Intranasal inoculations of naked or PLGA-PEI nanovectored DNA vaccine induce systemic and mucosal antibodies in pigs: A feasibility study

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Abstract:

Mucosa are the routes of entry of most pathogens into animals' organisms. Reducing the important global burden of mucosal infectious diseases in livestock animals is required in the field of veterinary public health. For veterinary respiratory pathogens, one possible strategy is the development of intranasal (IN) DNA vaccination. The aim of this study was to assess the feasibility of IN DNA vaccination in pigs, an important species in livestock production industry, and a source of zoonotic diseases. To achieve this goal, we used a DNA vaccine against pseudorabies virus (PrV) encoding the immunogenic glycoprotein B (pcDNA3-gB plasmid). When pigs were inoculated with the naked DNA vaccine through the IN route, PrV-specific IgG and IgA type antibodies were detected in porcine sera. Interestingly, mucosal salivary IgA antibodies against PrV were also detected, at similar levels to those measured following intramuscular injection (positive controls). Furthermore, the IN delivery of pcDNA3-gB combined with PLGA-PEI nanoparticles resulted in similar levels of antibodies but was associated with an increase in the duration of detection of mucosal IgA for 2 out of 3 pigs. Our results suggest that there is room to improve the efficacy of IN DNA vaccination in pigs through optimization of IN inoculations, for example by using nanoparticles such as PLGA-PEI. Further studies will be dedicated to optimizing and testing the protective potential of IN DNA vaccination procedures against PrV.

Keywords: DNA vaccination; swine; intranasal; antibodies; salivary IgA

1. Introduction

Respiratory pathogens of livestock animals are responsible for high economic losses for farms due to high morbidity and sometimes lethal respiratory diseases. In some rare cases,

49 animal pathogens can be transmitted and emerge in humans, as exemplified with the 2009
50 H1N1 influenza pandemic of swine origin (Hass et al., 2011). It is therefore necessary to
51 develop strategies to control animal infections, and vaccination represents one of the most
52 powerful tools available. Vaccines administered via the systemic route are often effective in
53 inducing broad and heterologous protective immunity, but there are exceptions. In these
54 cases, like for vaccination against swine influenza or porcine reproductive and respiratory
55 syndrome (Dhakal and Renukaradhya, 2019), mucosal vaccination may be beneficial.
56 Because respiratory pathogens enter the body through the respiratory mucosa, induction of
57 mucosal protective immune responses may limit entry of the pathogens and associated
58 infection (Kraehenbuhl and Neutra, 2013). Mucosae are located in various cavities of the
59 body, at the interface between the external and internal environments (*e.g* respiratory or
60 gastrointestinal tracts). They contribute to essential functions such as respiration, nutrition,
61 and reproduction, while playing a protective role by preventing the physical entry of
62 pathogens (Liebler-Tenorio and Pabst, 2006), and by mobilizing the innate and specific local
63 immune systems (Lawson et al., 2011; Liebler-Tenorio and Pabst, 2006; Lycke, 2012; Pabst,
64 2012). Moreover, a key part of mucosal immunity is mucosa-associated lymphoid tissues
65 (MALTs), that are functionally interconnected despite being anatomically separated, thus
66 allowing protection through IgA secretion can reach a broad range of distant mucosal
67 surfaces (McGhee and Fujihashi, 2012).

68 Most mucosal vaccination strategies involve the oral or nasal routes. Most mucosal
69 vaccines consist of attenuated pathogens, with no or very low virulence (Lycke, 2012; Pavot
70 et al., 2012) that mimic as much as possible the wild-type pathogens they are derived from
71 and can therefore enter the organism through the mucosa. Because these viruses remain alive,
72 reversion to virulence or interactions with other pathogens present at the time of inoculation
73 have been reported (Eclercy et al., 2019; Liu et al., 2018). Therefore, as a safety measure, it is
74 recommended to develop subunit vaccines. For example, vaccine antigens can be inserted

into viral or bacterial vectors, be administered as recombinant proteins (Wang et al., 2015) or as DNA vaccines (Farris et al., 2016; Oh et al., 2001). Among those, the DNA vaccine strategy presents many advantages, the main one being its simplicity. DNA vaccination consists in inoculating plasmid DNA encoding the vaccine antigen. These antigens are then produced in the vaccinated animals, inducing humoral and cellular immune responses (Dufour, 2001). In addition, DNA vaccines are non-infectious, easy to develop and to produce in high quantities, and they induce an immune response different from that of natural infection allowing differentiation of infected and vaccinated animals (DIVA). DNA vaccination has successfully been achieved in the veterinary field and several vaccines are available on the market (Clynav®, West Nile Innovator®, Apex-IHN® and Oncept® vaccines) (Dalmo, 2018). For intranasal (IN) DNA vaccination, the vaccine is either be inoculated in its naked form (Gomes et al., 2007) or combined with vectors (liposomal or nano/microparticulate structures) that serve as mucosal delivery systems (Csaba et al., 2009). It was previously shown in rodents that such nanovectors can protect the vaccine DNA from mucosal degradation and favor interaction with mucosal cells, antigen-presenting cells (APCs) or inductive sites of MALTs (Kraehenbuhl and Neutra, 2013). The two most common biopolymers, described for IN DNA vaccination purposes, are chitosan (Khatri et al., 2008) and poly(lactic-co-glycolic) acid (PLGA) (Du et al., 2015). In particular, PLGA has been optimized for a more efficient delivery and internalization of the DNA vaccine by mucosal surfaces (*e.g.* nasal cavity). For example, the combination of polyethylenimine (PEI) with PLGA enhances electrostatic interactions of PLGA with DNA plasmids (thanks to cationic nature of the PEI) and promotes its interaction with mucosal cells, and no cell cytotoxicity was reported (Shau et al., 2012). However, it is important to point out that the vast majority of IN DNA vaccine studies were performed using mice or guinea pigs (Ai et al., 2013; Du et al., 2015; Iqbal et al., 2003; Khatri et al., 2008; Nanda et al., 2014). Given the importance of respiratory pathogens in the veterinary field, and associated risks for zoonotic

diseases, there is a strong need to conduct new studies to determine the efficacy of IN DNA vaccination for livestock animals such as pigs.

Given that the respiratory tract is the most common route of viral entry, IN DNA vaccination strategies represent a novel and promising vaccination strategy. The pseudorabies virus (PrV) that uses nasal cells as a route of entry into the pig body (Babic et al., 1994) is an ideal viral model to test IN DNA vaccination in pigs. Also, intramuscular DNA vaccination is effective and can be used as positive control (Dory et al., 2005; Gerdts et al., 1997). Among the glycoproteins encoded by the PrV DNA vaccine, the glycoprotein B (gB) is of particular interest because it is highly immunogenic (Dory et al., 2009) and could be used to validate new DNA vaccine strategies. This protein has previously been used in our laboratory to evaluate the potential of electroporation for porcine DNA vaccination (Le Moigne et al., 2012).

In the present study, the plasmid encoding PrV-gB (pcDNA3-gB) associated with a plasmid pcDNA3-GM-CSF encoding granulocyte macrophage colony stimulating factor (GM-CSF used as an adjuvant (Somasundaram et al., 1999)) was administered intranasally in its naked form or associated with the nanovector PLGA-PEI. Naked DNA IN inoculation resulted in mucosal salivary IgA antibodies production. Furthermore, the vectored DNA vaccine appeared to extend the duration of IgA production compared to naked DNA alone. These promising results justify further studies to optimize IN DNA vaccination in pigs to prevent infectious respiratory diseases.

2. Materials and Methods

2.1 Plasmids

Plasmids pcDNA3-gB (encoding PrV-gB) (Dory et al., 2009), pcDNA3-GM-CSF (encoding granulocyte macrophage colony stimulating factor) (Somasundaram et al., 1999), and pTG11033 (encoding luciferase) (Lindberg et al., 2015) were produced in *E. coli* and

purified using a Maxiprep plus endotoxin-free DNA extraction kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The plasmids were resuspended in endotoxin-free tris-EDTA buffer. Plasmid concentrations and purities were assessed spectrophotometrically by measuring OD ratios at 260 nm/280 nm, and 260 nm/230 nm, respectively (Plate reader infinite 200 Pro, TECAN Ltd, Switzerland).

2.2 Preparation of plasmid-PLGA-PEI nanoparticles

PLGA-PEI nanoparticles were purchased from NanoVex Biotechnologies (Spain). The sizes of the particles were 200 ± 12 nm and the zeta potentials were 38.9 ± 0.4 mV. plasmid-PLGA-PEI nanoparticles were obtained by the adsorption of plasmid DNA onto PLGA-PEI surfaces, as previously described (Shau et al., 2012). Briefly, plasmid DNA and PLGA-PEI were diluted into ultrapure water and mixed at a weight DNA/weight PEI ratio of 12, vortexed for 30 sec and incubated for one hour at room temperature (RT). Freshly prepared nanoparticles were inoculated or studied *in vitro* within hours.

2.3 Assessment of humoral immune responses after IM injection or IN inoculation of a naked PrV-gB-based DNA vaccine

All the experimental protocols described in this paper were approved by the ethics committee for animal experimentation of ANSES/National Veterinary School of Alfort/University of Paris-Est Créteil (France) (Notice number 10/07/18-3), and by the French Ministry of Research under reference 15614-201806210855647_v2.

Two groups of 8–9-week-old specific pathogen-free (SPF) pigs were inoculated three times at 10-day intervals **(Fig. 1A)**. They weighed 24.5 ± 2.9 kg at the time of the first inoculation. Pigs in group #1 (n=3 pigs) were injected with 4 mL of the DNA vaccine

153 composed of 400 µg of pcDNA3-gB and 100 µg of pcDNA3-GM-CSF by the IM route in the
154 neck muscle. pcDNA3-GM-CSF was used as an adjuvant, as previously described
155 (Somasundaram et al., 1999). Pigs in group #2 (n=3 pigs) were inoculated with the same
156 DNA vaccine by the IN route (2 mL in each nostril) using an MAD100 Nasal^{TM} Intranasal
157 Mucosal Atomization Device (Teleflex Medical, Wayne, USA). Non-inoculated control pigs
158 made up an additional group, group #3 (n=2 pigs). All pigs were observed daily to detect any
159 adverse reactions. Body temperatures were measured daily and pigs were weighed weekly.
160 Blood sera were collected on days 0, 10, 20, 29 and 37 post-inoculation (pi). Salivary
161 samples were collected on days 29 and 37 pi. On day 38 pi, pigs were anesthetized with an
162 auricular intravenous injection of thiopental (1 g/50 kg body weight) and euthanized. All the
163 organs and the snout of each pig were observed during the autopsies to detect any problem
164 linked to inoculations.
165
166 **2.4 Assessment of humoral immune responses after IN inoculation of naked or**
167 **nanovectored PrV-gB-based DNA vaccines**
168 Four groups of 7–8-week-old SPF pigs were inoculated three times at 10-day intervals
169 **(Fig. 1B**). They weighed 25.9 ± 5.7 kg at the time of the first inoculation. Three groups of
170 three pigs were inoculated by the IN route using an MAD100 Nasal^{TM} Intranasal Mucosal
171 Atomization Device (Teleflex Medical, Wayne, USA) with 300 µg of pcDNA3-gB + 100 µg
172 of pcDNA3-GM-CSF (2 mL corresponding to half of the DNA vaccine in each nostril). Pigs
173 in groups #1 and #2 were inoculated with naked pcDNA3-gB and PLGA-PEI-pcDNA3-gB,
174 respectively. One control pig (group #3) was injected three times at the same time points with
175 the same naked DNA vaccine (4 mL in total) by the IM route. Finally, an additional group of
176 four non-inoculated pigs served as negative controls (group #4). Clinical observations and
177 euthanasia were performed as described above, except that euthanasia was performed on day
178 50 pi. Blood sera were collected the day before the first inoculation and on days 9, 20, 27, 37,

44 and 50 pi. Salivary samples were collected the day before the first inoculation and on days 10, 20, 30, 37 and 44 pi.

2.5 Determination of serum anti-PrV IgG and IgA antibody titers

An indirect ELISA for the determination of serum anti-PrV-specific IgG and IgA antibody titers was adapted from the protocol previously described (Le Moigne et al., 2012). Briefly, maxiSorp 96-well plates (Nunc, ThermoFisher, USA) were coated overnight with 1.5 µg/well of PrV glycoproteins diluted in 100 µL of 0.05 M carbonate buffer (Sigma-Aldrich, USA) at 4°C. The plates were washed twice with phosphate buffered saline (PBS) buffer pH 7.4 containing 0.05% tween 20 (PBS-T, Sigma-Aldrich, USA) prior to being blocked with 300 µL/well of blocking buffer [1% BSA (Eurobio, France) in PBS-T] for two hours at 37°C. After two washes with PBS-T, the plates were incubated for 1 h at 37°C with 100 µL/well of two-fold serial diluted serum samples in PBS-T. Each serum sample was analyzed in triplicate. After three washes, the plates were incubated for 1 h at 37°C with 100 µL/well of 1:20,000 diluted horseradish peroxidase (HRP)-conjugated goat anti-pig IgG (H/L) (Bio-Rad, USA) or 1:100,000 diluted HRP-conjugated goat anti-pig IgA AAI40P antibody (Bio-Rad, USA) for IgG or IgA determinations, respectively. These antibodies were diluted in 1% BSA/PBS-T. Finally, after three washes, 100 µL/well of tetramethylene benzidine (TMB) substrate (Pierce TMB substrate kit, ThermoFischer Scientific, USA) was added to the plates for 5 or 10 min for IgG or IgA determinations, respectively. The reactions were stopped by adding 100 µL/well of 2 M sulphuric acid (H_2SO_4, Sigma-Aldrich, USA). The plates were shaken for 1 min and the absorbance at 450 nm of each well was read on an Infinite 200 Pro plate reader (TECAN Ltd, Switzerland). Antibody titers were expressed as the highest dilution giving an OD value higher than the threefold OD of the control sera from the non-vaccinated and non-infected pigs.

205 **2.6 Determination of salivary anti-PrV IgA antibody titers**

206 An indirect ELISA was performed on salivary samples in triplicate as described above
207 for the serum samples, with minor modifications. 1:150,000 diluted AAI40P
208 HRP-conjugated goat anti-pig IgA antibody and 15 min of incubation with the
209 TMB-substrate were used.

210

211 **2.7 *In vitro* characterization of the nanovectored plasmids**

212 The plasmids were characterized as previously described, with some modifications
213 (Berchel et al., 2015; Le Gall et al., 2013). Twenty thousand cells/well of human bronchial
214 epithelial cells (HBE-16) were incubated in 96-well plates overnight (37°C, 5% CO_2) with
215 Eagle's minimal essential medium (EMEM) supplemented with 1% penicillin/streptomycin
216 antibiotics, 1% glucosamine (Gibco, ThermoFisher, USA) and 10% fetal bovine serum.
217 Then, the cells were incubated in triplicate for 72 hours with 40 µL/well of either naked
218 pTG11033 plasmid or PLGA-PEI-pTG11033. Controls consisted of EMEM or of PLGA-PEI
219 without DNA. Thereafter, the cultures were assessed for cytotoxic effects and the
220 transfection potential of each DNA or nanovector preparation.

221

222 **2.7.1 Cytotoxic effect evaluation**

223 Each preparation described above was evaluated for cytotoxic effects on cell
224 supernatants using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit, according to the
225 manufacturer's instructions (Promega, USA).

226

227 **2.7.2 Transfection potential evaluation**

228 After removing the remaining culture medium, 75 µL/well of passive lysis buffer (PLB
229 buffer, Promega, USA) were added. Then, 25 µL of each cell lysate were transferred to two
230 separates plates. The first plate (a transparent MaxiSorp 96-well plate, Nunc, Naperville,

231 USA) was used to determine total protein content using a BC Assay kit, according to the
232 manufacturer's instructions (Pierce, ThermoFisher, USA). The other plate (a white
233 MaxiSorpLumiNunc 96-well plate, Nunc, USA) was used to measure luciferase activities
234 using the Promega Firefly Luciferase Assay System (Promega, USA), according to the
235 manufacturer's instructions. An amount of 65 µL per well of Promega Luciferase Assay
236 Reagent was added. After 2 min incubation at RT in the dark, the luminescence was
237 measured with a luminescence Infinite 200 Pro plate reader (TECAN Ltd, Switzerland), with
238 reading and delay times of one second each. The results were expressed as relative light units
239 (RLUs) per milligram of total proteins.

240

241 **2.8 Statistical analyses**

242 For the *in vitro* experiments, a Mann–Whitney–Wilcoxon (U) test (non-directional
243 test) was used. Comparisons were performed at a limit of significance of 0.05.

244

245 **3. Results**

246 **3.1 PrV-specifc systemic and mucosal humoral responses induced after IN inoculation**
247 **of the naked PrV-gB-based DNA vaccine (pig experiment #1)**

248 Two groups of three pigs were inoculated three times at ten-day intervals with a naked
249 pcDNA3-gB-based DNA vaccine either through the IM or IN route, respectively **(Fig. 1A)**.
250 A third group of two pigs was not inoculated and served as a control group. None of the DNA
251 inoculated pigs had fever, showed side effects, and they all grew similarly to the
252 non-inoculated animals for the duration of the experiment. At necropsy, no lesions related to
253 the inoculation or injection procedures were observed.

254

255 **3.1.1 Determination of serum anti-PrV IgG and IgA following IM or IN inoculation of**
256 **the naked PrV-gB-based DNA vaccine**

In previous studies, we showed that pcDNA3-gB when inoculated intramuscularly induced strong production of systemic anti-PrV IgG antibodies (Dory et al., 2009; Le Moigne et al., 2012). Here, as expected, the three pigs injected with the PrV-gB-based DNA vaccine by the IM route exhibited an elevated serum anti-PrV IgG response that started to be detected on day 20 pi (10 days after the second injection), and the response remained high or increased after the third injection until the end of the pig experiment **(Fig. 2A)**. Maximum titers expressed in log2 base were between 13.5 (pigs #2 and #3) and 15.2 (pig #1). Serum anti-PrV IgA antibodies started to be detected on day 10 pi for pig #1, and on day 20 pi for the other two pigs **(data not shown**). The highest titers were observed on day 29 pi (between 6.3 and 7.3 log2). The two non-inoculated pigs produced no anti-PrV IgG and IgA **(Fig. 2A and data not shown)**.

For the group inoculated by the IN route with the naked PrV-gB-based DNA vaccine, pig #3 produced IgG antibodies against PrV from 20 days pi to the end of the pig experiment **(Fig. 2B)**. On day 37 pi, the titer was 9.3 log2 for this pig. Pig #2 in this group also exhibited an increasing level of PrV-specific serum IgG response, but it started only after the third inoculation (day 29 pi). It reached a titer of 6.3 log2 at the end of the pig experiment (37 dpi). For the third pig (pig #1), a transient and very low response was observed only on day 29 pi (4.4 log2). Concerning the induction of systemic anti-PrV IgA, pig #1 elicited such a response from 20 dpi (highest titer of 6.3 log2 on day 20 pi) **(data not shown)**. The other two pigs showed only a slight increase of the titer on day 37 pi (3.1 log2) **(data not shown)**.

3.1.2 Determination of anti-PrV sIgA in the saliva of pigs following IM or IN inoculation of the naked PrV-gB-based DNA vaccine

Thereafter, the presence of anti-PrV sIgA was determined in pig saliva collected on days 29 and 37 pi **(Fig. 3)**. No antibodies were detected in the samples from the two non-inoculated pigs. Antibodies were detected in two out of three pigs injected by the IM

route with the naked DNA vaccine. The titers of antibodies were 3.4 and 4.3 log 2, respectively. More interestingly, all the pigs inoculated through the IN route with the naked PrV-gB-based DNA vaccine had sIgA titers against PrV at least at the same order of magnitude as those of the pigs injected through the IM route with the same vaccine. Furthermore, pig #2 presented higher titers than those detected in the IM group (titers of 7.3 and 6.3 log2 on days 29 and 37 pi, respectively).

3.2 PrV-specifc systemic and mucosal humoral responses induced after IN inoculation of the PLGA-PEI-nanovectored DNA vaccine encoding PrV-gB (pig experiment #2)

In previous studies, researchers have used nanovectors to protect DNA vaccines from mucosal degradation. In the second part of this study, the PrV-gB-based DNA vaccine was associated with PLGA-PEI already known to enhance DNA vaccine efficacy, mainly in mice experiments.

3.2.1 *In vitro* characterization of the nanovectored DNA plasmids

The PLGA-PEI nanovectored DNA plasmids encoding luciferase were prepared as described above and incubated for 72 hours with the bronchial epithelial cell line HBE-16. Cytotoxic analyses performed on the supernatants of these cell cultures revealed that the preparation with PLGA-PEI had no toxicity for the cells (**Fig. 4A**). Furthermore, only the association of PLGA-PEI with the plasmid DNA encoding luciferase enabled the expression of luciferase within the cells (**Fig. 4B**). These results indicate that the nanovectors composed of PLGA-PEI are functional and can be safely administered to pigs.

3.2.2 Determination of serum anti-PrV IgG and IgA following IN inoculation of the PLGA-PEI-nanovectored PrV-gB-based DNA vaccine

Two groups of three pigs were intranasally inoculated three times at ten-day intervals with the naked PrV-gB-based DNA vaccine or PLGA-PEI PrV-gB-based DNA vaccine, respectively. As a control, an additional pig was intramuscularly injected at the same time points with the naked DNA vaccine. Finally, a fourth group of four non-inoculated pigs was added. No adverse reactions following any of the inoculations were observed throughout the pig experiment. Humoral immune responses against PrV were measured until day 50 pi **(Fig. 1B)**.

Specific IgG and IgA titers against PrV were measured in the sera of all the pigs every 7-10 days throughout the pig experiment. Confirming results from experiment #1 described above, anti-PrV IgG and IgA were detected in the sera of the pigs inoculated with the naked DNA vaccine through the IM **(data not shown)** and IN routes **(Fig. 5Afor IgG and data not shown for IgA)**. For the pigs inoculated with the PLGA-PEI-DNA vaccine, the systemic anti-PrV IgG and IgA immune responses was low for all three pigs (**Fig. 5B and data not shown, respectively**).

3.2.3 Determination of anti-PrV sIgA in the saliva of pigs inoculated with nanovectored pcDNA3-gB through the IN route

Compared to experiment #1, saliva was collected for a longer period from day 0 until day 44 pi (instead of only days 29 and 37 pi as in the first pig experiment). sIgA against PrV was detected in most of the pigs inoculated with the DNA vaccine after the second or third inoculation (naked DNA vaccine injected through the IM route **(Fig. 6A)** or inoculated through the IN route **(Fig. 6B)** and nanovectored PLGA-PEI-DNA vaccine **(Fig. 6C)**). However, for the majority of the pigs, the detection of these antibodies was transient since on day 44 pi, these antibodies were no longer detected **(Fig. 6A, 6B and pig #2 of Fig 6C)**.

There was two notable exceptions: sIgA against PrV was still detected in saliva in pigs #1 and #3 in the PLGA-PEI DNA vaccine group on day 44 pi (titers of 3.3) **(Fig. 6C)**.

4. Discussion

There is a need to develop new vaccine strategies against respiratory pathogens in pigs. Very promising results for IN DNA vaccination have been obtained using mice or guinea pigs (Ai et al., 2013; Du et al., 2015; Iqbal et al., 2003; Khatri et al., 2008; Nanda et al., 2014). Of specific interest, IN inoculation to mice of a naked plasmid encoding the same PrV-gB as the one used in the present study resulted in the induction of vaginal PrV-specific sIgA, but not in protection (Yoon et al., 2008). Unfortunately, there are very few data on mucosal DNA vaccination in large animals, and none on IN DNA vaccination in pigs. Furthermore negative results are common. For example, a DNA vaccine against the foot-and-mouth disease virus (FMDV) was not efficient in sheep when administered through the IN route, regardless of whether the vaccine was naked or not (Niborski et al., 2006).

The present study aims at generating preliminary results on the development of IN DNA vaccination in pigs. To do this, we used a DNA vaccine against PrV that induces immune responses after IM injection: a DNA vaccine encoding PrV gB (Dory et al., 2009; Le Moigne et al., 2012). This model is especially valuable for such study because (1) the virus enters the body through the nasal route (and therefore it is beneficial to generate protective immunity at this entry site), and (2) it has been shown that a plasmid encoding PrV-gB induced the production of mucosal sIgA against PrV in the vagina after IN inoculation of mice (Yoon et al., 2008). It can thus serve as a marker of induction of systemic and mucosal humoral immune responses to study new strategies to inoculate DNA vaccines in pigs. At this stage, it has to be pointed out that PrV-gB alone is not able to induce strong protective immunity against PrV-infection in pigs (van Rooij et al., 2000),

358 and this was also the case after IN inoculation in mice (Yoon et al., 2008). Therefore,
359 PrV-gB is fully adapted to the study we wanted to perform here since the main aim of the
360 present study was first to evaluate if IN inoculation of a DNA vaccine can induce the
361 production of immune responses, and more particularly mucosal immune responses. For
362 this reason, and because they are beyond our objective, protective studies were not
363 conducted. Before a PrV-challenge with a protective DNA vaccine encoding PrV-gB, -gC
364 and -gD (Dory et al., 2005; Gerdts et al., 1997; van Rooij et al., 2002) could be designed in
365 pigs, IN administration of DNA vaccine must first be optimized.

366 In the present study, the IM route of immunization served (1) to show that the PrV-gB
367 DNA vaccine is functional and induces the production of systemic humoral immune
368 response, and (2) to study whether sIgA against PrV are detected in the saliva of
369 intramuscularly injected pigs. In all cases, anti-PrV IgG and IgA were found in the sera of
370 these pigs; anti-PrV sIgA antibodies were detected in the saliva of 3 out of 4 pigs. To our
371 knowledge, this is the first time that such sIgA antibodies have been detected in the saliva
372 of pigs intramuscularly vaccinated with a DNA vaccine against PrV. Previously, such
373 antibodies were also detected in nasal secretions of pigs intramuscularly injected with a
374 commercial inactivated PrV vaccine (Geskypur®) (Le Luduec et al., 2016). Unfortunately,
375 the comparisons of the levels of antibodies obtained after the IM injection of our DNA
376 vaccine and Geskypur® are not possible since the sIgA levels were measured in two
377 different fluids.

378 IN inoculation of naked DNA vaccine has mainly been described as ineffective in
379 inducing immune responses or protection, with some notable exceptions. For example,
380 Svanholm *et al* reported that significant clinical protection against *Chlamydophila*
381 *pneumonia* was obtained after IN immunization of mice with a naked plasmid encoding
382 HSP-60 (Svanholm et al., 2000). Several other studies have reported induction of specific
383 systemic (serum IgG or IgA) or mucosal (sIgA) immune responses against β-galactosidase

(Csaba et al., 2006; Cui and Mumper, 2002), hepatitis B virus (Khatri et al., 2008) or *Streptococcus pneumoniae* (Xu et al., 2011) following IN naked DNA immunizations in mice. Herein, we observed the induction of immune responses after IN inoculation of the naked form of the PrV-gB DNA vaccine in pigs. This inoculation induced both systemic and mucosal humoral immune responses in all the vaccinated pigs (six pigs if we take into account both pig experiments). Even though the number of individuals used was too low to allow significance testing, it seems that systemic anti-PrV IgG and IgA levels is lower following IN inoculation than those measured in the IM group. In contrast, levels of sIgA in the saliva were around the same order of magnitude in the IM and IN groups. In fact, the highest levels of sIgA in the saliva were found for individuals of the IN groups in both pig experiments. But we observed a high inter-individual variations, especially in the IN groups that further limits our ability to interpret the data. That being said, it is noteworthy that all the 6 pigs inoculated with the naked DNA vaccine induced the production of sIgA against PrV in the salivary (instead of 3 out of 4 for the IM group), providing a strong evidence that IN DNA vaccination induces the production of respiratory mucosal immune responses against the targeted pathogen. Cao *et al* that inoculated pigs intranasally with an the attenuated PrV vaccine PrV K61 measured the sIgA titers in saliva around 8 log2 (Cao et al., 2011), i.e. slightly above the results we obtained here with the naked PrV-gB-based DNA vaccine, which shows that the titers that we obtained are common for the species. Further studies with a higher number of individuals are now necessary to determine if the IN route has a higher potency at inducing sIgA production. In addition, follow-up studies will need to include a thorough optimization procedure to reduce inter-individual variations and enhance the efficacy of this intranasally inoculated DNA vaccine by testing other adjuvants or modifying several other parameters (use of nanovectors, ways to inoculate, etc.).

Most researchers studying IN DNA vaccination combine their DNA vaccines with nanovectors to protect the DNA molecule from mucosal environment degradation until it reaches the cells involved in the development of immune responses. There are many types of nanovectors (for reviews see (Bernocchi et al., 2017; Xu et al., 2014)). For our study, we selected PLGA-PEI nanoparticles. Indeed, PLGA and/or PEI were previously successfully used in IN DNA vaccination in mice (Du et al., 2015; Torrieri-Dramard et al., 2011).

In the present proof of concept study, we selected a DNA/nanovector ratio previously found to be effective to transfect the bronchial lineage HBE-16 cell line in vitro. It did not ensure success of *in vivo* IN inoculation but we assumed it would increase chances of success (Ragelle et al., 2014). In association with PLGA-PEI, the duration of mucosal immune response in the saliva induced by the DNA vaccine was longer for two out of three pigs, compared to the three pigs inoculated intranasally with the naked DNA vaccine. This result may be anecdotal, but it reveals that mucosal humoral immune responses might be improved. Clearly, further studies are necessary to optimize the IN DNA vaccine to induce a high and sustained mucosal immune responses. We suggest that such studies should compare the efficiency of PLGA-PEI with other nanovectors, and optimize the other parameters such as the DNA/nanovector ratios, total quantity of DNA, adjuvants and inoculation conditions; inoculation conditions include anesthetized pigs (Janakova et al., 2002) or non-anesthetized pigs (Khatri et al., 2008), the volume of vaccine, number of doses, intervals between the doses, speed of inoculation, and specific devices for inoculation). Only such a thorough and comprehensive approach will allow us to reliably assess the protective immunity induced by IN DNA vaccine strategies.

5. Conclusion

The current study clearly showed that IN inoculation of a naked DNA vaccine encoding PrV-gB induces both a systemic and a mucosal humoral immune response against PrV in

pigs. To the best of our knowledge, this is the first report showing the induction of immune responses following IN inoculation of a naked DNA vaccine in pigs. Some immune response parameters appeared enhanced when the DNA vaccine was combined to PLGA-PEI nanovectors. Even though the inter-individual variability observed indicate that the optimized conditions were not reached in the present study, these results suggest that it is possible to improve DNA vaccine efficacy by varying its composition and inoculation conditions. Based on the promising results presented herein, we encourage future studies to build upon the present results and improve IN DNA vaccination in pigs.

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8. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends:

Figure 1: Schematic representation of the animal immunization experiments.

(A) Assessment of immune potentials in pigs inoculated intranasally with a naked PrV-gB DNA vaccine. **(B)** Assessment of immune potentials in pigs inoculated intranasally with a formulated PrV-gB DNA vaccine.

Figure 2: ELISA determination of systemic IgG antibody levels against PrV after inoculation with a naked PrV-gB-based DNA vaccine.

Pigs were inoculated three times with a naked pcDNA3-gB DNA vaccine, as indicated by the arrows. Pig sera were collected every 8-10 days throughout the pig experiment. IgG titers of the two non-inoculated pigs and the three pigs inoculated by the IM route are indicated in **(A)**. IgG titers of the three pigs inoculated by the IN route are indicated in **(B)**. Dilutions were performed in triplicate and the threshold of positivity was established as the OD mean of the negative controls ± 3 SD. Antibody titers are presented in log2 scales. Arrows topped with a number indicate the inoculations with DNA vaccine.

Figure 3: ELISA determination of salivary sIgA antibody levels against PrV after inoculation with a naked PrV-gB-based DNA vaccine.

Pigs were inoculated three times with the DNA vaccine at ten-day intervals. Pig saliva samples were collected at days 29 and 37 after the first inoculation (dpi). Black bars correspond to 29 dpi and dashed bars to 37 dpi. Saliva dilutions were performed in triplicate and the threshold of positivity was established as the OD mean of the negative controls ± 3 SD. Antibody titers are presented in log2 scales.

Figure 4: *In vitro* toxicity and cell transfection potentials of naked and PLGA-PEI associated plasmids.

HBE-16 cells were incubated with 1 µg of naked plasmid pTG11033 (Naked DNA), 1 µg of plasmid DNA pTG11033 adsorbed onto premade PLGA-PEI nanoparticles at a weight to weight ratio of 12 (PLGA-PEI-DNA). Cell viabilities **(A)** and luciferase expressions **(B)** were measured at 72 hours post-transfection. The untransfected cell condition served as a reference level (NT cells) corresponding to 100% viability (dashed line) (A) or to luciferase base level (B). In the latter case, the values expressed in relative light units (RLUs) per protein mg measured in the cell lysates are indicated. Mean results of three independent experiments are presented ± SD. Significant differences between each condition and their respective controls were determined with the non-parametric Mann–Whitney test (ab, $p < 0.05$).

Figure 5: ELISA determination of systemic IgG antibody levels against PrV after intranasal inoculation with a naked or PLGA-PEI associated PrV-gB-based DNA vaccine.

Pigs were inoculated three times with the DNA vaccine as indicated by the arrows. Pig sera were collected every 8-10 days throughout the pig experiment. Results for the three pigs inoculated with naked pcDNA3-gB are indicated in **(A)**. Results for the three pigs inoculated with PLGA-PEI-pcDNA3-gB are indicated in **(B)**. Dilutions were performed in triplicate and the threshold of positivity was established as the OD mean of the negative controls ± 3 SD. Antibody titers are presented in log2 scales. Arrows topped with a number indicate the inoculations with DNA vaccine.

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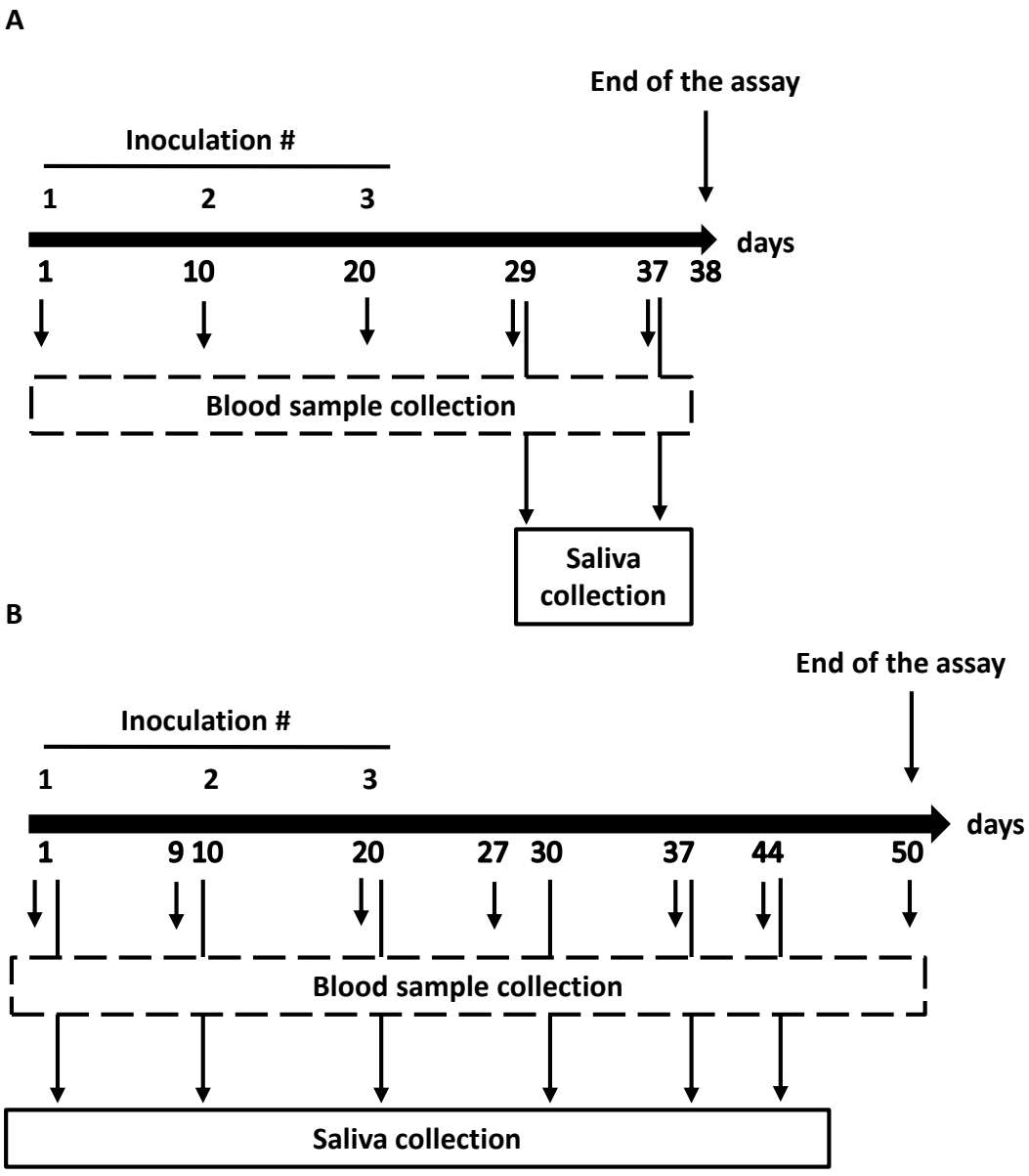

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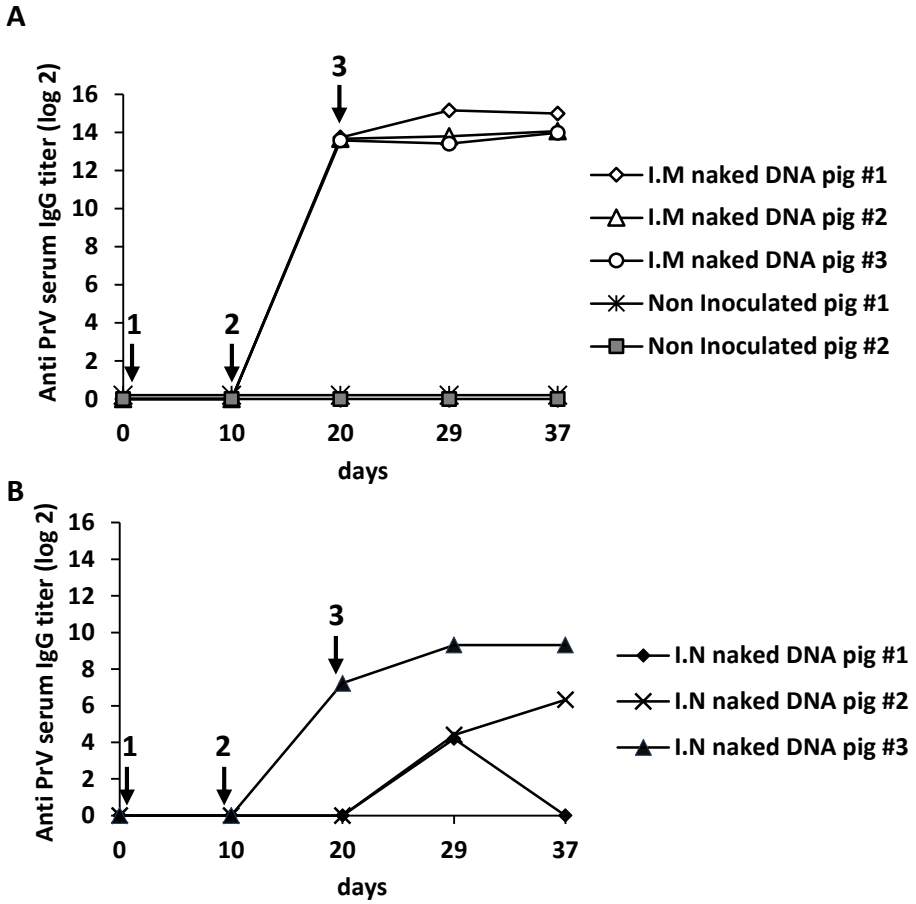

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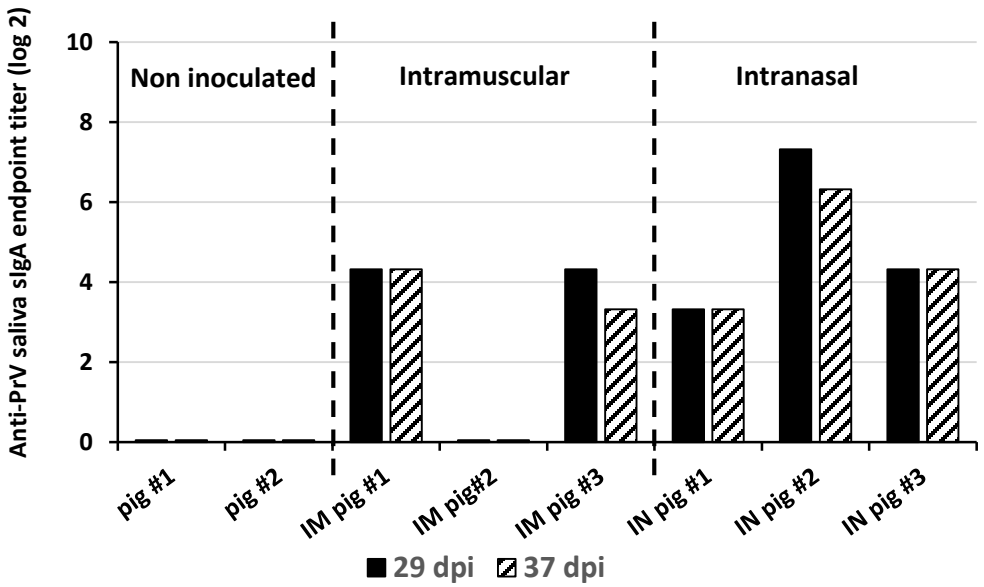

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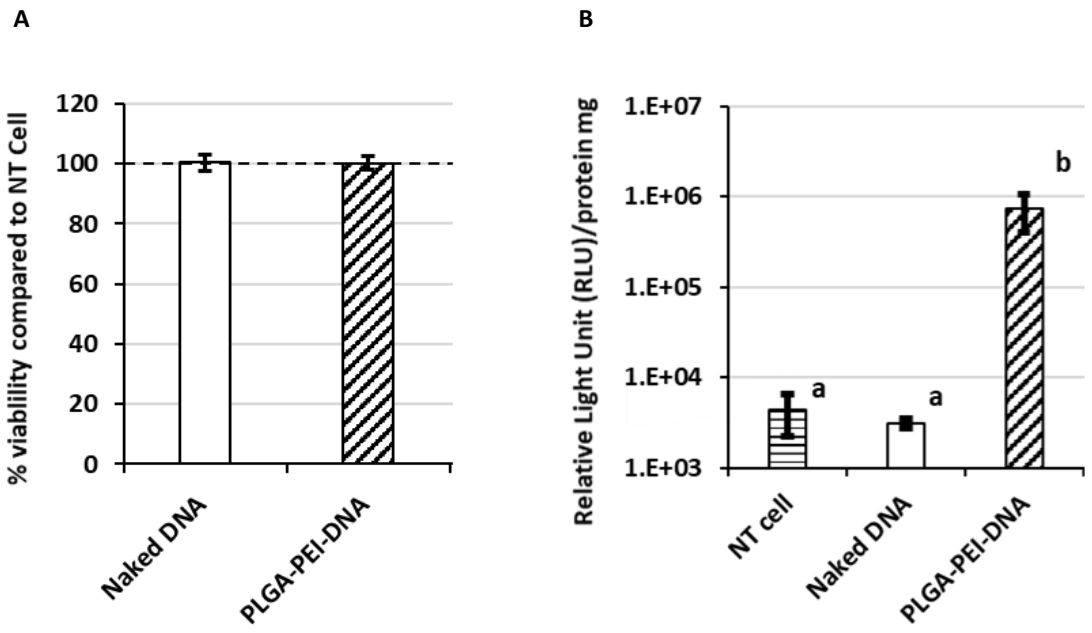

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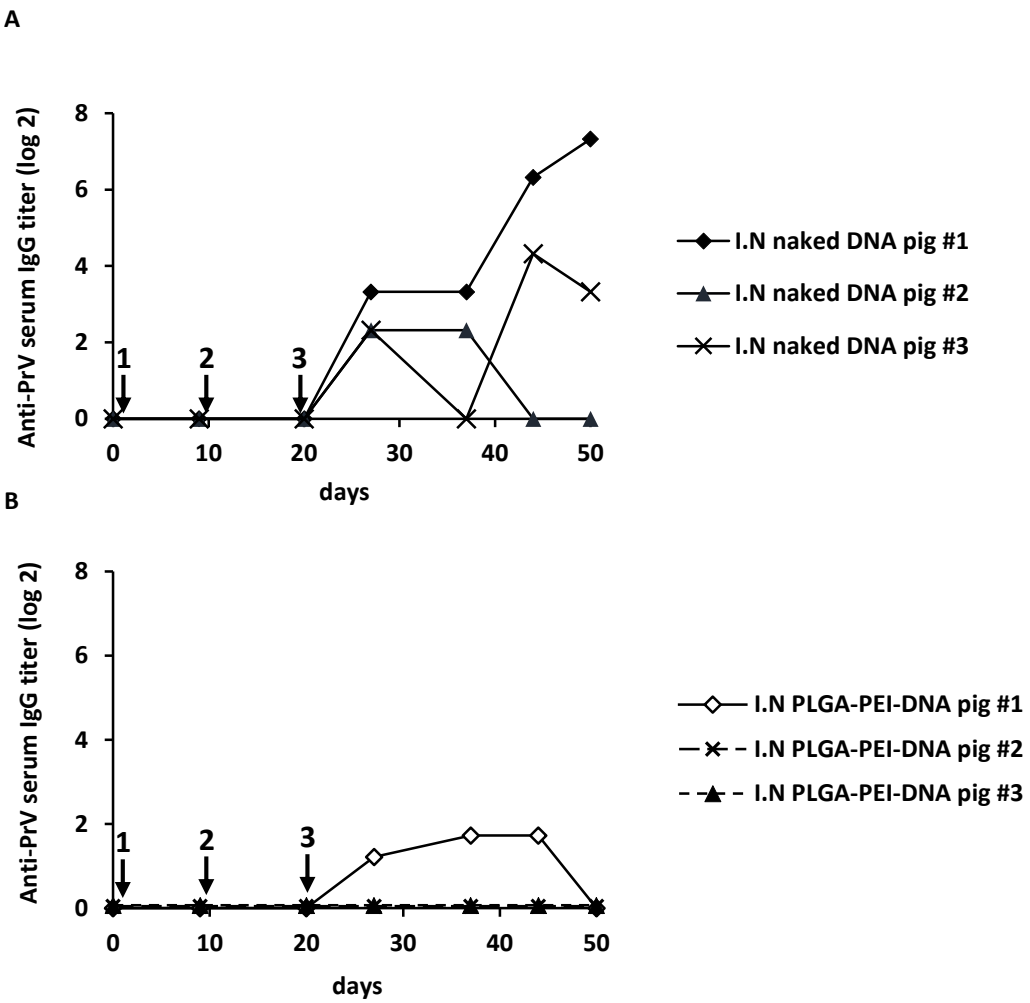

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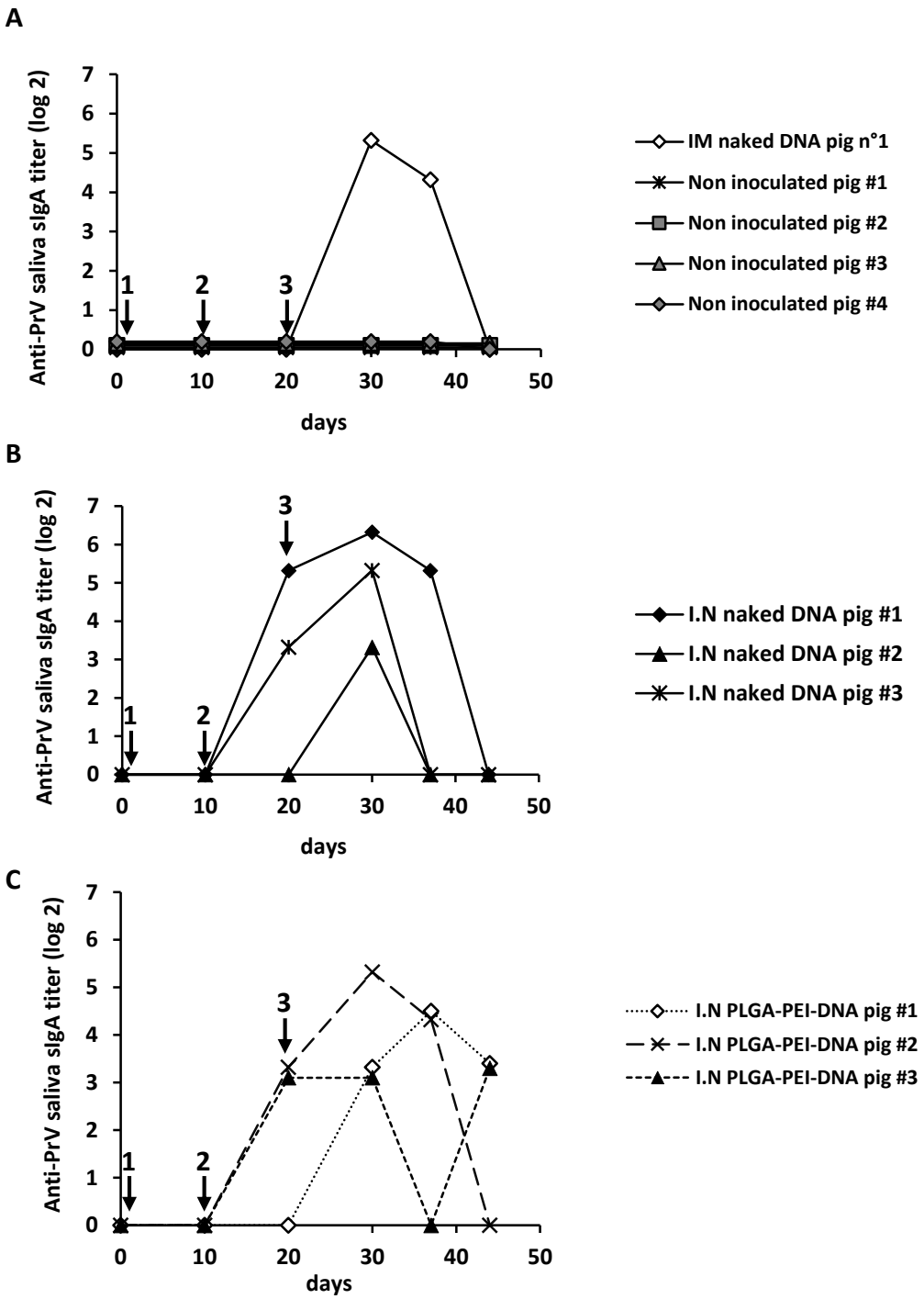

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