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Classical swine fever virus marker vaccine strain CP7_E2alf: Shedding and dissemination studies in boars

Carolin Dräger[a], Anja Petrov[a], Martin Beer[a], Jens P. Teifke[b], Sandra Blome[a,*]

[a] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany
[b] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald-Insel Riems, Germany

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ABSTRACT

Over the last decade, pestivirus chimaera CP7_E2alf has proven to be a most promising marker vaccine candidate against classical swine fever (CSF). To provide further background data for the risk assessment towards licensing and release, especially on presence of the vaccine chimaera in faeces, urine, and organs of the male reproductive tract, supplementary studies were carried out under controlled laboratory conditions. In detail, the shedding and dissemination pattern of Suvaxyn® CSF Marker ("CP7_E2alf") was assessed in 12 adult boars after single intramuscular vaccination with a tenfold vaccine dose. Four and seven days post vaccination, six animals were subjected to necropsy and triplicate samples were obtained from reproductive and lymphatic organs as well as urine, faeces, blood, and several additional organs and matrices. The sampling days were chosen based on pre-existing data that indicated the highest probability of virus detection. Upon vaccination, neither local nor systemic adverse effects were observed in the experimental animals. It was confirmed that primary replication is restricted to the lymphatic tissues and especially the tonsil. While viral genome was detectable in several samples from lymphatic tissues at four and seven days post vaccination, infectious virus was only demonstrated at four days post vaccination in one tonsil sample and one parotid lymphnode. Sporadic detection at a very low level occurred in some replicates of liver, lung, bone marrow, and salivary gland samples. In contrast, viral genome was not detected in any sample from reproductive organs and accessory sex glands, in faeces, urine, or bile.

The presented data on the dissemination of the vaccine virus CP7_E2alf in adult boars are supplementing existing safety and efficacy studies and indicate that the use of the vaccine is also safe in reproductive boars.

1. Introduction

Classical swine fever (CSF) remains one of the major threats for profitable pig production worldwide, and due to its impact, it is notifiable to the OIE [1]. In most industrialized pig production systems, prophylactic vaccination against the viral disease is banned and outbreaks are controlled through strict and legal binding hygiene measures (see e.g. European Union Council Directive 2001/89/EC and Commission Decision 2002/106/EC). In densely populated livestock areas and in larger outbreak scenarios, this policy can lead to tremendous numbers of animals that have to be culled in restriction zones. To avoid this, emergency vaccination could be implemented as laid down in the above-mentioned legislation. However, the option of emergency vaccination is hampered by limitations in the available subunit marker vaccines, and trade restrictions that are imposed on conventionally vaccinated animals [2]. To overcome these problems, research activities were directed towards the development of new generations of marker vaccines [3]. Over the last decade, several approaches have been followed and tested, among them chimeric pestiviruses such as "CP7_E2alf" [4–8].

After its first description as possible marker vaccine candidate against CSF in 2004 [4], "CP7_E2alf" was evaluated in the framework of two EU funded research projects and beyond. The chimaera with a cytopathogenic BVDV "CP7" backbone and the glycoprotein E2 of CSFV "Alfort 187" has proven safety for target and non-target species as well as against different challenge virus strains. Furthermore, genetic and serological DIVA concepts were established [9–11].

Given the fact that the vaccine is a genetically engineered virus, assessment of all safety aspects is crucial for field applications and the evaluation towards licensing. In this respect, one of the

* Corresponding author. Tel.: +49 38351 7 1144; fax: +49 38351 7 1275.
E-mail address: sandra.blome@fli.bund.de (S. Blome).

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outstanding issues was the question whether vaccine virus could be found in semen of vaccinated boars. Moreover, absence of shedding in faeces and urine still had to be demonstrated. These issues are going far beyond the standard requirements of the European Pharmacopoeia and were thus only done upon completion of the routine studies.

Here, a trial was conducted under controlled laboratory conditions with 12 boars.

As substitute for semen, all genitals and accessory sex glands were tested for vaccine virus. In addition, lymphatic and parenchymatous organs as well as a range of additional sample matrices including saliva, bile, and bone marrow, were screened for vaccine virus in order to provide additional data on distribution and primary replication sites.

2. Materials and methods

2.1. Study design

The study comprised 12 cross-bred boars aged 6–7 months. All animals were purchased from a commercial boar fattening farm and brought to the high containment facilities at the Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany. Upon arrival, the animals were randomly assigned to two stables/treatment groups (G1 and G2). All applicable animal welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration. The animal experiment was approved by the competent authority under reference number 7221.3-2-031/14.

After an acclimatization phase, animals were marked with an individual ear-tag and blood, faeces, and saliva samples (using dry cotton swabs; COPAN) were collected in order to evaluate the status of animals (freedom from pestiviruses and pestivirus antibodies). Thereafter, all animals were intramuscularly vaccinated with a tenfold vaccine dose of Suvaxyn® CSF Marker ("CP7_E2alf") into the right neck (1 ml solution, deep into the muscles behind the ear using a 2 ml syringe and a 20G needle). Upon vaccination, general health status and possible injection site reactions were assessed on a daily base. On day 4 post vaccination (dpv), six boars (G1) were slaughtered (electro-stunning and subsequent exsanguination) and subjected to post-mortem examination and sampling. Animals of G2 (six boars) were slaughtered and sampled 7 dpv.

At necropsy, the following sample materials were collected: EDTA blood, native blood for the preparation of serum, testis, epididymis, bulbourethral gland, vesicular glands, prostate gland, urine, faeces, liver, bile, lung, bone marrow, oropharyngeal fluid (using Genotubes; Prionics), salivary glands, spleen, and tonsils. In addition, the following lymphnodes were collected from the right side of the animals (side of vaccine injection): *Ln. mandibularis*, *Ln. retropharyngeus*, *Ln. cervicalis ventralis*, *Ln. cervicalis dorsalis*, *Ln. parotideus*, *Ln. popliteus*, and additional lymphnodes of the neck/head region (where applicable). For comparison, one animal (OM6) was sampled for these lymphnodes also on the left side.

2.2. Vaccine

For the trial, Suvaxyn® CSF Marker ("CP7_E2alf") batch T23488 (exp. 30-07-2015) was used together with the respective diluent as supplied (batch T30737, exp. 03-2018) by the manufacturer. The lyophilized vaccine was diluted in 1/10 of the diluent prescribed for routine reconstitution to obtain a tenfold vaccine virus dose. The resulting vaccine dose (1 ml per animal) had a titre of 10^7 tissue culture infectious doses 50% (TCID50) as defined by back titration.

2.3. Cells and viruses for laboratory tests

Cells and viruses for laboratory tests were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. The porcine kidney cell line 15 (PK15/5; CCLV-RIE0005), the Madin–Darby bovine kidney cells (MDBK; CCLV-RIE261), and also the sheep foetal thymus-R cells (SFT-R; CCLV-RIE0043) were obtained from the Collection of Cell Lines in Veterinary Medicine (CCLV, FLI Insel Riems, Germany).

Classical swine fever virus strain "Roesrath," BVDV strain "NADL" and Border disease virus (BDV) strain "S137/4" were obtained from the German National Reference Laboratory for CSF (FLI Insel Riems, Germany) for use in neutralization tests.

2.4. Laboratory investigations

2.4.1. Sample preparation and nucleic acid extraction

Serum samples were obtained from native blood by centrifugation at 2031 × *g* at 20 °C for 20 min. All tissue samples were cut in small pieces (size of a small lentil) and transferred to reaction tubes with 500 μl phosphate buffered saline (PBS) and a metal bead for homogenization using a TissueLyser II (Qiagen). The same was done with faecal material and saliva swab fragments. All samples were processed in independent triplicates (biological replicates). Subsequently, 250 μl of homogenate supernatants and body fluids were transferred to 750 μl of Trizol Reagent (Invitrogen). Downstream handling for nucleic acid extraction was done using the automated MagAttract® Virus Mini M48 Kit (Qiagen) on the King Fisher 96 Flex instrument (Thermo Scientific) as previously described [12]. All extractions contained an internal control RNA (IC2) [13].

2.4.2. Virus detection

Virus titrations were performed according to standard procedures as endpoint dilutions on PK15 cells to assess the administered vaccine titres. The titres expressed as $TCID_{50}$/ml were obtained by indirect immuno-peroxidase staining of heat-fixated cells which was performed 72 h post inoculation using a mouse-anti-CSFV-E2 monoclonal antibody mix and a polyclonal goat anti-mouse horseradish peroxidase conjugated secondary antibody (Thermo Fisher Scientific).

In order to ensure freedom from pestiviruses, blood, saliva, and faecal samples taken prior to vaccination were tested in a pan-pestivirus real-time reverse transcription polymerase chain reaction (RT-qPCR) as previously described [13]. These samples were also subjected to a CP7_E2alf specific RT-qPCR [4]. All samples taken after vaccination were tested primarily in the CP7_E2alf specific assay. All RT-qPCRs were performed with a Bio-Rad CFX 96 real-time detection system (Bio-Rad, Hercules, CA, USA). Results were recorded as quantification cycle (cq) values.

Virus isolation was carried out on RT-qPCR positive samples according to the standard screening protocols laid down in the Technical Annex of the European Commission Decision 2002/106/EC. Results were assessed after one passage using indirect immunofluorescence staining with a mouse-anti-CSFV-E2 monoclonal antibody mix and an Alexa Fluor® 488 conjugated goat anti-mouse IgG secondary antibody (Invitrogen). Results were visualized using an IX51 Olympus fluorescence microscope (Olympus).

2.4.3. Antibody detection

The pestivirus antibody free status of the animals was confirmed by testing all sera in a neutralization peroxidase-linked antibody assays (NPLA) according to the EU Diagnostic Manual, and the Technical Annex accompanying it. Neutralizing antibody titres against CSFV "Roesrath" were determined on PK15 cells, those directed against BVDV "NADL" on MDBK cells, and against BDV "S137/4" on