINAL REPORT

Procedures must be recorded in working protocols, following Good Clinical Practices (GCP). Observations should be made over a period of time appropriate for the LPR and its adverse events should be documented and included in the final report. Reasonable attempts should be made to determine causality for the adverse event(s).

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April 5th, 2014

C A M E V E T

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TRÁMITE I

DATE: SEPTEMBER 27 TH, 2013

***POTENCY FOR BOVINE VACCINES CONTAINING  
INACTIVATED BOVINE ROTAVIRUS GROUP A CAUSAL  
AGENT OF CALF NEONATAL DIARRHEA***

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# ***POTENCY FOR BOVINE VACCINES CONTAINING INACTIVATED BOVINE ROTAVIRUS GROUP A CAUSAL AGENT OF CALF NEONATAL DIARRHEA***

## **1. INTRODUCTION**

The syndrome named neonatal calf diarrhea or calf scour is a multifactorial disease affecting calves under three months of age. Calf scour represent an important sanitary problem in cattle livestock worldwide. Calf scour can be due to infectious and non infectious causes; however infectious diarrheas are the ones with causing highest mortality (Blanchard, 2012).

Diarrhea etiology involves viral, bacteria and parasites, including Rotavirus, Coronavirus, pathogenic *Escherichia coli*, *Salmonella spp.*, *Clostridium perfringens*, *Cryptosporidium spp.* and coccidia (Blanchard, 2012). Rotavirus group A (RVA) is considered the main cause of calf diarrhea worldwide (Badaracco et al., 2012; Blanchard, 2012; Cho et al., 2013), affecting calves under eight week of age. The susceptibility is reduced as the age increases. RVA infection is restricted to the mature enterocytes of the tip of the villi of the small intestine of neonatal calves. After three month of age the enteric epithelial cells are replaced by non susceptible cells (Blanchard, 2012; Dhama et al., 2009).

Rotaviruses are classify in G-types an P-types according to the variability of the the two most external capsid proteins VP7 (glycoprotein) and VP4 (spike and protease sensitive). The RVA circulating in bovine carry the G-types: G1, G2, G3, G4, G5, G6, G8, G10 y G15; and the P-types P[1], P[5], P[11], P[14], P[17] y P[21]. However, only G6, G10 y G8 in combination with P[5], P[11] y P[1] are epidemiologically relevant (Badaracco et al., 2011, 2012; de Verdier Klingenberg et al., 1999; Fukai et al., 2004; Garaicoechea et al., 2006).

The preventive measures to control neonatal calf diarrhea due to bovine RVA are focused in reducing the severity of the disease and the amount of infectious virus shed to the environment. The vaccination of the pregnant dams during the last stage of pregnancy (60 and 30-45 days before calving) is recommended in order to increase the transference of specific maternal antibodies to the newborn calf via colostrums intake (Kaplon et al., ; Parreño et al., 2004; Saif and Fernandez, 1996).

The international regularoy agencies (APHIS, USA; EMEA-CVMP, UE; OIE; VICH) had not given any recommendation regarding the production and potency testing of RVA vaccines. However, giving the sanitary impact of this neonatal disease in the cattle industry in America, **it is important to work in the development and implementation of control test in lab animal models that can be used for testing the potency not only of veterinary vaccines used for cattle and equines but also for human infants.**

The aim of the present guide is the recommendation of a guinea pig model as alternative method for RVA vaccine potency testing of each vaccine batch to be released into the market. The test has been statistically validated with the target species and possesses a substantial agreement with the results obtained in bovines. The Antibody titer obtained in vaccinated guinea pigs can be used as a predictive parameter to estimate the antibody titer induced by the vaccine in the vaccinated cows. Additionally, the efficacy of vaccines classified as of satisfactory and no satisfactory potency by the guinea pig model were tested in a calf model on RVA infection and disease (Parreño et al., 2012). The guinea pig model represents a proper predictive tool to estimate the protection rate conferred by colostral antibodies in calves fed colostrums from vaccinated and non-vaccinated cows, further challenged with bovine RVA (Parreño et al., 2004).

Regarding animal health, international organisms encourage the development of *in vitro* tests, to avoid and reduce to the minimum the use of animals for experimental control tests (Hendriksen, 2009). In the particular case of the vaccines used for the prevention of neonatal calf diarrhea, inactivated, aqueous or oil adjuvanted, containing one or more RVA strains combined with other bacterial (*E. coli*, *Salmonella spp*) and viral (bovine coronavirus) agents, the application of these techniques is possible and worth exploring them. However, given the disadvantage that each formulation (group of inactivated antigens and adjuvant, vaccines in subunits and DNA vaccines) needs to be standardized, an *in vivo* test is still considered inevitable to assess potency of these products (Taffs, 2001).

## **2. POTENCY CONTROL IN GUINEA PIGS: AIMS**

This guide describes a procedure for *in vivo* vaccine potency testing in lab animals (guinea pigs) which also predict the efficacy of the vaccines to prevent RVA diarrhea in calves. The number of animals employed are n= 6 per vaccine and 4 witnesses/placebos. Guinea pigs do not possess a RVA infecting them and are naturally seronegative for antibodies to RVA. Guinea pigs, unlike other laboratory animals such as mice, have the advantage of being bigger in size, thus allowing paired serum sampling without risking their lives. Furthermore, with the volume of sample obtained, the quality of all viral antigens contained in polyvalent vaccines can be assessed. In some cases, such vaccines can contain two RVA strains plus a coronavirus. Finally, serological evaluation is independent from the type of adjuvant (oil or water) and from the amount and quality of inactivated viruses contained in the formulation.

Regarding animal welfare, this test is according to the 3R (Hendriksen, 2009).

For the validation of the model international recommendations were followed (EMEA/P038/97, 1998; Taffs, 2001). Experimental and commercial vaccines containing RVA combined with other pathogens causing calf diarrhea (*E. coli*, *Samonella*, bovine Coronavirus, among others), formulated in aqueous and oil adjuvants were tested in parallel in guinea pigs and bovines. Vaccine immunogenicity measured in guinea pig as the

anti-RVA IgG antibody (Ab) titer at 30 days post vaccination was correlated with the IgG1 Ab titer to RVA in bovines reached at 60 days post vaccination –in concurrence with calving moment. IgG1 isotype is selectively transferred from the cow’s serum to the colostrum and the IgG1 absorbed by the calves after colostrum intake is associated with protection rates in experimentally RVA challenge calves (Parreño et al., 2004). The ELISA techniques used in this protocol were validated under 17025 ISO quality standards and the procedure is detailed in ANNEX I.

The statistical analysis conducted showed almost perfect to substantial agreement between the guinea pig model and the bovines to predict the potency of a RVA vaccine. The technical details of the validation are summarized in ANNEX II. This guinea pig model can be used to determine the potency of each RVA vaccine batch to be release to the market. It is a useful and practical tool for the vaccine companies and for the animal health authorities to guarantee the potency and efficacy of biological product by a harmonized control.

## **2.1 Test design**

### *2.1.1 Guinea Pigs*

A minimum of 6 animals are used for each vaccine to be tested, animal should be older than 30 days of age and possess a weight of  $400 \pm 50$  grams. The animal can be males and females, but each group may have animals of the same sex. Quarantine period should be at least seven (7) days after arrival. Animal are seronegative to antibodies to bovine RVA.

### *2.1.2 Procedure*

Guinea pigs are immunized with two doses of vaccine ( 21 days apart), by subcutaneous route and a dose volume corresponding to 1/5 of the bovine dose. The animals remain under control during 30 at first dose (0 days post vaccination -DPV) and 9 days post-booster (30 DPV). Together with the guinea pigs inoculated with the tested vaccine (n=6), two other groups of guinea pigs are included, one inoculated with a reference vaccine of known potency (n=6) and a group of witness, non vaccinated control animals (n=4). At 30 DPV, all animals are sampled, sera are tested for RVA-specific antibodies by ELISA (ANNEX I). Complementary, serum samples can be tested by viral neutralization assay, technique to evaluate the neutralizing Ab titer to each serotype of RVA included in the vaccine.

### 2.1.3 Interpretation

The regression analysis conducted between the IgG Ab titer to RVA in guinea pigs and IgG1 to RVA in bovines determined by ELISA in the validation of the model demonstrated that Ab response induced by vaccination in both species is directly proportional to the RVA antigen concentration of the vaccine. Guinea pigs, because their seronegative condition, showed higher power than the bovines to discriminate between vaccine containing different antigen dose (dose-response assay, ANNEX II) (Parreño et al., 2012).

From the results obtained in the regression equation and using classification three analysis, two split points and three vaccine categories were estimated, ANNEX II (Parreño et al., 2012).

The guinea pig model significantly discriminated between vaccines with RVA concentrations of  $10^4$ ,  $10^5$  y  $10^6$  FFFU/dose or higher. Vaccines inducing RVA Ab titer higher than 4.0 in guinea pig are considered of satisfactory potency, while vaccines inducing RVA Ab titers higher than 4.46 are classified as of very satisfactory potency. Vaccines inducing RVA Ab titer higher than 4.0 in guinea pig induced an increment in the Ab IgG1 titer to RVA of 0.40 regarding their base line Ab titer. Vaccines with Ab titers in guinea pigs higher than 4.46 induced an increment in the IgG1 anti-RVA Ab titer in the vaccinated bovines of 0.75 regarding their base line Ab titer and reached a final IgG1 Ab titer to RVA of 3.70 at calving (60 dpv).

The estimated split points were used to evaluate the concordance between the model and bovines to classify the vaccines ANNEX II (Parreño et al., 2012).

Regarding the vaccine potency obtained in guinea pigs and vaccinated cows and its efficacy to protect calves against RVA diarrhea, the conducted studies indicated that calves receiving 1 liter of colostrum of unvaccinated cows (IgG1 ab titer to RVA higher than 3.53), reach IgG1 Ab titer in serum to RVA between 2.4 and 3.01 and develop severe diarrhea. Calves receiving 1 liter of colostrum from vaccinated cows (IgG1 ab titer to RVA higher than 3.70) reach IgG1 Ab titer in serum to RVA between 4.21 and 4.81 and showed a significant reduction in the severity of the diarrhea (Parreño et al., 2012).

Species	VACCINE POTENCY			
	Non satisfactory	Intermediate	Satisfactory	Very Satisfactory
GUINEA PIG	$\bar{y} < 1.93$	$1.93 \leq \bar{y} < 4.0$	$4.0 \leq \bar{y} < 4.46$	$\bar{y} \leq 4.46$

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BOVINE  
(T60-T0 IgG1 Ab titer)

$\bar{Y} < 0.33$

$0.33 \leq \bar{Y} < 0.40$

$0.40 \leq \bar{Y} < 0.75$

$\bar{Y} \leq 0.75$

---

Table 1. Split points determined by ELISA expressed as the log<sub>10</sub> of the reciprocal of the analyzed serum dilution that results positive in the assay. 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 manufacturer'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 at calving. 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 lab 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.

The guinea pig model succeeded in adequately predicting not only vaccines immunogenicity, but also the efficacy to prevent against RVA diarrhea in experimentally challenged calves (Parreño, 2012). The proposed test does not need complex technology nor infrastructure, just an animal facility with guinea pigs and common serological techniques (ELISA, alternatively VN) of routine use in virology laboratories, appropriately harmonized with international norms (9CFR, OIE, EMEA) and preferably validated following norms ISO-IEC 17025.

#### *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 quality results to be the expected value (higher than 4.0 in guinea pigs), and unvaccinated control animals (controls) remain seronegative for Ab against RVA-1 throughout the experience.

#### *2.1.5 Calculation*

All serums of animals immunized with the vaccine under control obtained 30 DPV will be tested by ELISA. The Ab titer to RVA of each sample is expressed as the log<sub>10</sub> of the inverse of the highest dilution of sera positive for Ab to RVA. FIVE (5) sera with the highest titers obtained 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 at 30 dpv must be equal or higher than **4.0**.

### **3. Harmonization of assays for the region**

A positive and negative control serum panel and reference vaccines will be elaborated and made available for regional users to harmonize the results obtained for each assay laboratory adopting the control method. Reference vaccine will allow to declare an assay as properly conducted while the panel of reference sera could be used as positive control of the recommended technique (ELISA) and as positive standards for the standardization of alternative assays (VN).

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## **Annex II**

### **CAMEVET Guideline**

**Development and statistical validation of a guinea pig model as an alternative method for potency testing of bovine rotavirus vaccines**

Poster presentation at:

## **11<sup>th</sup> International Symposium on Double-Stranded RNA Viruses**



**San Juan, Puerto Rico  
November 27 – December 1, 2012**



## Section

### Vaccines and Biotherapies

- P1-18      **Sukumar Kandasamy** (Ohio State Univ, USA)  
Vitamin-A deficiency impairs antibody responses to human rotavirus vaccines and protection in a neonatal gnotobiotic piglet model
- P1-19      **Sung-sil Moon** (CDC, USA)  
Dose sparing and enhanced immunogenicity of inactivated rotavirus vaccine administered to skin using a microneedle patch
- P1-20      **Viviana Parreño** (Virology Institute, INTA, Argentina)  
Statistical validation of a guinea pig model as an alternative method for bovine rotavirus vaccines potency testing
- P1-21      **Hugo Reis Resque** (Oswaldo Cruz Inst, Brazil)  
Detection of unusual G8 and G12 rotavirus strains in vaccinated and non-vaccinated children from northeastern Brazil
- P1-22      **Gabriella Santoro** (Univ of Rome, Italy)  
Thiazolides inhibit rotavirus morphogenesis and interfere with virus-induced NF- $\kappa$ B activity
- P1-23      **Yuhuan Wang** (CDC, USA)  
Inactivation of rotavirus by heat and formaldehyde
- P1-24      **Alejandra Badaracco** (Virology Institute, INTA, Argentina)  
Discovery and molecular characterization of a group A rotavirus strain detected in an Argentinean Vicuña (Vicugna vicugna)
- P1-25      **Houping Wang** (CDC, USA)  
Interference between G1 Rotarix and G2 DS-1 strain in human intestinal cells

## **Development and statistical validation of a guinea pig model as an alternative method for potency testing of bovine rotavirus vaccines**

Viviana Parreño<sup>1</sup>; Maria V. Lopez<sup>2</sup>; Maria Vena<sup>3</sup>; Daniela Rodriguez<sup>1</sup>; Jorge Fillippi<sup>3</sup>; Celina Vega<sup>1</sup>; Gisela Marcoppido<sup>1</sup>; Laura Marangunich and F. Fernández<sup>1</sup>

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<sup>(2)</sup> Facultad de Agronomía, UBA

<sup>(3)</sup> Biogénesis Bagó S.A.

<sup>(4)</sup> Universidad Nacional de Tres de Febrero, UNTREF, Argentina

### **Abstract**

Bovine A Rotavirus Group (RVA) is the major cause of neonatal diarrhea in calves, worldwide. The prevention of RVA neonatal calf diarrhea is based on passive immunity strategies. Cows are vaccinated during pregnancy with RVA vaccines to increase the maternal passive immunity transferred to their calves. Vaccine commercialization requires immunogenicity test in the target species. The aim of this study was the development of a guinea pig model as a method for RVA vaccine potency testing statistically validated to predict vaccine immunogenicity and efficacy in bovines. Briefly guinea pigs were immunized with two doses of vaccine, 21 days apart and sampled at 30 days post vaccination (dpv). Rotavirus IgG antibody (Ab) response to vaccination in guinea pigs was measured by ELISA and statistically compared to the IgG1 Ab response in vaccinated cows. The guinea pig model showed a dose–response according to the RVA antigen concentration in the vaccine. It was able to discriminate among vaccines containing 1 log<sub>10</sub> difference in its RVA concentration with very good repeatability and reproducibility (CV<20%). A classification tree analysis of the Ab titers obtained in guinea pigs and bovines allowed vaccine classification in three potency categories: satisfactory, intermediate and unsatisfactory. Concordance analysis using experimental and commercial vaccines showed almost perfect agreement between the model and bovines (Weighted Kappa: 0.825). Calves fed colostrum from vaccinated/unvaccinated cows showed different protection rates according to the category of the vaccine. The obtained results indicate that the developed guinea pig model represents a reliable tool to estimate batch-to-batch vaccine potency and to predict efficacy of RVA vaccines used in cattle.

### **1. Introduction**

Bovine Rotavirus Group A (RVA) is the major cause of neonatal diarrhea in calves worldwide. The prevention of the disease is based on passive immunity strategies. Cows are vaccinated during the late stage of pregnancy with RVA inactivated vaccines to increase the maternal passive immunity transferred to their calves via colostrum intake. The vaccines used to prevent RVA calf scours in Argentina are aqueous or oil formulations including G6P[5] and G10P[11] inactivated RVA

together with different combinations of the following antigens: E coli, Salmomella, Clostridium and Corovavirus. The Virology Institute, INTA conducted the development and statistical validation of a lab animal model to be used as an alternative method for vaccine potency testing, instead of cattle, that will allow the vaccine producers and the National Veterinary Health Authorities (SENASA) to control the immunogenicity of the RVA vaccines released to the market.

Guinea pigs raised under controlled conditions possess the advantage that are naturally seronegatives for antibodies (Ab) to RVA, but develop a strong Ab response after RVA immunization resulting in an excellent model for vaccine potency testing.

The aim of this study was to develop a guinea pig model as a method for RVA vaccine potency testing statistically validated to predict the immunogenicity and efficacy of RVA vaccines in bovines. The potency of the vaccine obtained in the guinea pig model was compared with the potency obtained in cattle and used as predictive tool to estimate the efficacy of the vaccines to prevent RVA diarrhea in neonatal calves.

## **2. Materials and Methods**

### **2.1. Experimental Design, vaccines and statistical analysis:**

#### ***2.1.1- Dose-Response assay : Kinetic of Ab responses, discriminatory capability, split points***

Two sets of four water-in-oil vaccines containing increasing concentration of bovine RVA were formulated with the reference strains UK (G6P[5]) and B223 (G10P[11]) (Fig. 1). Viral antigens were BEI inactivated. Each set of these “reference vaccines” was tested in three independent field trials in bovines (n=5 per group). Heifers and cows were immunized with two doses (3ml) of vaccine 30 days apart. In parallel, each set of vaccines was administered to groups of 5 guinea pigs in two independent assays. Guinea pigs received two doses of vaccine corresponding to 1/5 of the bovine dose (0,6 ml, 21 days apart) (Fig. 2). The minimum number of repetitions (animals per group; n=5) was calculated to achieve a statistical power of at least 83% to discriminate between vaccines containing bovine RVA concentrations differing in one  $\log_{10}$  (Pryseley, 1999; Westfall, 1999).

The obtained results were used:

- i) to establish the optimal sampling time for comparison between the guinea pig model and bovine,
- ii) to evaluate the ability of each species to discriminate among vaccines of different antigen concentration (ANOVA),
- iii) to estimate the splits point for vaccine classification by Regression and Classification Tree analysis (Di Rienzo, 2002).

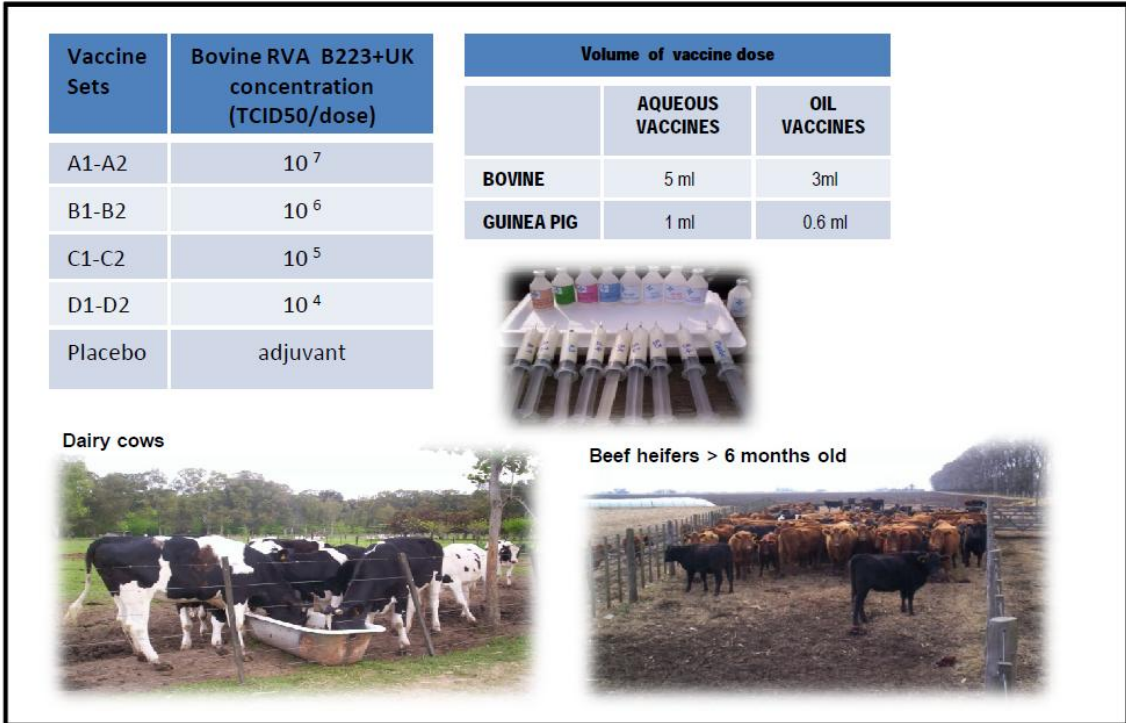
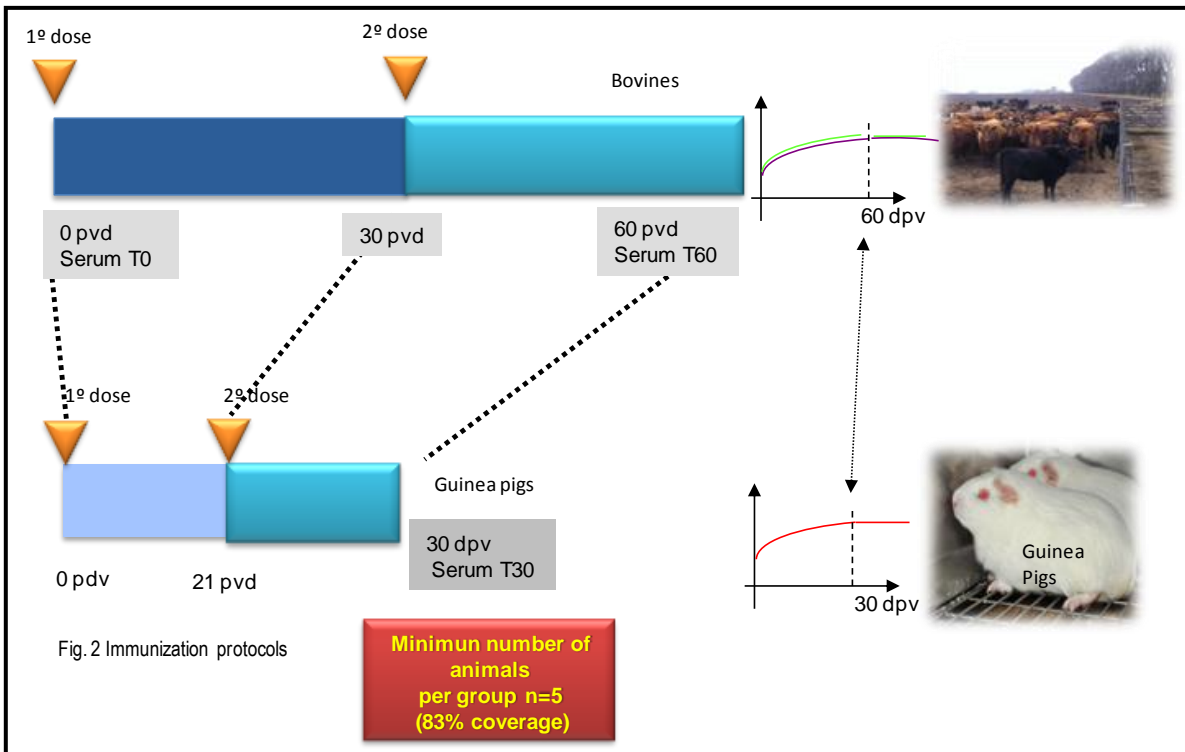


Figure 1. Dose-Response assay: reference vaccines



### **2.1.2- Concordance analysis**

The calculated split points were further used for vaccine classification in three categories: “low”, “intermediate” and “satisfactory” potency. A total of 33 vaccines (16 experimental and 17 commercial) were tested in parallel in cattle and guinea pigs. Concordance between the model and the target species to classify the vaccines was evaluated by weighted Kappa (Viera, 2005). The analysis was conducted using MedCalc software 12.3.0.0.

### **2.2- Serology**

Rotavirus IgG Ab response to vaccination in guinea pigs was measured by ELISA and statistically compared to the IgG1 Ab response in vaccinated cows. Both ELISA assays were standardized under ISO 17025 standards, the validation parameters are summarized in Table 1.

IgG1 isotype was specifically determine as a market of quality control of bovine RVA vaccines, since it is the specific isotype that is actively transferred from serum to colostrum in the bovine mammary gland and represent the main isotype of passive maternal antibodies transferred to the neonatal calves after colostrum intake.

Table 1. ELISA for the titration of RVA-specific antibodies in guinea pigs and bovine sera

<b>Validation Parameters</b>	<b>IgG anti-RVA Guinea pig</b>	<b>IgG1 anti-RVA Bovine</b>
<b>Cut off</b>	11%PP	12%
<b>Sensitivity</b>	98.5%	97.7%
<b>Specificity</b>	97.7%	100%
<b>Intermediate precision (3 years)</b>	24%	13.5%
<b>Accuracy (ROC analysis)</b>	98.5%	100%

### **2.3- Rotavirus Challenge experiment in neonatal calves**

Colostrum deprived calves were entered to isolation boxes and were fed 1 liter of colostrum from cows vaccinated with a satisfactory and an intermediate RVA vaccine within the first 6 hours after birth and then fed Ab free milk. Colostrum deprived calves were assigned as negative control group. All animals were challenged orally with  $10^5$  FFU of RVA G6P[5]. Calves fed colostrum from cows vaccinated with the satisfactory vaccine showed significantly higher protection against RVA diarrhea than calves fed colostrum of cows vaccinated with a vaccine of intermediate potency. This latter group did not differ from the colostrum deprived calves. All animals shed virus after the experimental infection, only calves fed colostrum from cows vaccinated with the satisfactory

vaccine showed a significant delay in the onset of virus shedding compared with the colostrum deprived calves.

### 3. Results

#### 3.1.1 Dose Response: discriminatory power and kinetics of the Ab response

Both species showed a dose–response according to the RVA antigen concentration in the vaccine and were able to discriminate among vaccines containing 1 log<sub>10</sub> difference in its antigen concentration (Table 2). When studying the increment in the Ab titer after vaccination, bovine were able to discriminate between vaccines containing 10<sup>7</sup> from those with 10<sup>6</sup> -10<sup>5</sup> and 10<sup>4</sup> TCID<sub>50</sub> RVA/dose. The guinea pig model was able to discriminate among vaccines 10<sup>6</sup> or higher from 10<sup>5</sup> and 10<sup>4</sup>. In both species vaccines containing 10<sup>4</sup> TCID<sub>50</sub> did not induce Ab responses to RVA.

Table 2. Antibody responses to RVA after vaccination

Vaccine RVA concentration TCID <sub>50</sub> /dose	BOVINE			Guinea pig	
	n	ΔIgG1 RVA Ab titer T60-T0	Average IgG1 RVA Ab titer t at 60 pvd (calving)	n	Avera RVA IgG Ab titer at 30 pvd
10 <sup>7</sup>	15	1.04 A	4.00	20	4.79 A
10 <sup>6</sup>	14	0.63 B	3.52	18	4.67 A
10 <sup>5</sup>	15	0.44 B	3.23	17	4.00 B
10 <sup>4</sup>	14	0.10 C	3.25	16	0.70 C
Placebo/non vaccinated	17	-0.03 C	2.91	20	0.30 C

Mean in the same column with different uppercase letter differs significantly. One way ANOVA, Bonferroni, p<0.0001

#### 3.1.2 Classification Tree analysis and split points for vaccine classification

The classification tree analysis of the Ab titers obtained in guinea pigs and bovines (Fig 3a) allowed vaccine classification in three potency categories. The split points in guinea pigs were estimated as the value that better discriminates the vaccines previously classified by bovines (Fig 3b).

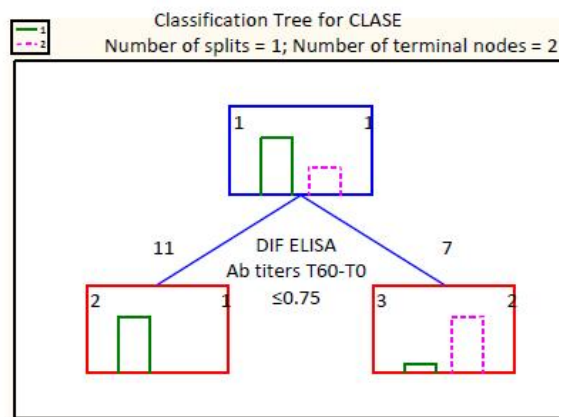
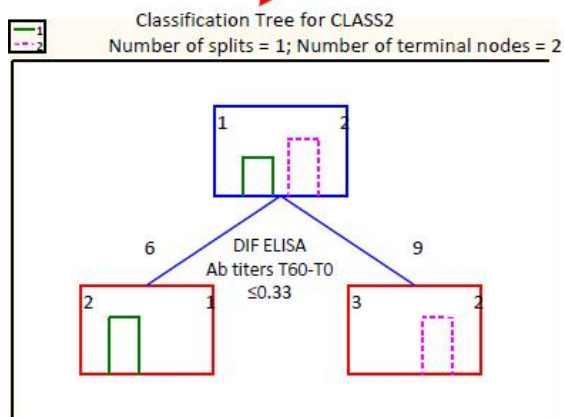
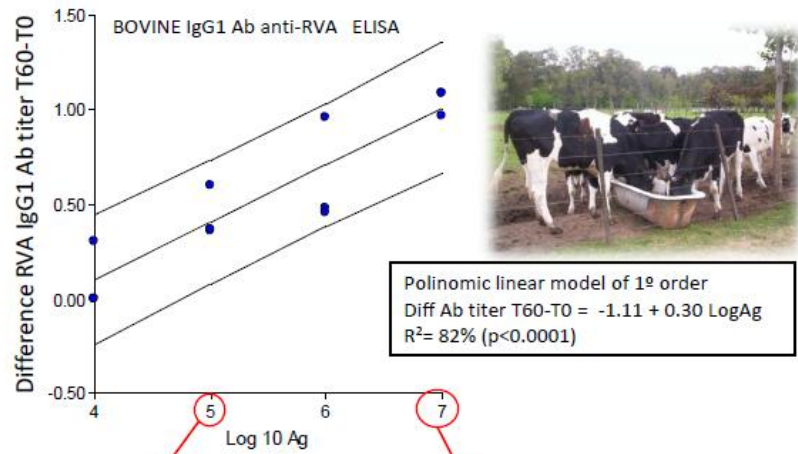


Fig. 3a. Split points estimated in bovine

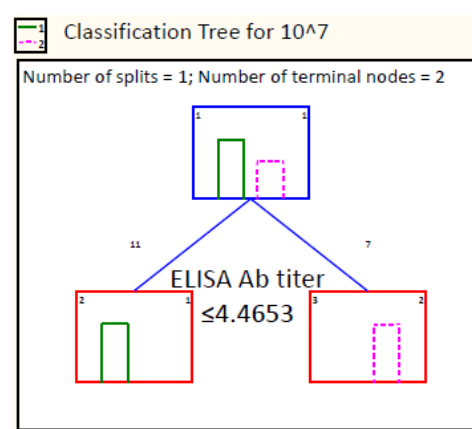
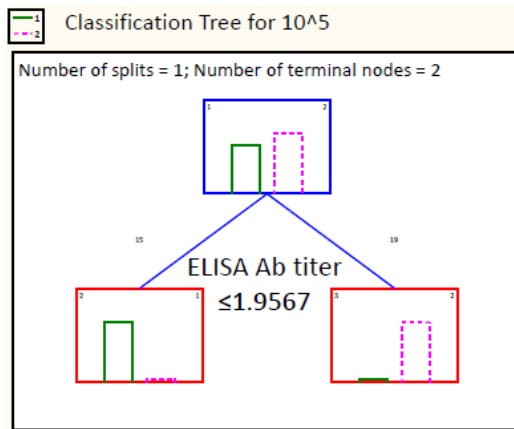
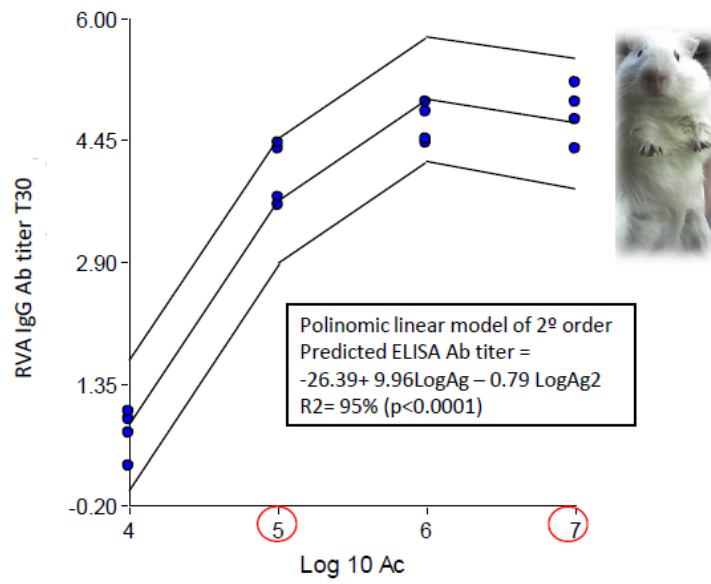


Figure 3b. Splits points estimated for guinea pigs based in the classification made by bovines



In bovines, the split points were estimated in terms of increment of Ab titer after vaccination as well as the final Ab titer reached at 60 pvd (calving) (Table 3). The Ab titer increments after vaccination (T60-T0) obtained in bovines were compared with the Ab titer induced by the vaccines in guinea pigs.

Table 3. Splits points for Vaccine classification

Animal species	Vaccine classification according to RVA ELISA Ab titer (RVA concentration /dose)		
	Low Potency (<10 <sup>5</sup> )	intermediate Potency [10 <sup>5</sup> -10 <sup>7</sup> )	satisfactory potency [≤10 <sup>7</sup> )
<b>GUINEA PIG</b> (IgG Ab titer 30 dpv)	x < 1.96	1.96 ≤ x < <b>4.46</b>	<b>4.46</b> ≤ x
<b>BOVINE</b> Diff T60-T0 IgG1 Ab titer IgG1 Ab titer (60 dpv)	X < 0.33 X < 3.53	0.33 ≤ X < <b>0.75</b> 3.53 ≤ X < <b>3.70</b>	<b>0.75</b> ≤ X <b>3.70</b> ≤ X

### 3.3 Concordance analysis

Concordance analysis using 43 comparison lines, including experimental and commercial vaccines showed **almost perfect agreement** between the lab animal model and bovines. When only the commercial vaccines and negative control were tested and the references vaccines are excluded from the analysis the concordance remains with **substantial agreement** (Weighted Kappa: 0.762). When the analysis is conducted using the final IgG1 anti-RV Ab titer at calving (60 pvd) there are substantial agreement between the model and the target species (Weighted Kappa: 0.752).

Table 4. concordance analysis including all vaccines tested

ELISA; n=43			
Guinea Pig \ Bovine	LOW Xdif t60-t0 < 0.33	INTERMEMEDIATE 0.33 ≤ x < 0.75	SATISFACTORY 0.75 ≤ x
LOW x < 1.935	<b>16</b>	0	0
INTERMEDIATE 1.95 ≤ x < 4.46	0	<b>8</b>	5
SATISFACTORY 4.46 ≤ x	0	2	<b>12</b>

Sample size 43; 43 comparison lines . Placebo / non vaccinated control groups = 10; Experimental vaccines of known concentration = 4; Doses-response reference vaccine = 12 Commercial vaccines = 17;  
Weighted Kappa 0.825; p<0.0001; **Almost Perfect agreement**

### **3.4 Efficacy of vaccines classified in different categories in calves experimentally challenged with bovine RVA**

Colostrum deprived calves were entered to isolation boxes and were fed 1 liter of colostrum from cows vaccinated with a satisfactory and an intermediate RVA vaccine within the first 6 hours after birth and then fed Ab free milk. Colostrum deprived calves were assigned as negative control group. All animals were challenged orally with  $10^5$  FFU of RVA G6P[5]. Calves fed colostrum from cows vaccinated with the satisfactory vaccine showed significantly higher protection against RVA diarrhea than calves fed colostrum of cows vaccinated with a vaccine of intermediate potency. This latter group did not differ from the colostrum deprived calves. All animals shed virus after the experimental infection, only calves fed colostrum from cows vaccinated with the satisfactory vaccine showed a significant delay in the onset of virus shedding compared with the colostrum deprived calves (Table 5).

### **Conclusions**

The obtained results indicate that the validated guinea pig model represents a reliable, economic and rapid tool, aligned with the 3R principle, to estimate batch-to-batch RVA vaccine potency.

- ✓ The potency obtained by the model represents a predictive value of the vaccine potency in pregnant cows and vaccine efficacy to prevent RVA diarrhea in neonatal calves.
- ✓ The model is under evaluation by the Argentinean animal health authorities (SENASA) to be use as the official test for RVA vaccine quality control.
- ✓ Its implementation will help to guarantee the presence of effective products in the local market and can be extended to control vaccine quality in the region.

Table 5. Challenge experiment in calves

Treatment	RVA IgG Ab Titer guinea pigs	RVA IgG1 Ab titer cow at calving	n calves	RVA IgG1 Ab titer calves 0 pid	Diarrhea				Virus shedding			
					% animal affected	Onset (days)	Duration (days)	Severity	%animal affected	Onset (days)	Duration (days)	Average RVAtiter (FFU/ml)
Satisfactory vaccine	4.94	4.81	6	4.52 A	67%	5.5 A	0.83A	4 A	100%	3.33 B	8	3.7
Intermediate vaccine	4.36	3.61	9	2.94 B	100%	2.2 B	7.44B	20 B	100%	2.11 AB	8	4.0
Colostrum deprive calves	na	neg	10	neg	100%	1.6 B	8.30B	24 B	100%	1.6 A	6	4.4

- Los recursos del CAMEVET se originan en el pago de inscripciones a los Seminarios.
- El presente Balance responde al período del 31 de diciembre de 2014 al 31 de octubre de 2015.
- Se presentan los ingresos y gastos tanto en dólares USA como en Pesos argentinos

**Cuenta en dólares hasta Octubre 31, 2015**

<b>Ingresos</b>	<b>31/12/2014 - 31/10/2015</b>
Recursos disponibles al 31 de diciembre 2014	USD 66,468.00
<b>Subtotal de Ingresos</b>	<b>USD 66,468.00</b>
<b>Nota: Total correspondiente al cierre de año 2014 que incluye, las inscripciones del Seminario en Canadá 2014 USD 26700 y lo acumulado de años anteriores.</b>	
<b>Egresos</b>	
<b>Gastos fijos (Salarios )</b>	
Secretario CAMEVET (Dr. Enrique Argento)	USD 4,000.00
Secretaria Asistente ( Srta. Ana Maria Sgammini)	USD 3,000.00
Gastos Admin. Por uso de las Oficinas de la OIE (150/mes)	USD 1,500.00
<b>Subtotal Gastos Fijos</b>	<b>USD 8,500.00</b>
<b>Gastos para la Reunión Anual de CAMEVET</b>	
Financiación a Puntos Focales para Reunión Anual de CAMEVET (Cuba , costo de tkt)	USD 818.00
Financiación para Oradores Reunión Anual de CAMEVET	0
Gastos Staff CAMEVET (Viáticos y gastos misceláneos)	0
Compras de Materiales para la Reunión Anual de CAMEVET	0
<b>Subtotal</b>	<b>USD 818.00</b>
<b>Gastos de Participación en Otros Eventos</b>	
Participación en reuniones del VICH Outreach Meetings	USD 2,589.00
OIE Conference Regional Commission for the Americas	
<b>Subtotal</b>	<b>USD 2,589.00</b>
<b>Otros Gastos</b>	
Internet ( Dominio de CAMEVET)	USD 10.00
<b>Subtotal</b>	<b>USD 10.00</b>
<b>Gastos Variables</b>	
Cambio de Dólares a Pesos Argentinos	USD 4,000.00

<b>Subtotal</b>	USD 4,000.00
<hr/>	
<b>Subtotal de Gastos</b>	USD 15,917.00
<hr/>	
<b>Total de Ingresos menos Egresos hasta Octubre 2015</b>	<b>USD 50,551.00</b>

**Nota: Viáticos -PF Cuba serán entregados durante el Seminario USD 953.00**

**Cuenta en Pesos hasta Octubre 31, 2015**

<b>Ingresos</b>	<b>31/12/2014 - 31/10/2015</b>
Recursos disponibles al 31 de diciembre de año 2014	ARS 12,405.00
Inscripción al Seminario CAMEVET 2015	ARS 60,000.00
Devolución de Gastos	ARS 0.00
Cambio de Dólares a Pesos Argentinos*	ARS 57,800.00
<b>Subtotal</b>	<b>ARS 130,205.00</b>

**\*Corresponde a 4000 dólares USA cambiado a ARS para compra de pasajes aéreos de Seminario y reuniones VICH**

**Nota: Del saldo de Inscripción en pesos ARS 60,000 el día 5 de noviembre se descontaron ARS 4,000 por pago duplicado de una inscripción correspondiente al Laboratorio OVER de Argentina. Total de inscripciones recibidas hasta el 6 de Noviembre ARS 56,000 pesos**

**Egresos**

**Gastos para la Reunión Anual de CAMEVET**

Gastos por compra de tiquetes aéreos (Dr. Argento – Sta. Ana Sgaminni)	ARS 28,167.00
<b>Subtotal</b>	<b>ARS 28,167.00</b>

**Gastos de Participación en Otros Eventos**

Gastos por compra de tiquetes aéreos -Dr. Argento – Vich*	ARS 44,043.70
<b>Subtotal</b>	<b>ARS 44,043.70</b>

**\*Nota: Reuniones de Vich Outreach Forum**

1. 24-25 de febrero, Washington DC USA
2. 25 - 29 de octubre, Tokio Japón

**Otros Gastos**

Varios (Clases de Inglés Secretaria)	ARS 3,900.00
Misceláneos ( Placa de Reconocimiento)	ARS 1,288.00
<b>Subtotal</b>	<b>ARS 5,188.00</b>

<b>Subtotal de Gastos</b>	ARS 77,398.70
<b>Total de Ingresos menos Egresos hasta Octubre 2015</b>	<b>ARS 52,806.30</b>

### Informe de ingresos y gastos CAMEVET Hasta 31 de Octubre 2015

<b>Dólares disponibles al 31 de octubre del corriente año</b>	50,551.00 USD
<b>Pesos disponibles al 31 de octubre del corriente año</b>	52,806.30 ARG*
(*Incluye inscripciones al XXI Seminario en pesos Argentinos)	

#### Ingresos

##### Inscripción al XXI Seminario Guatemala 2015

Inscripción en Pesos 14 participantes	56,000.00 ARG*
Inscripción en Dólares 115 Participantes*	40,975.00 USD

\* Se aplicaron diferentes categorías de inscripción y descuentos a patrocinadores.

**Nota:** Las inscripciones en pesos en total hasta 31 de Octubre fueron ARS 60,000.00 pero el 5 de Noviembre se hizo una devolución de ARS 4,000.00 pesos. A Organización Veterinaria Regional SRL por doble pago de la inscripción de Sr. Diego Eborraz.

**Nota:\*** De las inscripciones en dólares se emitió el pago de financiación Dr. Lemes Anaya por el concepto de full per diem por 6 días -USD 953.

#### Previsión de gastos 2015 – 2016

<b>Existencias al 31 de octubre de 2015</b>	<b>ARS 52,806.30</b>	<b>USD 50,551.00</b>
<b>Ingresos</b>		<b>USD 40,022.00</b>
<b>Totales al 13 de noviembre</b>	<b>ARS 52,806.30</b>	<b>USD 90,573.00</b>

<b>Salario secretario CAMEVET (Dr. Enrique Argento)</b>	<b>USD 4,000.00</b>
<b>Salario secretaria Asistente (Srta. Ana Maria Sgammini)</b>	<b>USD 3,000.00</b>
<b>Gastos Admin. Por uso de las Oficinas de la OIE (150/mes)</b>	<b>USD 1,500.00</b>
<b>Participación en reunión de VICH, Bruselas, junio de 2016 (Ticket aéreo y viáticos)</b>	<b>USD 3,500.00</b>
<b>Internet (renovación anual dominio @camevet.org)</b>	<b>USD 10.00</b>
<b>Total presupuesto de gastos</b>	<b>USD 12,010.00</b>

## Inscripciones Argentina 2015

No.	Nombre	Organización	País	Pago
1	Carlos Rufrano	CLAMEVET	ARGENTINA	ARS 4,000.00
2	Bruno Forti	CLAMEVET	ARGENTINA	ARS 4,000.00
3	Jorge Armando Dale	CLAMEVET	ARGENTINA	ARS 4,000.00
4	Patricia Garcia D'Auro	CDV	ARGENTINA	ARS 4,000.00
5	Martin Costi	Laboratorio WEIZUR	ARGENTINA	ARS 4,000.00
6	Gustavo Belligni	VITAFORMA	ARGENTINA	ARS 4,000.00
7	Carlos Francia	CAPROVE	ARGENTINA	ARS 4,000.00
8	Eduardo Lopez	CAPROVE	ARGENTINA	ARS 4,000.00
9	Juan Carlos Sosa	CHINFIELD S.A.	ARGENTINA	ARS 4,000.00
10	Mrs. Milena Aguirre	OVER	ARGENTINA	ARS 4,000.00
11	Diego Esbarraz	OVER	ARGENTINA	ARS 4,000.00
12	Niels Scherling	BROUWER S.A.	ARGENTINA	ARS 4,000.00
13	Rodolfo Perotti	BROUWER S.A.	ARGENTINA	ARS 4,000.00
14	Federico Luchter	CAPROVE	ARGENTINA	ARS 4,000.00
<b>Pago de inscripción en pesos</b>				<b>ARS 56,000.00</b>

## Inscripciones en dólares americanos 2015

Nº	Participante y/o Organismo	Pago USD	Recibo Nº
1	Agribands Purina Perú S.A	350	0025
2	Ilender Peru S.A	350	0026
3	Virbac México S.A	350	0027
4	Lauda S.A.P	350	0028
5	Phibro Animal Health	350	0029
6	Alba Codutti	350	0030
7	Regulatory Affairs Global	350	0031
8	Raquel Pinto López	350	0032
9	Leonardo Costa	350	0033
10	Phibro Animal Health	450	0034
11	Real & CIA Ltda	450	0035
12	Vecol S.A	450	0036
13	Heel Colombia Ltda	350	0037
14	Francisca Galvez	350	0038
15	Chemie S.A	350	0039
16	Prondil S.A	350	0040
17	Prondil S.A	350	0041
18	Laboratorios Norvet	350	0042
19	Zoetis Mexico S.A de C.V	350	0043
20	Zoetis Costa Rica C.R.L	350	0044
21	Rebexa Group	700 (2 inscripciones por 350)	0045

22	Biokhemia	350	0046
23	Distrivet S.A	350	0047
24	Compañía Libeles S.A	350	0049
25	Laboratorios Pasteur S.A	350	0050
26	Saint CyR S.R.L	350	0051
27	Biozoo S.A de C.V	225 (Patrocinador plata 50% descuento)	0052
28	Químicas Veterinarias S.A	900 (2 inscripciones por 450)	0053
29	Adriana Karkaukas	350	0054
30	Laboratorio Labyes	350	0055
31	Ceva	350	0056
32	Ceva	350	0057
33	Ceva	350	0058
34	Alcames Laboratorios	350	0059
35	Proagro S.A	1050 (3 inscripciones por 350)	0060
36	Vetinova	350	0061
37	Desarrollo y Nutricion Animal S.A	350	0062
38	Alexandra Luna Orta	350	0063
39	Itamis Garcia	350	0064
40	Karina Rosales Bosch	350	0065
41	Diasmarys Salinas	350	0066
42	Tecnocalidad S.A	450	0067
43	Coaspharma S.A.S	450	0068
44	Farbiovet	350	0069
45	Global Vet	225 (Patrocinador plata 50% descuento)	0070
46	Pet Kraft	225 (Patrocinador plata 50% descuento)	0071
47	Agrozona Guatemala S.A	450	0072
48	Flavia Schubert	350	0073
49	Virback	350	0075
50	Cargill Alimentos	350	0076
51	Cargill Alimentos	350	0077
52	Biogenesis Bago S.A	350	0078
53	Biogenesis Bago S.A	350	0079
54	Calox	350	0080
55	Mariana Nunes la Paz	350	0081
56	Luara Garcia	350	0082
57	Phibro Animal Health	350	0083
58	Biomeda	350	0084
59	Aracelly Espinosa	175 (Patrocinador plata 50% descuento)	0085
60	Agrovet Market	350	0086
61	Agrovet Market	350	0087



62	Cámara de insumos Agropecuarios	350	0088
63	Cev	350	0089
64	Lapisa	450	0090
65	Laura Lorenzetti	350	0091
66	Corex S.A de C.V	350	0093
67	Corex S.A de C.V	350	0094
68	Heloiza Pereira	350	0095
69	Arlení Concha Paula	350	0096
70	Silvia Melgar	350	0097
71	Hallmark S.A	350	0098
72	Luisa Vargas	350	0099
73	Flavia Fernández	350	0100
74	Faryvet	350	0101
75	Vedilab	700 (2 inscripciones por 350)	0102
76	Liliana Revolledo	350	0103
77	Ricardo Pinto	350	0105
78	Valeria Capurro	450	0106
79	Intervet Colombia	350	0107
80	Vicar Farmaceutica	450	0108
81	Bayer S.A	350	0109
82	Bayer S.A	350	0110
83	Bayer S.A	350	0111
84	Mundo Animal	350	0112
85	Laboratorios Hipra S.A	350	0113
86	Ourofino Saude Animal	1050 (3 inscripciones por 350)	0114
87	Carval	350	0115
88	Mustad Argentina S.A	350	0116
89	Laboratorios Biomont S.A	175 (patrocinador plata 50% descuento)	0118
90	Clea Camargo	450	0119
91	Labipharma S.A	175 (patrocinador plata 50% descuento)	0120
92	Alanac	175 (patrocinador plata 50% descuento)	0121
93	Liliana Torres	350	0122
94	Intervet México S.A de C.V	350	0124
95	Investigación Aplicadas S.A de C.V	450	0125
96	Bioboi Homeopatia Animal	450	0126
97	Labovet Productos Veterinarios Ltda	1350 (3 inscripciones por 450)	0127
98	Laboratorios Tornel	350	0129
99	Labiana Life Sciences	350	0130
100	Marcelo Allignani	350	0131
101	Laboratorios Biomont S.A	450	0133
102	Laboratorios Biomont S.A	350	0134

103	Asociacion Nacionales de Laboratorios Veterinarios A.C	350	0135
104	Globalvet S.A.C	350	0136
105	Globalvet S.A.C	350	0137
106	Boehringer Ingelheim Perú S.A.C	350	0138
	<b>TOTAL</b>	<b>40.975,00</b>	