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Biological characterization of a recombinant pseudorabies virus

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Abstract

In a previous study we obtained and characterized *in vitro* a novel pseudorabies virus (PRV) variant named gIp2 with a TK, gI/gE, 11k and 28k negative phenotype and a duplication of PK gene. The main objective of the present study was to determine the safety and efficacy, as a vaccine candidate, of this recombinant PRV. For this purpose, we used 24 PRV seronegative three weeks old piglets that were divided into five groups of treatment. Piglets of groups A and B were immunized twice with $10^{6.5}$ and $10^{5.5}$ $TCID_{50}$ of gIp2, respectively; pigs of group C were vaccinated twice with MLV vaccine *Auskipra GN*® and pigs of groups D and E were not immunized and served as infected and uninfected controls, respectively. Four weeks after the second immunization pigs of groups A, B, C and D were challenged by intranasal inoculation of 10^6 $TCID_{50}$ of the wild-type NIA-3 strain of PRV. No adverse reactions or clinical signs were observed in any group after immunization, indicating that the application of up to 10 times the conventional dose included in a commercial vaccine (i.e. $10^{5.5}$ $TCID_{50}$) of gIp2 was safe in piglets. Additionally, the inoculation of gIp2 induced an immune response able to provide clinical and virological protection against pseudorabies virus after challenge. In conclusion, the use of gIp2 in piglets as a vaccine virus is safe and induces an immunity comparable to that exerted by commercially available vaccines.

Additional key words: immunization, protection, safety, vaccine.

Resumen

Caracterización biológica de un virus de la enfermedad de Aujeszky recombinante

En un estudio anterior, nuestro grupo de investigación había obtenido y caracterizado *in vitro* una nueva variante del virus de la enfermedad de Aujeszky (PRV) denominado gIp2, que presenta un fenotipo TK, gI/gE, 11K y 28K negativo, así como una duplicidad en el gen que codifica la PK. El principal objetivo de este estudio fue determinar la seguridad y eficacia, como candidato vacunal, de este virus recombinante. Para ello utilizamos 24 lechones de tres semanas de vida seronegativos al PRV, que fueron divididos en cinco grupos distintos. Los lechones de los grupos A y B fueron inmunizados dos veces con $10^{6.5}$ y $10^{5.5}$ $DI_{50}CT$ del virus gIp2; los lechones del grupo C fueron inmunizados dos veces con la vacuna comercial *Auskipra GN*® y, finalmente, los lechones de los grupos D y E permanecieron sin inmunizar y se utilizaron como testigos infectados y sin infectar, respectivamente. Cuatro semanas después de la segunda inmunización los lechones de los grupos A, B, C y D fueron desafiados por vía intranasal con 10^6 $DI_{50}CT$ de la cepa virulenta NIA-3. Tras la inmunización no se observaron reacciones adversas ni signos clínicos en ninguno de los grupos, indicando que la aplicación de hasta 10 veces la dosis convencional de una vacuna comercial (i.e.$10^{5.5}$ $DI_{50}CT$) del virus gIp2 es segura en lechones. Además, la inoculación del virus gIp2 indujo una respuesta inmune capaz de estimular una protección clínica y virológica tras el desafío. En resumen, la utilización del virus gIp2 como virus vacunal en lechones es segura e induce una inmunidad comparable a la producida por vacunas comerciales.

Palabras clave adicionales: inmunización, protección, seguridad, vacunas.

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Abbreviations used: ADG (average daily gain); ANOVA (analysis of variance); CPE (cytopathic effect); DMEM (Dulbecco's modified Eagle medium); dpc (days post-challenge); ELISA (enzyme-linked immunosorbent assay); FBS (foetal bovine serum); FCE (food conversion efficiency); gE, gI and gG (glycoproteins E, I and G, respectively); MLV (modified-live virus); NIA-3 (Northern Ireland Aujeszky-3 strain of PRV); PK (protein-kinase); PK-15 (porcine kidney 15); PRV (pseudorabies virus); SN (seroneutralization assay); $TCID_{50}$ (tissue culture infective dose 50); TK (timidin-kinase).

Introduction

Pseudorabies is one of the most relevant pig diseases that affect swine industry. The causative agent is a linear double-stranded DNA virus, pseudorabies virus (PRV), which has spread worldwide causing huge economical losses. The most remarkable signs of PRV infection are severe, and usually lethal, nervous disorders in young piglets, fever, depression, respiratory and nervous symptoms in weaned and fattening pigs, and reproductive failure and nervous signs in adults.

Several modified live virus (MLV) vaccines against PRV have been developed by attenuation on cell cultures, chemical or thermal treatment and by genetic engineering techniques. In the last years, the improvement achieved on molecular biology and genetic engineer has permitted to increase the knowledge about the involvement of PRV proteins in virulence and attenuation. The role of timidin-kinase (TK) and the glycoprotein E (gE) proteins in PRV attenuation has been widely studied. gE is a membrane protein, non-essential for virus replication and involved in PRV dissemination through epithelial cells in the primary replication site, respiratory tract and in neuronal synapses. Therefore, the lack of expression of gE protein has been shown to attenuate the PRV by reduction of the virulence and neuronal colonization. Nonetheless, it does not reduce the level of immunological stimulation, that seems to be similar to the pathogenic Northern Ireland Aujeszky-3 (NIA-3) strain of PRV. Moreover, the application of gE- vaccines reduce field strain latency after challenge. On the other hand, TK protein has an essential role for viral replication, being indispensable for infective virion production in non-dividing cells such as neurons and blood mononuclear cells. Consequently, the deletion of the gene coding for TK protein results in a significant reduction of the virulence and neuronal colonization. Recent studies have shown that deletion of genes coding for gE and TK proteins could have a synergic effect on PRV attenuation.

Studies carried out by Fernández *et al*. (1999) described the development and *in vitro* characterization of a novel PRV variant named gIp2, produced by genetic recombination between two PRV isomers. During the recombination process a part of the U_S component was deleted resulting in a TK, gI/gE, 11k and 28k negative phenotype. The level of replication of gIp2 has been evaluated *in vitro*, showing half of the replication capacity of the wild strain NIA-3 in Vero cell line, derived from monkey kidney, and 35 times lower than NIA-3 and similar to the vaccine strain Bartha in SH-SY5Y cells, derived from human neuroblastoma. Moreover, a duplication of protein-kinase (PK) gene was produced during the recombination process. The effect of this diploidy on pathogenesis is still unknown. However, the PK protein seems to be involved in blockade of apoptosis in infected cells and its inactivation leads to a reduction of the level of *in vitro* replication, that could be related to the reduction of the protective immune-properties. Consequently, the diploidy of US3-encoded PK gene could play an important role in the development of an appropriate immune response against PRV infection.

The main objective of the present study was the characterization of the biological function of the recombinant PRV gIp2, in order to determine its safety and efficacy as a vaccine candidate and as a viral vector for heterologous protein expression. An additional objective was to determine the possible influence of the PK protein duplication on the immune-protective properties of gIp2 against PRV infection.

Material and methods

Virus and cell culture

Experiments were performed using the recombinant PRV gIp2, which has been propagated onto porcine kidney (PK-15) cell line, using Dulbecco´s modified Eagle´s medium (DMEM) supplemented with antibiotics and 5% foetal bovine serum (FBS). Infectivity titers were determined in PK-15 cell cultures and adjusted with DMEM to $10^{6.5}$ and $10^{5.5}$ tissue culture infectious doses 50 ($TCID_{50}$) mL^{-1}.

The pseudorabies MLV vaccine *Auskipra GN*® (Laboratorios Hipra, Spain), which is derived from Bartha K-61 strain of PRV, was used following the manufacturer's instructions.

Challenge was performed using the virulent NIA-3 strain of PRV. Growth was carried out onto PK-15 cultures using DMEM supplemented with antibiotics and 5% FBS. Infectivity titers were determined in PK-15 cultures and adjusted with DMEM to 10^6 $TCID_{50}$ mL^{-1}.

Animals and facilities

Twenty-four 3-week-old crossbred (Landrace x Large White) pigs with no detectable PRV serum antibody

titers measured by ELISA test (HerdChek PRV gB, IDEXX Laboratories, Spain) were included in this experiment. The pigs were randomly divided into five groups, assigned to one treatment group and housed in isolated pens with a concrete floor and automatic watering system.

Experimental design and sample collection

The study comprised five experimental groups. Five pigs, members of group A (numbered 1, 2, 3, 4, 5), and five pigs of group B (numbered 6, 7, 8, 9, 10) were immunized twice on days 0 and 21 with $10^{6.5}$ and $10^{5.5}$ $TCID_{50}$ of gIp2, respectively. Five pigs from group C (numbered 11, 12, 13, 14, 15) were vaccinated twice, on days 0 and 21, with MLV vaccine *Auskipra GN*® following manufacturer's instructions. Five pigs from group D (numbered 16, 17, 18, 19, 20) and four pigs from group E (numbered 21, 22, 23, 24) were not immunized and served as infected and uninfected controls, respectively.

Four weeks after the second immunization (day + 0) pigs from groups A, B, C and D were challenged by intranasal inoculation of 10^6 $TCID_{50}$ of the wild-type NIA-3 strain of PRV.

All pigs were examined daily for clinical signs and rectal temperatures were measured. The clinical signs observed were classified as non-specific signs, respiratory signs, cutaneous signs and nervous signs. Non-specific signs were evaluated using the following scoring system: depression received a score of 1 when present, anorexia a score of 2 and letargia a score of 3; for the respiratory signs, sneezing was scored 1, coughing and nasal secretions were scored 2, superficial breathing was scored 3 and abdominal breathing was scored 4; for cutaneous signs, cyanosis was scored 1; finally, nervous signs were evaluated following this scoring system: shivering was scored 1, nistagmous was scored 2, incoordination was scored 3, opistotonous and paddling were scored 4 and paralysis was scored 5. Additionally, food intake, average daily gain (ADG) and food conversion efficiency (FCE) index were recorded.

Blood samples were collected in serum-clot vacuum tubes on each vaccination day and then 3, 6, 9, 14 and 21 days post-vaccination, at challenge and 3, 6, 9 and 16 days post-challenge (dpc). Serum was obtained from all blood samples and stored at -80ºC until its utilization for virus isolation and serological assays.

Dry cotton-tipped nasal swabs were used to collect nasal exudates on the same day of each blood collection. Swabs were immersed in 2 mL of DMEM and stored at -80ºC until they were used for virus isolation.

Sixteen days after challenge (D+16) all pigs were euthanized and tissue samples were collected, including lung, tonsils, thymus, ileum, brain, adrenal gland, and submandibular, superficial inguinal, mediastinic and mesenteric lymph nodes. All these tissues were stored at -80ºC until they were processed for virus isolation.

Virus isolation and virus titration

The aliquots of sera were thawed at room temperature, diluted 1:4 in DMEM and passed through a filter with 0.45 µm pores. Nasal swabs immersed in DMEM were thawed at room temperature, homogenized and passed through a filter with 0.45 µm pores. We homogenized 1 g of each tissue sample with 9 mL of DMEM, centrifuged at 1200 g for 20 min at 4ºC and the supernatant was passed through a filter with 0.45 µm pores.

All samples were added in duplicate (200 µL $well^{-1}$) onto PK-15 cell monolayers seeded in 24-well plates and incubated for 90 min at 37ºC to facilitate adsorption. The cultures were washed twice with DMEM and fresh DMEM containing 5% FBS was added. The culture plates were incubated at 37ºC in a humidified atmosphere containing 5% CO_2. Monolayers were examined for cytopathic effect (CPE) on days 4, 5 and 6 post-inoculation.

We used DMEM as the diluent for virus titration. Serial 10-fold dilutions of experimental PRV and positive clinical samples were made in 96-well microtiter plates previously seeded in PK-15 cultures. The cell cultures were incubated at 37ºC, in a humidified atmosphere of 5% CO_2 in air. Monolayers were examined for CPE on days 4, 5 and 6 post-inoculation. The number of positive wells per dilution was recorded. Titers were calculated as previously described and were expressed as $TCID_{50}$ mL^{-1} for fluid samples or $TCID_{50}$ g^{-1} for tissue samples, after adjusting for the dilutions made when preparing the samples for virus isolation.

Serological examinations

Serum samples were examined for PRV-specific antibodies using the ELISA kit HerdChek PRV gB (IDEXX

Laboratories, Spain) and gE protein antibodies using the ELISA kit *INGEZIM ADVgI*® (Ingenasa, Spain).

Seroneutralization assay (SN) was performed onto PK-15 cell cultures. Sera were two-fold diluted, using DMEM as diluent, in 96-well tissue culture plates and 200 $TCID_{50}$ of NIA-3 strain of PRV were added to each well. The culture plates were incubated for 1 hour at 37ºC in a humidified atmosphere containing 5% CO_2. After that time, 100 µL of a suspension containing 2 x 10^5 PK-15 cells mL^{-1} were added to each well. The plates were then incubated at 37ºC in a humidified atmosphere containing 5% CO_2 and were examined for CPE on days 4, 5 and 6 post-inoculation.

Statistical analysis

Analysis of variance (ANOVA) and Duncan´s multiple range test were used to compare clinical scores, body weight, ADG, PRV titers and neutralizing antibodies among the different groups. T-student was used to analyze differences on rectal temperature before and after each immunization and challenge. Statistical analysis was performed using SPSS software package and results were considered statistically significant when $p < 0.05$.

Results

Clinical examinations

Experimental immunization did not produce any clinical sign or side effect. However, clinical signs associated to PRV infection were detected in animals from groups A, B, C and D after challenge. These clinical manifestations were more intense in pigs of group D and included letargia, severe respiratory disorders, cyanosis, hyper-salivation and other neurological signs, as incoordination and paddling. The clinical signs were so severe that led to death of 100% of the animals, three of them on day +3 (*i.e.* 3 dpc) and the other two on day +5 (*i.e.* 5 dpc). Less severe clinical signs were observed after challenge in pigs of groups A, B, and C. Nonetheless, systemic and respiratory signs were observed in all groups. Particularly, depression or letargia were observed in most animals of group A between days +2 and +4 and in most animals of groups B and C between days +2 and +5. Later on, systemic signs were observed in two, one and one pigs of group B on days +6, +7 and +8, respectively and in two and three pigs of group C on days +7 and +8, respectively. Additionally, mild respiratory signs were observed in some pigs of groups A and B from day +1 to day +5 and in some pigs of group C from day +1 to day +9. All these signs tended to be milder in groups A and B compared to group C, to the point that the differences in mean values of clinical signs were statistically significant on day +1 (*i.e.* 1 dpc) for groups A and B ($p=0.003$), on day +5 (*i.e.* 5 dpc) for group A ($p=0.002$) and on day +7 (*i.e.* 7 dpc) for groups A and B ($p=0.011$). Moreover, a significant reduction in clinical sign duration was observed in groups A and B in comparison to group C ($p<0.001$).

Rectal temperatures measured after immunization showed a slight increase in comparison to pre-immunization data only on day 1 (*i.e.* 1 day after first immunization) in groups A ($p=0.014$), B ($p=0.031$) and C ($p=0.040$), though a maximum value of 39.7ºC was only recorded in one or two animals per group. After challenge, significant increases in rectal temperature were recorded on day +1 (*i.e.* 1 dpc) in group B ($p=0.014$) and on days +2, +3 and +4 (*i.e.* 2, 3 and 4 dpc) in group A ($p<0.001$; $p=0.004$; $p=0.015$, respectively), B ($p<0.001$; $p=0.001$; $p<0.001$), C ($p<0.001$; $p=0.003$; $p=0.003$) and D ($p<0.001$; $p=0.030$; $p=0.010$) (Figure 1).

All uninfected-control pigs remained in normal health status throughout the experiment.

Productive performance

None of the immunization protocols produced any negative repercussions on productive parameters (body weight, ADG, food intake and FCE). After challenge, the death of all pigs from group D on days +3 and +5 (*i.e.* 3 and 5 dpc) made impossible to compare previously immunized (groups A, B and C) and non-immunized-challenged (group D) groups. Although a detriment in food intake, which was lower in group A, and a statistically significant reduction on ADG were observed in groups A, B and C in comparison to group E ($p=0.001$), the body weight measured and the FCE calculated after challenge were similar among all groups.

Virus isolation

PRV was not isolated from any serum sample collected throughout the experimental period.

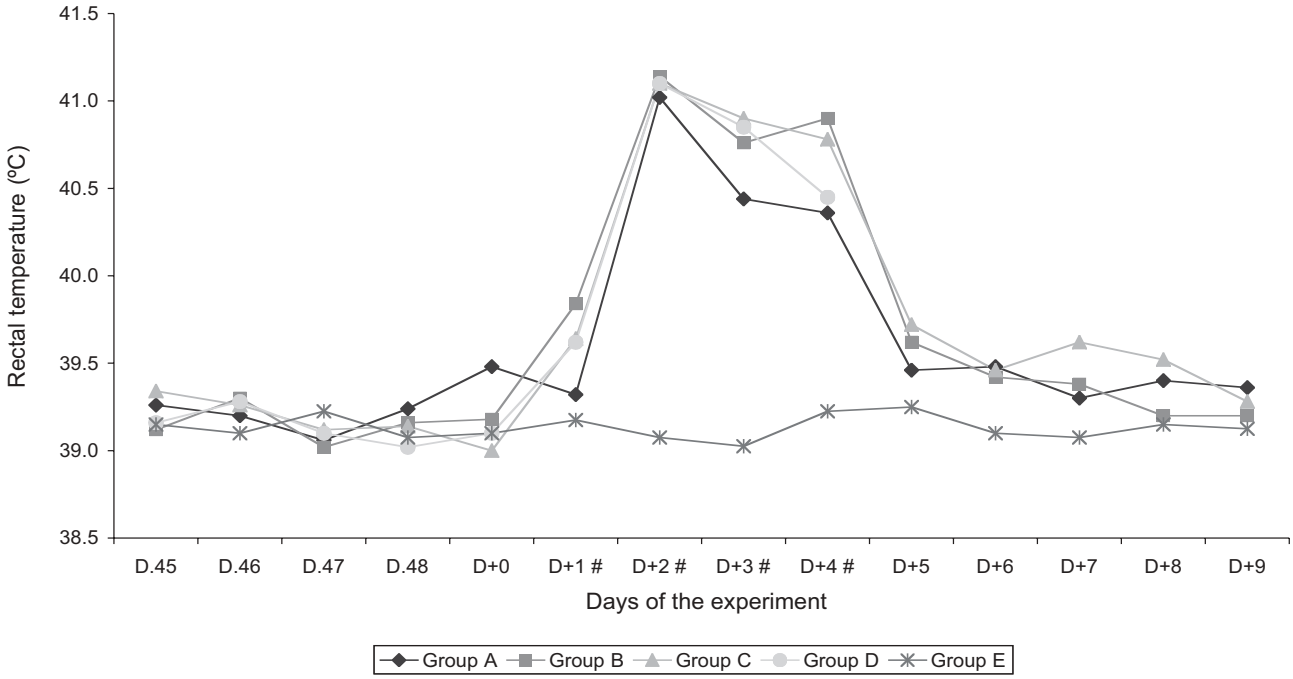

Figure 1. Average rectal temperature measured after challenge with NIA-3 strain of PRV. The sign "#" expresses statistically significant differences between groups ($p<0.05$).

Virus isolation was achieved from nasal swabs taken from all the animals from groups A, B and C on day 3 (*i.e.* 3 days after first immunization), from one pig from group C on day 9 (*i.e.* 9 days after first immunization) and from one pig from group A and one from group B on day 24 (*i.e.* 3 days after second immunization) (Table 1). After challenge, PRV was isolated on day +3 (*i.e.* 3 dpc) from all the nasal swabs collected from pigs of groups A, B, C and D and on days +9 and +16 (*i.e.* 9 and 16 dpc) from two and one pigs belonging to group C, respectively. However, there were not statistically significant differences in PRV titer detected in nasal secretions between groups (Table 1).

PRV was not detected in any tissue sample collected from groups A, B, C and E. PRV was isolated from pigs belonging to group D from lung, brain, adrenal gland, tonsils, ileum and submandibular, mesenteric, mediastinic and inguinal lymph nodes (Table 2).

Serology

ELISA results showed that all immunized animals (groups A, B and C) had seroconverted on day 21 (*i.e.* 21 days after first immunization). Due to the death of all the animals from group D between days +3 and +5 (*i.e.* 3 and 5 dpc), we could only collect two serum samples after challenge, on day +3, that were negative by ELISA test. Animals from group E remained seronegative until the end of the experiment.

Antibodies against PRV gE protein were not detected before challenge. After challenge four out of five animals from groups A, B and C had seroconverted by day +16 (*i.e.* 16 dpc). Due to the death of all the animals from group D, it was only possible to analyze serum samples taken from two pigs on day +3 (*i.e.* 3 dpc), that were negative for antibodies against gE.

Twenty-one days after the first immunization, pigs from groups A, B and C had serum neutralizing antibodies titers ranging from 1 to 4 $\log_2$. Previous to challenge exposure, the titer of neutralizing antibodies reached by pigs of group A was significantly higher than that attained by the pigs of the rest of the groups. At challenge, mean neutralizing antibody titers of pigs from groups A, B and C were 6, 4.57 and 4.04 $\log_2$, respectively (Figure 2). After PRV infection, antibody titers achieved by pigs from groups B and C were significantly higher than those of group A, reaching at the end of the experiment mean values of 9.59, 9.56 and 8 $\log_2$, respectively (Figure 2).

Table 1. PRV isolation from nasal swabs collected pre- and post- challenge

Group	Animal	Day of the experiment															
		1st immunization					2nd immunization							Challenge			
		D.0	*D.3*	*D.6*	*D.9*	*D.14*	*D.21*	*D.24*	*D.27*	*D.30*	*D.35*	*D.42*	*D+0*	*D+3*	*D+6*	*D+9*	*D+16*
A	1	-	+	-	-	-	-	-	-	-	-	-	-	+ (3)	-	-	-
	2	-	+	-	-	-	-	-	-	-	-	-	-	+ (4.8)	-	-	-
	3	-	+	-	-	-	-	+	-	-	-	-	-	+ (3.6)	-	-	-
	4	-	+	-	-	-	-	-	-	-	-	-	-	+ (4.3)	-	-	-
	5	-	+	-	-	-	-	-	-	-	-	-	-	+ (3.3)	-	-	-
B	6	-	+	-	-	-	-	-	-	-	-	-	-	+ (4.2)	-	-	-
	7	-	+	-	-	-	-	-	-	-	-	-	-	+ (4)	-	-	-
	8	-	+	-	-	-	-	+	-	-	-	-	-	+ (6)	-	-	-
	9	-	-	-	-	-	-	-	-	-	-	ND	-	+ (6.3)	-	-	-
	10	-	+	-	-	-	-	-	-	-	-	-	-	+ (4.5)	-	-	-
C	11	-	+	-	+	-	-	-	-	-	-	-	-	+ (4.3)	-	+ (< 2)	-
	12	-	+	-	-	-	-	-	-	-	-	-	-	+ (4.5)	-	+ (< 2)	-
	13	-	+	-	-	-	-	-	-	-	-	-	-	ND	-	-	+ (< 2)
	14	-	+	-	-	-	-	-	-	-	-	-	-	+ (4.5)	-	-	-
	15	-	+	-	-	-	-	-	-	-	-	-	-	+ (4.5)	-	-	-
D	16	-	-	-	-	-	-	-	-	-	-	-	-	+ (3.8)	ND	ND	ND
	17	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND
	18	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND
	19	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND
	20	-	-	-	-	-	-	-	-	-	-	-	-	+ (5.5)	ND	ND	ND
E	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

In parenthesis: viral titer expressed as log10 $TCID_{50}$ mL^{-1}; ND: non-determined

Discussion

This study assessed the safety of the recombinant PRV gIp2 and its ability to induce protection against the highly virulent NIA-3 strain of PRV, under experimental conditions.

In the safety experiment, we included two groups of pigs exposed by the intramuscular route to different doses of gIp2, one of them 10 times higher than mean doses normally used in commercial vaccines, and a positive control, consisting in a group of pigs that were vaccinated with a commercial MLV vaccine (*Auskipra GN*®). The immunization of the pigs did not cause remarkable adverse reactions in any of the groups and only transient increases in body temperature, similar to those reported by other authors after inoculation of piglets with other PRV MLV vaccines, were recorded in some animals of groups A and C.

The absence of clinical manifestations, productivity detriment and viremia after immunization, as well as the shedding pattern in nasal swabs, similar between gIp2 and the MLV vaccine *Auskipra GN*®, allows us to conclude that our recombinant PRV gIp2 is, at least, as safe as other PRV vaccine strains. This bio-safety is probably related to the deletion of the genes coding for gE and TK proteins in gIp2. In this sense, it has been proven that gE protein is involved in PRV dissemination through epithelial cells in the primary replication site and the respiratory tract and the complex gI/gE in the trans-synaptic spread of the virus. Consequently, the elimination of the gI/gE complex expression leads to a reduction in virulence and neuro-invasiveness. On the other hand, the absence of the TK protein has been related to attenuation because it is essential to produce infectious viral particles in non-dividing cells, such as neurons or blood mononuclear cells.

Table 2. PRV isolation from tissue samples collected at necropsy

Group	Animal	Lung	Brain	Adrenal	Tonsils	Ileon	Thymus	[a] Sub Ln	[b] Mes Ln	[c] Med Ln	[d] Ing Ln
A	1	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-
B	6	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-
C	11	-	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	-	-	-
D	16	+ (2.5)	-	-	+ (4.6)	-	ND	+ (4.5)	-	+ (3.5)	+ (<2)
	17	+ (<2)	-	ND	+ (4)	-	ND	+ (3.6)	-	+ (2.6)	-
	18	+ (<2)	-	-	+ (6.1)	-	ND	+ (4.5)	-	-	-
	19	+ (<2)	-	-	+ (3.6)	+ (2.5)	ND	+ (2.2)	-	+ (2)	-
	20	+ (4.5)	+ (2.6)	+ (3)	+ (4.5)	-	ND	+ (3.5)	-	+ (3)	+ (3.3)
E	21	-	-	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	-	-	-
	23	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-

In parenthesis: viral titer expressed as log10 $TCID_{50}$ g^{-1}; ND: non-determined; [a]:submandibular lymph node. [b]:mesenteric lymph. [c]:mediastinic lymph node. [d]:superficial inguinal lymph node.

The second objective of our study was to evaluate the immune-protective properties of the recombinant PRV gIp2. For this purpose, we exposed the immunized piglets to 10^6 $TCID_{50}$ of the highly virulent PRV strain NIA-3. The results obtained indicate that the clinical and virological protection conferred by gIp2 is equal to that elicited by the commercial vaccine *Auskipra GN®*, or even higher when the dose of gIp2 to which the pigs are exposed is high (i.e. $10^{6.5}$ $TCID_{50}$). In terms of clinical manifestations, our results point out that all immunogens used in our study are able to confer some degree of protection. In this sense, although hypertermia, lasting 4 or 5 days, was observed after challenge in pigs of all immunized groups, this finding is consistent with previous reports that have described hypertermia for 4-5 days in vaccinated pigs, in contrast to hypertermia of up to 10 days in no-immunized pigs. Additionally, although systemic and respiratory signs were found in pigs of all challenged groups, the signs observed were milder in immunized groups than in the non-immunized group, in which all animals died as a consequence of exposure to wild-type PRV NIA-3. These results are also similar to those obtained by other authors who observed the presence of mild clinical signs between days 2 and 7 after challenge of vaccinated pigs. Moreover, clinical protection seems to be dependent on the amount of antigen to which the animals are exposed. In this sense, the group of pigs exposed to a higher dose of gIp2 showed a significant reduction in the severity and a significant shortening in the duration of clinical manifestations compared to the rest of immunized pigs.

The virological protection provided by gIp2 was determined by PRV detection in serum and peripheral tissues. PRV was never detected in any serum sample of any of the experimental groups. In the same way,

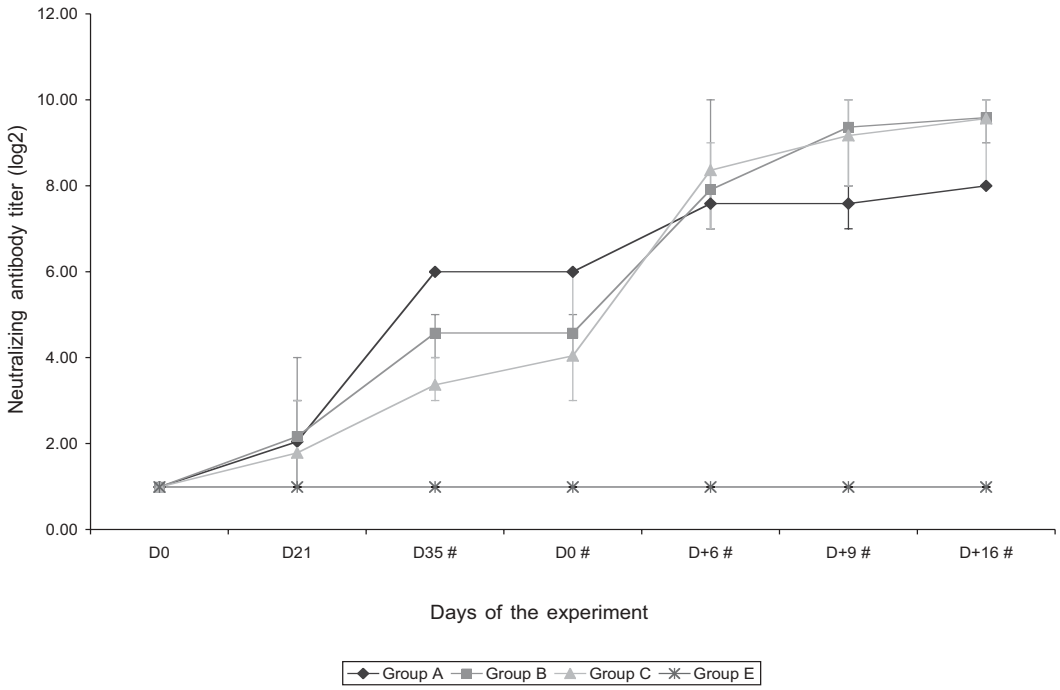

Figure 2. Geometric mean of the titer of neutralizing antibodies against PRV expressed as log2. Neutralizing antibody titers lower than 2 are considered negative. The sign "#" expresses statistically significant differences between groups ($p<0.05$).

no virus was isolated from any tissue sample of any of the previously immunized pigs, which is in agreement with previous reports of viral distribution in vaccinated animals. However, PRV was detected in several tissue samples of non-immunized-challenged controls, especially in lung, tonsils and different lymph nodes, as has been previously described after challenge of non-immunized animals. The colonization of tissues in the absence of viremia in this group could be explained by the ability of PRV to spread from the primary replication site associated to leucocytes, lymph and throughout neurons. Specifically, PRV can access to endothelial cells from blood monocytes by fusion of plasmatic membrane in the process of tissue colonization.

In addition, we studied the immune response elicited by vaccination. Generally speaking, our recombinant PRV induces an immune response similar to that generated by the commercial vaccine used in our experiment or that reported by others after immunization with gE-, TK- or gI-/gE-/TK- strains or PRV. However, it is remarkable that the group of pigs exposed to a higher dose of gIp2, i.e. group A, experienced a marked increase in the titer of neutralizing antibodies after the second immunization, to the point that a significantly higher mean titer of neutralizing antibodies was observed in this group at the time of challenge, compared to the other two immunized groups. On the contrary, the titer of neutralizing antibodies increased only moderately in this group after challenge while an abrupt increase was observed in pigs from groups B and C. The different patterns observed in the development of neutralizing antibodies could be related to protection. Although we do not know the reasons for the differences observed, our hypothesis is that the higher antigen mass to which pigs of group A were exposed for immunization might have induced a higher titer of neutralizing antibodies before challenge. Then, the high titer of neutralizing antibodies present at challenge might have caused a more efficient blockage of PRV infection, which would have led to a significant reduction in the viral replication in the primary replication sites and a reduction in the amount of antigen present in the host, which, in turn, might have caused a lower stimulation of the immune system and might explain the moderate increase of neutralizing antibodies observed after challenge.

Finally, it was expected that antibodies against gE protein of PRV could not be detected before challenge and the verification of this property validates the suita-

bility of gIp2 as a MLV candidate because it allows to discriminate between vaccinated and infected animals. However, gE seroconversion after challenge was expected because the pigs were exposed to a tremendous amount of virulent PRV to demonstrate the efficacy of the tested vaccines.

In summary, the results of our study shows that immunization of 3-week old piglets with the recombinant PRV gIp2 is safe and induces a immunity capable of conferring protection against PRV infection similar to that conferred by commercially available MLV vaccines. Moreover, a better protection is achieved when a higher dose of gIp2 is used in the immunization protocol, probably because of a stronger stimulation of the immune system that induces higher titers of neutralizing antibodies at challenge, which are capable of protecting more effectively against pseudorabies infection.

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References

ARELLANO P.E., MORRISON R.B., MOLITOR T.W., THAWLEY D.G., 1992. High- and low-challenge exposure doses used to compare intranasal and intramuscular administration of pseudorabies virus vaccine in passively immune pigs. J Am Vet Med Assoc 200, 685-691.

BRACK A.R., KLUPP B.G., GRANZOW H., TIRABASSI R., ENQUIST L.W., METTENLEITER T.C., 2000. Role of the cytoplasmic tail of pseudorabies virus glycoprotein E in virion formation. J Virol 74, 4004-4016. doi:10.1128/JVI.74.9.4004-4016.2000.

DE BRUIN M.G., DE VISSER Y.E., KIMMAN T.G., BIANCHI A.T., 1998. Time course of the porcine cellular and humoral immune responses in vivo against pseudorabies virus after inoculation and challenge: significance of in vitro antigenic restimulation. Vet Immunol Immunopathol 65, 75-87. doi:10.1016/S0165-2427(98)00175-5.

DONALDSON A.I., WARDLEY R.C., MARTIN S., HARKNESS J.W., 1984. Influence of vaccination on Aujeszky's disease virus and disease transmission. Vet Rec 115, 121-124.

FAVOREEL H.W., VAN DE WALLE G.R., NAUWYNCK H.J., METTENLEITER T.C., PENSAERT M.B., 2003. Pseudorabies virus (PRV)-specific antibodies suppress intracellular viral protein levels in PRV-infected monocytes. J Gen Virol 84, 2969-2973. doi: 10.1099/vir.0.19281-0.

FERNANDEZ A., MENENDEZ DEL CAMPO A.M., FERNANDEZ S., CAMACHO A., CASTRO J.M., TABARES E., 1999. Conversion of US3-encoded protein kinase gene from pseudorabies virus in a diploid gene located within inverted repeats by genetic recombination between the viral genome isomers. Virus Res 61, 125-135. doi:10.1016/S0168-1702(99)00035-0 .

FERRARI M., BRACK A., ROMANELLI M.G., METTENLEITER T.C., CORRADI A., DAL M.N., LOSIO M.N., SILINI R., PINONI C., PRATELLI A., 2000a. A study of the ability of a TK-negative and gI/gE-negative pseudorabies virus (PRV) mutant inoculated by different routes to protect pigs against PRV infection. J Vet Med B Infect Dis Vet Public Health 47, 753-762. doi:10.1046/j.1439-0450.2000.00407.x.

FERRARI M., GUALANDI G.L., CORRADI A., MONACI C., ROMANELLI M.G., LOSIO M.N., CANTONI A.M., PRATELLI A., 2000b. The response of pigs inoculated with a thymidine kinase-negative (TK-) pseudorabies virus to challenge infection with virulent virus. Comp Immunol Microbiol Infect Dis 23, 15-26. doi:10.1016/S0147-9571(99)00019-3 .

FERRARI M., MARRUCHELLA G., MORE-MARSAN P., SONCINI M., BARIGAZZI G., 2000c. Properties of a thymidine kinase (TK) negative and glycoproteins I/E (gI/gE) negative mutant of pseudorabies virus. Proc. 16th International Pig Veterinary Society Congress. Melbourne, Australia, pp. 557-557.

FERRARI M., METTENLEITER T.C., ROMANELLI M.G., CABASSI E., CORRADI A., DAL M.N., SILINI R., 2000d. A comparative study of pseudorabies virus (PRV) strains with defects in thymidine kinase and glycoprotein genes. J Comp Pathol 123, 152-163. doi:10.1053/jcpa.2000.0406.

GEENEN K., FAVOREEL H.W., OLSEN L., ENQUIST L.W., NAUWYNCK H.J., 2005. The pseudorabies virus US3 protein kinase possesses anti-apoptotic activity that protects cells from apoptosis during infection and after treatment with sorbitol or staurosporine. Virology 331, 144-150. doi:10.1016/j.virol.2004.10.027.

HUSAK P.J., KUO T., ENQUIST L.W., 2000. Pseudorabies virus membrane proteins gI and gE facilitate anterograde spread of infection in projection-specific neurons in the rat. J Virol 74, 10975-10983. doi:10.1128/JVI.74.23.10975-10983.2000.

KIMMAN T.G., DE WIND N., DE BRUIN T., DE VISSER Y., VOERMANS J., 1994. Inactivation of glycoprotein gE and thymidine kinase or the US3-encoded protein kinase synergistically decreases in vivo replication of pseudorabies virus and the induction of protective immunity. Virology 205, 511-518. doi:10.1006/viro.1994.1672.

KIT S., 1990. Genetically engineered vaccines for control of Aujeszky's disease (pseudorabies). Vaccine 8, 420-424. doi:10.1016/0264-410X(90)90240-M.

LABARQUE G.G., NAUWYNCK H.J., MAES D.G., PENSAERT M.B., 1999. Protection of fattening pigs against challenge with Aujeszky's disease virus after a successive intranasal/intramuscular vaccination. Vet Q 21, 104-107.

MCFERRAN J.B., DOW C., 1975. Studies on immunisation of pigs with the Bartha strain of Aujeszky's disease virus. Res Vet Sci 19, 17-22.

MCGREGOR S., EASTERDAY B.C., KAPLAN A.S., BENPORAT T., 1985. Vaccination of swine with thymidine kinase-deficient mutants of pseudorabies virus. Am J Vet Res 46, 1494-1497.

MENGELING W.L., BROCKMEIER S.L., LAGER K.M., VORWALD A.C., 1997. The role of biotechnologically engineered vaccines and diagnostics in pseudorabies (Aujeszky's disease) eradication strategies. Vet Microbiol 55, 49-60. doi:10.1016/S0378-1135(96)01306-5.

MULLER T.F., TEUFFERT J., ZELLMER R., CONRATHS F.J., 2001. Experimental infection of European wild boars and domestic pigs with pseudorabies viruses with differing virulence. Am J Vet Res 62, 252-258. doi:10.2460/ajvr.2001.62.252.

NAUWYNCK H.J., 1997. Functional aspects of Aujeszky's disease (pseudorabies) viral proteins with relation to invasion, virulence and immunogenicity. Vet Microbiol 55, 3-11. doi:10.1016/S0378-1135(96)01299-0.

NAUWYNCK H.J., ZONNEKEYN V., PENSAERT M.B., 1997. Virological protection of sows upon challenge with Aujeszky's disease virus after multiple vaccinations with attenuated or inactivated vaccines. Zentralbl Veterinarmed B 44, 609-615.

NAUWYNCK H.J., FAVOREEL H.W., PENSAERT M., 2000. Functions of viral glycoproteins in the pathogenesis of Aujeszky´s disease virus in pigs. Vet Res 31, 116-117.

OGG P.D., MCDONELL P.J., RYCKMAN B.J., KNUDSON C.M., ROLLER R.J., 2004. The HSV-1 Us3 protein kinase is sufficient to block apoptosis induced by overexpression of a variety of Bcl-2 family members. Virology 319, 212-224. doi:10.1016/j.virol.2003.10.019.

PETROVSKIS E.A., TIMMINS J.G., GIERMAN T.M., POST L.E., 1986. Deletions in vaccine strains of pseudorabies virus and their effect on synthesis of glycoprotein gp63. J Virol 60, 1166-1169.

QUINT W., GIELKENS A., VAN O.J., BERNS A., CUYPERS H.T., 1987. Construction and characterization of deletion mutants of pseudorabies virus: a new generation of 'live' vaccines. J Gen Virol 68, 523-534.

REED J.J., MUENCH T.H., 1938. A simple method to estimating fifty per cent end points. Am J Hyg 27, 493-497.

TIRABASSI R.S., ENQUIST L.W., 1998. Role of envelope protein gE endocytosis in the pseudorabies virus life cycle. J Virol 72, 4571-4579.

TIRABASSI R.S., ENQUIST L.W., 2000. Role of the pseudorabies virus gI cytoplasmic domain in neuroinvasion, virulence, and posttranslational N-linked glycosylation. J Virol 74, 3505-3516. doi:10.1128/JVI.74.8.3505-3516.2000.

VAN DE WALLE G.R., FAVOREEL H.W., NAUWYNCK H.J., METTENLEITER T.C., PENSAERT M.B., 2003. Transmission of pseudorabies virus from immune-masked blood monocytes to endothelial cells. J Gen Virol 84, 629-637. doi:10.1099/vir.0.18796-0.

VAN ROOIJ E.M.A., HAAGMANS B.L., GLANSBEEK H.L., DE VISSER Y.E., BOERSMA W., BIANCHI A.T.J., 1999. DNA vaccine coding for glycoprotein B of PRV induces cytotoxic T cell responses in pigs against PRV. Virus Res 31, 132-133.

VANNIER P., 1985. Experimental infection of fattening pigs with pseudorabies (Aujeszky's disease) virus: efficacy of attenuated live- and inactivated-virus vaccines in pigs with or without passive immunity. Am J Vet Res 46, 1498-1502.

VILNIS A., SUSSMAN M.D., THACKER B.J., SENN M., MAES R.K., 1998. Vaccine genotype and route of administration affect pseudorabies field virus latency load after challenge. Vet Microbiol 62, 81-96. doi:10.1016/S03781135(98)00200-4.

YANG M., CARD J.P., TIRABASSI R.S., MISELIS R.R., ENQUIST L.W., 1999. Retrograde, transneuronal spread of pseudorabies virus in defined neuronal circuitry of the rat brain is facilitated by gE mutations that reduce virulence. J Virol 73, 4350-4359.

ZUFFA A., SALA J., CERNIK K., 1982. Immunity of pigs vaccinated by live or inactivated Aujeszky vaccines against experimental infection. Zentralbl Veterinarmed B 29, 663-675.