Short communication: a modified *Vaccinia virus* Ankara-based *Porcine circovirus* 2 vaccine elicits strong antibody response upon prime-boost homologous immunization in a preclinical model

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Abstract

Porcine circovirus 2 (PCV2) infections are related to a number of syndromes and clinical manifestations, generally known as *Porcine circovirus*-associated diseases, which are related to losses in the swine industry. There are commercially available vaccines and new vaccines being tested, however, persistency of the PCV2 as an important pig pathogen, and the growing number of affected farms in different countries have suggested that there is room for vaccine improvement. In this study, we describe the construction and testing of a recombinant live vaccine based on a modified *Vaccinia virus* Ankara (MVA) vector expressing the PCV2b capsid protein (CAP). Using a two-dose homologous vaccination regimen, in mice, we demonstrated that the vaccine induced high titers of anti-PCV2 antibodies. The vaccine is stable upon lyophilization, and, together with the good immunogenicity potential observed, the results support further evaluation of the MVA-CAP vaccine in the target species.

Keywords *Porcine circovirus* 2 · Capsid protein · Modified *Vaccinia virus* Ankara (MVA) · Recombinant vaccine · Homologous vaccination regimen

Abbreviations

PCV2	<i>Porcine circovirus</i> 2
CAP	Capsid protein
PCVAD	<i>Porcine circovirus</i> -associated diseases
PMWS	Postweaning multisystemic wasting syndrome
MVA	Modified <i>Vaccinia virus</i> Ankara
MVA-CAP	Recombinant MVA expressing CAP

Highlights

- A MVA-vectored PCV2 vaccine was designed and tested on a preclinical model.
- Two homologous doses of the vaccine elicited strong antibody response.
- The lyophilized vaccine was stable for up to one year when stored at 4 °C.

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Introduction

Modifications of consume habits associated to possible health and economic advantages have led to an increase in the global swine farming and commercialization of pig products [1]. The constant expansion of the swine industry, especially in developed countries, has been characterized by the reduction of farm numbers and a correspondent increase in herd sizes [1]. As a result, pig farming in increasingly higher densities allows for the transmission of pathogens and the dissemination of diseases [2]. One of the most economically important pathogens for the swine industry worldwide is the *Porcine circovirus* 2 (PCV2), a non-enveloped, single-stranded DNA virus that shows a 40–60% average prevalence in affected herds, reaching up to 100% seropositivity in determined geographic settings [2]. PCV2 is a member of the *Circoviridae* virus family, *Circovirus* genus [3], and is associated with the development of several clinical manifestations collectively known as *Porcine circovirus*-associated diseases (PCVAD) [4, 5]. Due to its high prevalence in herds and pathogenic effects in infected animals, PCV2 is currently recognized as one of the most important viruses in the global swine industry [6].

Although several strategies for the production of an immunogenic vaccine for PCV2 have been explored [7–10], the ongoing PCV2 global spread suggests that there is still room for vaccine development or improvement [11]. In the veterinary field, the modified *Vaccinia* virus Ankara (MVA) has featured in the production of vaccine platforms against different diseases such as leishmaniasis [12–14], BoHV-1 [15], bluetongue disease [16], African horse sickness [17, 18], among others. Nonetheless, the most significant use of MVA in the veterinary field is related to anti-rabies vaccines [19].

This study described the development and evaluation of a MVA-based recombinant vector capable of expressing the PCV2 capsid protein (CAP). The immunogenicity of the produced vaccine was evaluated in a mouse experimental model.

Materials and methods

Construction of recombinant virus MVA-CAP

The cDNA coding for the PCV2b CAP protein was produced synthetically, based on PCV2b gene sequence deposited in GenBank (DQ364650.1). The synthetic gene (CAP-PCV2) was inserted into the *Sma* I site of a transfer plasmid under the control of the mH5 early-late *Vaccinia* virus promoter [20]. The transfer plasmid contains a cDNA coding for the green fluorescent protein (GFP) regulated by a *Vaccinia* virus late promoter, which allows for the selection of recombinant viral clones in infected cells (Fig. 1).

The recombinant MVA vector (MVA-CAP) construction was based on the homologous recombination between the transfer plasmid and the wild-type MVA genome, in BHK-21 cells (Baby Hamster Kidney fibroblasts, originally derived from the American Type Culture Collection – ATCC® CCL-10™) infected with MVA at a multiplicity of infection (MOI) of 1.0. Correct construction of the transfer plasmid was assessed through DNA sequencing. Visible fluorescent plaques containing the recombinant viruses were selected through several rounds of plaque isolation and subsequently propagated in BHK-21 cells. Recombinant virus purification was performed in 36% (w/v) sucrose cushion (in 10 mM Tris-HCl, pH 9.0), and the titer was determined by a plaque-based assay. The expression of the transgenic CAP protein by the MVA vector was evaluated by flow cytometry. Briefly, BHK-21 cells infected with the recombinant vector were dislodged from plates, counted, fixed in paraformaldehyde, and subsequently labeled with anti-PCV2 (VMRD/PAB-PCV2) and anti-pig IgG (TRITC – abcam/ab6912) antibodies. Results were acquired using a FACSCanto™ II (BD Biosciences) flow cytometer and the BD FACSDiva 6.1 acquisition software. Data were analyzed with FlowJo software (Tree Star Inc.). The gating strategy to detect the presence of the CAP protein is shown in Fig. 2a.

Mice infections

Immunizations were performed in Balb/C female mice, 8–12 weeks old, treated with albendazole (ricobendazole) for 14 days and later divided into two groups: animals inoculated

Fig. 1 Genetic elements in the construction of the MVA-CAP recombinant MVA. The construction of the MVA-CAP recombinant vaccine vector was based on the homologous recombination between the transfer plasmid containing the nucleotide sequence coding for the PCV2b capsid protein (CAP-PCV2) (which is under control of the modified H5 *Vaccinia* virus promoter – mH5) (a and b) and the MVA DNA (c). The transfer plasmid also contains a copy of the green fluorescent protein cDNA (GFP) and flanking regions (MVA-Flank 1 and 2) to direct homologous

recombination to the major deletion region III on the MVA genome. A linearized version of the transfer plasmid (portion) is shown on b. Other genetic elements such as restriction enzyme sites, origin of replication, and the ampicillin resistance gene are shown on a. The major deletion regions on the MVA genome are depicted on c (I to VI), including a detail of the deletion region III showing the homologous flanks. A detail of genetic composition on the MVA genome after homologous recombination is shown on d

with a MVA-GFP virus (expressing only GFP) [21] ($N = 18$) and animals inoculated with MVA-CAP recombinant virus ($N = 23$). Animals were anesthetized with a solution of ketamine, xylazine, and PBS and then immunized with 10^7 plaque forming units (pfu) of the recombinant viruses through the intramuscular route, in a prime-boost immunization regimen. The boost was carried out 14 days after the first dose, and the animals were euthanized 21 days after the boost. Blood was collected through the brachial plexus, and the obtained sera was stored at $-20\text{ }^{\circ}\text{C}$ until use.

Immunizations and animal handling were in accordance with the ethical principles of animal experimentation adopted by the Ethics Committee for Animal experiments at UFMG (CEUA/UFMG – protocol 273/2008).

Evaluation of humoral response by indirect ELISA

Sera from immunized animals were subjected to an enzyme-linked immunosorbent assay (ELISA) [*Porcine circovirus* (PCV2) ELISA test kit (Accurate Chemical & Scientific Corporation)], according to manufacturer's instructions. The test evaluated the generation of humoral immune responses upon immunization with the MVA-CAP recombinant vaccine. Results were analyzed using *GraphPad Prism 4* (GraphPad Inc., San Diego, CA, USA) and *one-way ANOVA* test, as well as *Tukey* multiple comparison test to analyze the variance between groups. Values of $P < 0.05$ were considered statistically significant.

Evaluation of the recombinant vaccine shelf life upon lyophilization

For the production of lyophilized vaccine, BHK-21 cells were infected at MOI of 1, and the recombinant virus was purified, as described previously. Purified MVA-CAP was diluted in 10-mM Tris-HCl plus stabilizing solution. The final suspension was lyophilized, aliquoted, stored at $4\text{ }^{\circ}\text{C}$, and monthly tested for viability in cultured BHK-21 cells.

Results

Expression of CAP protein by the MVA vector

The expression of the PCV2b capsid protein by the recombinant MVA-CAP was evaluated by flow cytometry. Cell subsets were divided into low, medium, and high according to the cell size and granularity in the cell populations (Fig. 2a). Expression of the CAP protein was readily detected (Fig. 2b). In addition, the “high” subset presented a better expression of the analyzed antigen than the subsets “medium” and “low,” respectively, suggesting that the viral infection possibly triggers cellular changes that may interfere with cell

morphology. We used a MVA-GFP virus – consisting of the MVA backbone expressing the GFP protein – as the experiment negative control. As expected, only background fluorescence was observed.

Anti-PCV2 serological responses in immunized BALB/C mice

Sera from mice immunized either with MVA-CAP or MVA-GFP viruses were subjected to an ELISA test for the evaluation of antibody production against the recombinant vaccine expressing the CAP protein of PCV2b. As demonstrated on Fig. 3, significant titers of anti-PCV2 antibodies were produced in MVA-CAP immunized animals whereas only background signal was detected in the MVA-GFP inoculated group. The *Tukey* multiple pairwise comparison test was used to analyze the variance between groups; statistically significant differences ($P < 0.05$) were found between MVA-GFP and MVA-CAP vaccinated groups, and between MVA-CAP and the ELISA test-included negative control. No statistical differences were observed between MVA-CAP group, and the test-included positive control.

Evaluation of the vaccine shelf-life

Stability is a necessary feature for any immunogen to reach the market. If a given vaccine loses too much activity as it goes through the commercial chain up to the final user, its potential becomes certainly limited. To look into that, we lyophilized the MVA-CAP immunogen and tested the recombinant virus viability once a month over a 12-month period. Fig. 4 shows that there was no significant virus titer loss during the evaluated time, indicating that the lyophilized vaccine remained stable after 12 months of storage at $4\text{ }^{\circ}\text{C}$.

Discussion

Vaccines remain as the most cost-effective and safe method in preventing animal diseases [22]. In the context of PCVAD, one illustrative example is that after the introduction of vaccines against PCV2 in Europe, Canada, and the USA, the mortality rates associated to PCVAD decreased significantly [5]. Nevertheless, the spread of the disease to other parts of the globe suggests that there is still room for anti-PCV2 vaccine development and improvement. In this preclinical study, we evaluated the protective potential of an MVA-based recombinant live-vaccine expressing the capsid protein of PCV2.

The generation of antigen-specific antibody titers is considered the first correlate of protection against most viral pathogens [23]. Indeed, the generation of anti-PCV2 antibodies has been considered as the most important indication of protective immunity conferred by vaccines against PCV2 [24–27].

Fig. 2 Analysis of CAP protein expression in cells infected with the MVA vector. **a** Representative graph of the gating strategy used in the flow cytometric assay; subsets low, medium, and high granularity (SSC) and size (FSC) are represented. **b** In black, Vector MVA-CAP; in gray, Vector MVA-GFP as control (MVA-GFPc); in white, CC – non-infected cells used as controls. A statistically significant difference ($p < 0.05$) was observed between the control group MVA-GFPc vs MVA-CAP and between the MVA-CAP vs CC group. Statistical differences were also observed between the subset's low vs high and medium vs high

Diminished levels of anti-PCV2 specific antibodies have been associated with the development of circovirus-associated diseases and increased susceptibility to infection [28]. Studies describing the humoral responses generated after PCV2 infection indicate that the seroconversion occurs between 10 and 28 days postinfection or vaccination [29, 30]. In our preclinical study, mice seroconverted efficiently 35 days post-immunization, presenting high anti-PCV2 IgG titers.

The capability of MVA-based vectors in generating specific antibodies to the transgenic protein product upon vaccination has been demonstrated for human and animals alike, in many studies. However, many authors suggest that anti-vector antibodies, generated after the priming dose, may compromise the boost efficiency during a homologous regimen vaccination. Nonetheless, Ferrer and colleagues (2011) [15] have demonstrated the efficiency of the homologous immunizations with a recombinant MVA against the *Bovine herpesvirus* type 1. Other groups have also presented results suggesting the efficiency of homologous prime-boost vaccinations against veterinary relevant diseases employing MVA recombinant vectors [17, 31, 18]. Our group has also demonstrated the efficiency of the homologous prime-boost immunization against *Dengue virus* infections using a MVA vector expressing the viral envelope protein [32, 33]. In the context of PCV2 infections, Aravindaram and co-workers (2009) [34] have demonstrated the protective potential of a MVA-based vaccine in swine's. However, in this case, authors employed a heterologous prime-boost regimen in which priming consisted of

Fig. 3 Evaluation of the antibody responses in mice inoculated with MVA-CAP and with MVA-GFP by commercial ELISA. The assay was performed 21 days after the booster dose. Statistically significant differences are represented. MVA-GFP (animals inoculated only with MVA-GFP. $N = 18$), MVA-CAP (animals inoculated with the recombinant virus MVA-CAP. $N = 23$), C-POS (kit positive control), and C-NEG (kit negative control). All experiments were performed in triplicate

* GraphPad Prism5 - Oneway ANOVA, post-test Tukey.

Vaccine Shelf-Life

Fig. 4 Shelf-life of lyophilized vaccine over time. Purified MVA-CAP stocks were lyophilized and kept at 4 °C for 12 months. Aliquots were titrated monthly in cultured cells. Titers are expressed in pfu/mL

naked DNA vectors delivered through a gene gun and boost was a recombinant MVA expressing PCV2 genes [34]. Despite the efficacy demonstrated in that study, and in many other studies employing heterologous prime-boost vaccination strategies, the use of homologous vaccine regimens is preferably over heterologous protocols whenever possible, as the first strategy reduces costs and simplifies the management of herds when compared to the latter [35].

One key aspect of vaccines and other products aimed at reaching the veterinary market – specially farming products – is the stability of the goods exposed to harsh conditions. This is necessary as such products usually have to endure longer travel times in the commercial chain, as well as not always pristine storage conditions, especially when freezing is necessary. In this respect, we demonstrated that the MVA-CAP vaccine is stable for up to 12 months when lyophilized and stored at a common refrigerator (4 °C).

In conclusion, we have tested a MVA-based PCV2b live recombinant vaccine that was able to generate robust antibody

responses upon homologous prime-boost regimens in a pre-clinical setting. We believe that these results support further development of the proposed immunogen and clinical testing in swine.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This study is in accordance with the ethical principles of animal experimentation adopted by the Ethics Committee for Animal experiments (CEUA/UFGM – protocol 273/2008).

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