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A simple method for measuring porcine circovirus 2 whole virion particles and standardizing vaccine formulation

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ABSTRACT

Porcine Circovirus 2 (PCV2) is involved in porcine circovirus-associated disease, that causes great economic losses to the livestock industry worldwide. Vaccination against PCV2 proved to be very effective in reducing disease occurrence and it is currently performed on a large scale. Starting from a previous model concerning Foot-and Mouth Disease Virus antigens, we developed a rapid and simple method to quantify PCV2 whole virion particles in inactivated vaccines. This procedure, based on sucrose gradient analysis and fluorometric evaluation of viral genomic content, allows for a better standardization of the antigen payload in vaccine batches. It also provides a valid indication of virion integrity. Most important, such a method can be applied to whole virion vaccines regardless of the production procedures, thus enabling meaningful comparisons on a common basis. In a future batch consistency approach to PCV2 vaccine manufacture, our procedure represents a valuable tool to improve in-process controls and to guarantee conformity of the final product with passmarks for approval. This might have important repercussions in terms of reduced usage of animals for vaccine batch release, in the framework of the current 3Rs policy.

1. Introduction

Porcine Circovirus 2 (PCV2) is a small, non-enveloped virus, containing 1768 bp of single-stranded circular DNA and belonging to the family *Circoviridae* [1]. The virus agent shows limited nucleotide divergence and variability among strains, which underlies the existence of two main genotypes, named PCV2a and PCV2b, respectively [2]. PCV2 is considered the main causative agent in the development of several diseases and syndromes of pigs, collectively referred to as porcine circovirus-associated disease (PCVAD). This complex includes postweaning multysistemic wasting syndrome, respiratory disease, porcine dermatitis and nephropathy syndrome, enteritis, and reproductive failure [3,4]. In order to reduce the important economic losses due to PCVAD, several vaccines have been developed and successfully employed worldwide since 2006. Although all commercial PCV2 vaccines are based on the PCV2a genotype, they show important differences not only concerning antigen and adjuvant types, but also dosage, time and route of administration, and target animal (sow or piglet or both). Four different vaccines were licensed in Europe because of a demonstrated efficacy in reducing mortality, weight loss, PCV2 excretion, viral load and virus persistence in tissues [5,6]. Circovac® (Merial S.A.S., Lyon, France), the first vaccine approved in Europe, contains inactivated PCV2; Ingelvac® CircoFLEX (Boehringer Ingelheim, Ingelheim/Rhein, Germany) and Porcilis® PCV (Intervet International BV, Boxmeer Netherlands) are based on viral ORF2 protein expressed in a baculovirus system; Suvaxyn® PCV2 (Fort Dodge Animal Health Limited, Southampton, United Kingdom) contains inactivated PCV1 chimeric virus expressing the PCV2 ORF2 protein.

The above differences in terms of immunogenic components and formulation do not allow for an easy comparison of PCV2 vaccines in terms of immunizing dose and efficacy, which represents a great limitation for a future "batch consistency" policy underlying batch release procedures. Indeed, the batch consistency approach has become a mandatory step in vaccine development [7], being thus considered as a valid alternative to animal testing [8]. In practice, such a strategy implies that a vaccine product, already shown to be safe and effective, has defined antigen payload

Abbreviations: PCV2, Porcine Circovirus type 2; PCVAD, porcine circovirus-associated disease; FMDV, Foot-and Mouth Disease Virus; BPL, beta-propiolactone; SGA, sucrose gradient analysis.

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and quality features that are similar in homogenous batches, as shown by strict in-process controls throughout the whole production cycle. Therefore, the product profile can be precisely described by a set of parameters (biochemical, immunological and/or functional) to be investigated by analytical *in vitro* methods. In particular, these in-process controls should identify the presence of a defined antigen payload and relevant quality features, to be offset against precise passmarks for batch approval.

Owing to the above, this study aimed to develop a simple model for assessing PCV2 antigen payload and integrity for inactivated vaccines based on whole virions. On the basis of a previous model concerning in-process controls of Foot-and Mouth Disease Virus (FMDV) antigens for vaccine production [9], we developed an integrated procedure based on sucrose gradient analysis (SGA) of inactivated, concentrated PCV2 antigens, toward a better standardization of antigen payload in vaccine batches.

2. Materials and methods

2.1. Virus and cells

Circovirus-free PK15c28 cells (porcine kidney cells, IZSLER cell bank code BS CL 179) were cultured in Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Confluent PK15c28 cell cultures were trypsinized, counted and adjusted to a final concentration of 300,000 cells/ml in the above medium. Next, they were infected in 50 ml Falcon tubes (Becton Dickinson, US) with PCV2 (Bio Bank Veterinary Resources, IZSLER, code VIR RE RSCIC 151), at multiplicity of infection (MOI) 0.1 for 30 min at 37 °C. Cells were then transferred into cell culture flasks of different sizes for 4 days until confluence. PCV2-infected cells were lysed by 2 freeze/thaw cycles at −20 °C and clarified by centrifugation in a JA-20 rotor (Beckman Coulter, USA) at 8000 rpm for 15 min at 4 °C. Virus titers were determined by direct immunofluorescence in PK15c28 cells grown in 96-well microtiter plates. Briefly, 1.5×10^4 cells/well and serial dilutions of the virus (both at 50 μL/well) were grown at 37 °C in 5% CO_2 for 24 h. Then, 100 μL/well of complete medium supplemented with 1000 U/ml of swine recombinant IFN-γ (AbD Serotec, Oxford, UK) were added to semi-confluent monolayers. After 3 more days of culture, medium was discarded, monolayers were washed once with PBS, fixed with 80% acetone and stained with an anti-PCV2 polyclonal antiserum conjugated to fluorescein isothiocyanate (VMRD, Pullman, US, cat CJ-F-PCV2), and examined for fluorescence by ultraviolet (UV) microscopy. 50% end-point titers were determined as previously described [10].

2.2. Virus inactivation, concentration and centrifugation

Clarified (10,000 rpm, 10 min) PCV2 was treated with beta-propiolactone (BPL, Sigma–Aldrich, US, cat P5648) twice in three days (0.05% final) at 4 °C. 10 days after the start of the procedure, inactivation was confirmed on PK15c28 cells by direct immunofluorescence and flow cytometry analyses, using the aforementioned polyclonal antiserum to PCV2. Then, inactivated viral suspension was concentrated through a 10,000 MWCO Hydrosart Vi*vaflow* 200 tangential flow membrane (Sartorius Stedim Biotech GmbH, Germany). Concentrated PCV2 was pelleted either in a JA-20 rotor (Beckman Coulter, USA) at 20,000 rpm for 16 h at 4 °C, or in a T-880 rotor (Du Pont Sorvall) at 60,000 rpm for 75 min at 10 °C. The pellet was resuspended overnight at 4 °C in sterile PBS and stocked at −80 °C.

2.3. Sucrose gradient analysis and DNA extraction

Pelletted and clarified (10,000 rpm, 10 min) PCV2 in 0.5 ml aliquots was layered onto linear, continuous 10–25% sucrose gradients prepared in 40 mM sodium phosphate buffer (pH 7.4). Control gradients were loaded with PBS and mock antigen (cryolysate of BPL-treated PK15c28 cells). Gradients were centrifuged at 35,000 rpm in a SW-40 rotor (Beckman Coulter) for 3 h at 10 °C. They were chased by a 50% sucrose solution from the bottom at 1 ml/min by means of a gradient fractionator (Brandel, United Kingdom). Absorbance at 254 nm was monitored in a 5 mm flow cell (Uvicord SII and Rec102 recording apparatus, Pharmacia Biotech). 1 ml, high-absorbance fractions with a sucrose concentration consistent with 57 S PCV2 were promptly collected and diafiltered with sterile PBS through Amicon Ultra-15 Centrifugal Filter membranes (Millipore, Germany). The same procedure was applied to control sucrose gradients (PBS and cell cryolysate). Optical densities of diafiltered fractions at 260 nm were read in a 10 mm pathlength spectrophotometer cuvettes before and after DNA extraction (QIAamp DNA Blood Mini Kit, Qiagen, Netherlands), respectively. Fractions of control sucrose gradients (see above) were used as absorbance blanks. Extracted DNA concentration was assessed by a Qubit® 2.0 Fluorometer (Invitrogen, UK).

2.4. Estimate of PCV2 viral particles

Total DNA was extracted and PCV2 DNA copies in sucrose gradient fractions were calculated as follows: (Avogadro's constant) × (calculated DNA quantity)/(PCV2 DNA molecular weight, i.e. 548,847 for a ssDNA genome of 1768 bp, see PCV2a Reference Sequence: NCBI accession number AF055392). The number of PCV2 DNA copies was considered equal to the number of PCV2 virions. This was converted into mass (ng) based on a molecular weight of PCV2 particles approximately equal to 2,348,847, i.e. 1,800,000 Da for 60 copies of capsid ORF2 protein +548,847 Da of ssDNA, which implies a 4.28:1 protein/DNA ratio. On the basis of these parameters, we were able to analyze the relationship between PCV2 antigen mass and the areas under absorbance curves (obtained by triangulation of the UV absorbance peaks).

3. Results and discussion

The main objective of this study was to find a predictive process parameter related to PCV2 antigen mass that could be used towards consistent vaccine production procedures. The virus purification system developed in our study was based on fundamental physical properties of PCV2 virions, in particular the sedimentation coefficient, determined to be 57S (Svedberg units) by Allan and co-workers in 1994 [11]. This enabled us to postulate a consistent procedure for transient separation of PCV2 virions in continuous sucrose gradients. To this purpose, we evaluated different sucrose gradient concentrations and centrifugal forces and times. Centrifugation of inactivated virus through linear 10–25% sucrose gradients for 180 min under the aforementioned conditions allowed viral particles to be separated in clearly identifiable peaks (see Fig. 1) and to be easily recovered. PCV2 could be unambiguously identified by electron microscopy (Fig. 1A), an ELISA based on monoclonal antibodies to PCV2 [12] and Real-time PCR for ORF2 gene (internal SOP at IZSLER, Brescia, Italy). Also, antigen production methods were refined to obtain a good virus yield. In this respect, the presence of FCS was mandatory in agreement with a fundamental requirement of S-phase cells for successful, high-titered replication of PCV2. Infection of PK15c28 cells without FCS gave rise to empty viral particles located at lower sucrose concentrations (electron

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