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

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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 intestinte 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 recommnedation of a guinea pig model as alternatve method for RVA vaccine potency testing of each vaccine batch to be release into the market. The test have been statistically validated with the target species and possess 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 represent a proper predictive tool to estimate the protection rate conferred by colostral antibodies in calves feed 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 it is 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 absorved 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 ± 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 antobdies to bovine RVA.

2.1.2 Procedure

Guinea pigs are immunized with two doses of vaccine (21 days appart), by subcutaneal 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 de 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).

Regardong 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 receiuving 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).

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

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 log10 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 manufacter´s recommendations, and are sampled at 0 and 60 dpv. This latter point corresponded to the peak or plateau of Ab titers reached by aqueous or oil vaccines, respectively 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 log10 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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