RESEARCH REPORT
SWINE HEALTH

Title: Do capsid mutants with the PCV-2 genotype variations induce virulence differences in vitro and in vivo? - **NPB # 08-268**

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Industry summary
The porcine circovirus of type 2 (PCV-2) is involved in porcine circovirus diseases such as post-weaning multisystemic wasting syndrome (PMWS), reproduction disorders, porcine dermatitis and nephropathy syndrome. All these disease cause a serious economic problem to the swine industry. PMWS outbreaks that occurred in 2005 in North America were assumed to be linked to the emergence of PCV-2 belonging to the PCV-2b genogroup. However, no clear evidence was found between this PCV-2b genogroup and a higher virulence. The objectives of the present work were to compare the virulence of a strain of PCV-2a genogroup to that of a strain of PCV-2b genogroup and to check if a specific signature of each genogroup located in the capsid protein, unique component of the external structure of the virus, could be a factor of virulence. The results showed that the PCV-2b strain was more virulent than the PCV-2a one and that modifications of the capsid motif attenuated the virulence. Altogether this findings put a new perspective on possible improvement when designing future PCV-2 vaccines.
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Scientific abstract
The porcine circovirus of type 2 (PCV-2) is the causal agent of post-weaning multisystemic wasting syndrome (PMWS) and is associated to other diseases grouped under the term porcine circovirus associated diseases. Two main genogroups a and b were identified during the outbreaks of PMWS in 2005 in North America, and it was speculated that PCV-2b isolates were more virulent than those of PCV-2a. This work was aimed to investigate if PCV-2 genetic variations were responsible of virulence and check if a motif specific of each PCV-2 genogroup located in the capsid protein contributes to a difference in virulence. Two clones, PCV-2a and PCV-2b, representative of the PCV-2 genogroups were produced as well as two mutants, PCV-2a (PCV-2b motif) and PCV-2a (PCV-2b motif) displaying an inversion of the capsid genogroup-specific motif into the backbone of the parental strains. Both clones and mutants were infectious *in vitro*. The experimental trial on SPF piglets demonstrated that the PCV-2b clone was more virulent than the PCV-2a one and that the PCV-2a (PCV-2b motif) was highly attenuated while the PCV-2b (PCV-2a motif) was moderately attenuated in virulence. These results suggest that the amino acids between positions 86 and 91 of the viral protein are involved in the virulence of PCV-2 and that this motif is thereby a putative biomarker of virulence between the two genotypes.

Introduction

Porcine circovirus of type 2 (PCV-2) is a member of the genus *Circovirus* and of the *Circoviridae* family. It is the aetiological agent of post-weaning multisystemic wasting syndrome (PMWS). This disease emerged in all pig-producing areas in the late 1990's and affects 5 to 13 week-old pigs. The characteristic clinical signs include wasting, dyspnea, lymph node enlargement and less frequently pallor and diarrhea. PCV-2 is also associated with other diseases including porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC) and reproductive failure (19). All these syndromes are now referred to as PCVD (Porcine Circovirus Diseases) or PCVAD (Porcine Circovirus Associated Diseases). However, PCV-2 needs infectious co-factors (PRRS, Parvovirus, etc…) or non-infectious ones (massive cross-fostering, mixing in post-weaning facilities, immunostimulation, etc…) to trigger PMWS. PCV-2 is a small icosahedral non-enveloped virus with a single stranded circular DNA genome of 1767 or 1768 nucleotides. The viral genome presents three major ORFs: ORF1 encodes Rep and Rep' essential for the viral replication, ORF2 the capsid protein and ORF3 a protein involved in apoptosis. The PCV-2 capsid protein is the sole component of the viral capsid, the external virus structure and is the main immunogenic protein of the virus (3) with at least three epitopes (17, 20, 26).

Genomic analysis of the PCV-2 showed a widespread distribution of the virus in the pig population and a high homology of the isolates either from PMWS-affected farms or from healthy ones (6). High similarities were also found among isolates found in archived tissues and current isolates. PCV-2 isolates clustered in two main genogroups a and b (6, 10, 15, 25). In 2005, North America has experienced outbreaks of PMWS with a high level of mortality. The sequencing of the PCV-2 showed that the viruses belong in majority to PCV-2b genogroup, which was absent before in these countries (5, 10). A shift from PCV-2a to PCV-2b genogroup is also observed in Europe (7, 27). Phylogenetic analyses revealed that all strains of each genogroup shared a distinct amino acid sequence (from 86 to 91) in the capsid protein. The capsid motif specific of PCV-2 genogroup is TNKISI for the PCV-2a genogroup and SNPRSV for PCV-2b genogroup.

The last PMWS outbreak and the shift of genogroup raise the question about the relationship between virulence and PCV-2 genogroup. The hypothesis of a higher virulence of PCV-2b strains remains a debate with conflicting results (14, 21). Despite all the research on the virus and on the disease, the PCV-2 factors of virulence are still unknown and particularly those linked to the only protein involved in the capsid structure, the Cap protein.

Objectives

The objective of the study was to assess if PCV-2 genetic variability is responsible of virulence variations and more precisely if the capsid motif specific of each PCV-2 genogroup is involved in the virulence.

Materials and methods

Cell and virus

Circovirus-free pig kidney cell line (PK15) was grown in EMEM containing 5% foetal calf serum and penicillin /streptomycin sulphate (100U/mL each) at 37°C in the presence of 5% (v/v) CO_2.

The PCV-2a (23) and PCV-2b (GenBank accession number AF201311) (12) isolates were used in this study and both are able to induce diseases *in vivo* (PMWS or reproduction disorders).

Construction of the PCV-2a parental infectious clone

The construction of PCV-2a infectious clone was carried out in two stages. A long fragment corresponding to almost the complete genome of PCV-2a was amplified by PCR (primers PCV-2a I for and PCV-2a I rev in Table I) from DNA extracted from PCV-2a infected PK15 cells and cloned into the PCR4 vector (Invitrogen). In parallel, the PCV-2a II forward and reverse primers (Table I) were used to amplify the Rep gene by PCR and next cloned into the same vector (Invitrogen). Both clones were subsequently digested by the *Nde*I and *Not*I enzymes. The Rep gene purified on

agarose gel, was then ligated with the linearized plasmid with the PCV-2a long fragment. The ligation product was used to transform electro-competent (XL1 blue) bacteria. The integrity of the 1.5 copies of the PCV-2a genome was confirmed by sequencing.

Construction of the PCV-2b parental infectious clone

The PCV-2b parental infectious clone was constructed from a previous PCV-2b infectious clone composed of two copies of PCV-2b genome cloned in tandem in the pBluescript vector (12). A couple of primers (PCV-2b I for and PCV-2b I rev in Table I) were designed in the Rep gene allowing the amplification of 0.5 and 1.5 copies of the PCV-2b genome from the PCV-2b dimer clone. The fragment of 1.5 copies was then purified on 1% agarose gel and cloned subsequently into the PCR4 vector (Invitrogen) in order to obtain the PCV-2b infectious clone with one copy of the Cap gene. The sequence was checked by sequencing the whole PCV-2b sequence.

Construction of the two mutants of the genogroup-specific capsid motif

The sequences of each PCV-2 infectious clones were used as a template for the design of primers. The mutants were obtained by site-directed mutagenesis in one or two steps using the QuikChange® II site-directed mutagenesis kit (Stratagene) according the manufacturer's instructions. The basic procedure uses a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation i.e. the nucleotide substitutions encoding the amino acid modifications in the genotype-specific motif. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended by PCR in order to generate a mutated plasmid. Following that step, the product is treated with *Dpn*I endonuclease that digests the methylated and hemimethylated parental DNA template. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue competent cells. The primers used for the mutagenesis are listed in Table II. Each mutant clone was checked by sequencing the 1.5 copies of the insert.

***In vitro* transfection of PK15 cells**

The infectivity of the two parental clones and of the two mutants clones *in vitro* was assessed by transfecting PK15 cells and then by infecting new fresh PK15 cells with the supernatants of transfection. PK15 cells were grown in 6-well plates until 80% of confluency and then transfected with 1µg of DNA using Lipofectamine 2000 formulation (Invitrogen), according to the manufacturer's recommendations. Mock-transfected cells with GFP-pcDNA3.1 vector and pcDNA3.1 were included as controls. The supernatants were collected 24h post-transfection and stored at -80°C. The cells were fixed 24h after transfection in 80% cold acetone for 10 min at -20°C. Cells expressing the capsid of each parental clones and of each mutant clones were revealed by immunoperoxydase monolayer assay (IPMA) with polyclonal anti-PCV-2b antibody. The number of transfected cells is determined by counting fluorescent cells 24 hours after transfection of PK15 cells with the plasmid expressing the green-fluorescent protein (GFP-pcDNA3.1).

Detection of PCV-2 antigens and of infectious virus

PCV2 titration was assessed by an immunoperoxidase monolayer assay (IPMA) using a protocol described previously (12) with a pig anti-PCV-2b polyclonal serum. The viral titers of the collected supernatants as well as in several organs (tonsils, tracheo-bronchial and inguinal lymph nodes) were estimated by 10-fold serial dilutions according the Kaerber's method (13).

Experimental inoculation of SPF piglets with parental infectious clones and of mutants

Fourty 6-week-old Large White specific pathogen-free (SPF) piglets were used, derived from the AFSSA SPF herd (4).

They were randomly divided into 6 groups housed in 5 separated rooms of the air-filtered level-3 biosecurity-facilities, each room containing 2 pens of 4 pigs. Control piglets (group 1) and control immuno-stimulated piglets (group 2) were in the same room but in separate units. Except the

control group 1, all the animals were immuno-stimulated with 2 mg of keyhole limpet hemocyanin emulsified in 4 mL of incomplete Freund adjuvant (KLH/ICFA) at 2 and 7 days post-inoculation (dpi) by intramuscular (IM) injection in four sites (both sides of the neck and both hips). The animals of groups 3 to 6 received 400µg of DNA by the IM route of parental clones: PCV-2a in group 3 or PCV-2b in group 4; or of mutant clones: PCV-2a (PCV-2b motif) in group 5 or PCV-2b (PCV-2a motif) in group 6. Pigs were daily examined for clinical signs (rectal temperature, cough, individual weight, feed consumption) until the end of the experiment at 35 dpi. Blood samples were weekly taken for PCV-2 serology and real-time PCR. One pig that received PCV-2b DNA died at 14 dpi. The necropsy revealed an uroperitoneum due to a rupture of the bladder. Euthanasia of the other pigs was carried out between 35 and 40 dpi by anaesthesia followed by exsanguinations and all the pigs were necropsied for examination of organs. Tissue samples (tracheo-bronchial, inguinal, mesenteric, and axillary lymph nodes; tonsil, spleen, ileum, lung and liver) were collected for laboratory investigations and stored at -80 °C. The experiment was performed in accordance with EU and French regulations on animal experimentation.

Macroscopic lesions and histo-pathology

At necropsy, the macroscopic lesions were assessed. Enlargement of lymph nodes were scored from 0 (normal) to 3 (severe). Samples from tracheo-bronchial and inguinal lymph nodes, tonsils, lung and liver were fixed in formalin for histological examination. The microscopic lesions were assessed on haemalumeosin-safranin stained tissue sections. The lymphocyte depletion and presence of inflammation were assessed in lymphoid organs and scored as explained above. The severity of the microlesions in lung and liver were equally estimated. The presence of PCV2 antigens was detected by immunochemistry on fixed tissue sections (tracheo-bronchial and inguinal lymph nodes, tonsils, lung and liver) as previously described (12) using a rabbit anti-PCV-2 polyclonal serum.

PCV-2 serology and quantitative PCR

Serum samples were tested for PCV-2 antibodies by a PCV-2 specific ELISA (Blanchard et al, 2003). The number of PCV-2 genomes was assessed in serum and in lymphoid organs (tonsils, tracheo-bronchial and inguinal lymph nodes) by a real-time PCR based on TaqMan technology according to a procedure already described (12). The primers and the probe used in this quantitative PCR allowed the amplication of the fragment of 64 base pairs in the PCV-2 capsid gene of both constructions. Only the reverse primer presents a mismatch of two bases with the PCV-2a sequence.

Statistical analysis

PCV-2 antibody responses, lesion scores and infectious PCV-2 titers were analyzed by the nonparametric U-test of Mann and Whitney using the SYSTAT 9 computer software package (SPSS Inc., Richmond, CA, USA). The PCV-2 genomic loads in serum and organs were statistically analyzed using an adapted method which associates a Bernouilli model for the zero values and a log normal model for the positive values (23).

Results

PCV-2 parental clones and PCV-2 mutant clones expressed capsid antigens and are infectious *in vitro*.

Two parental clones representative of PCV-2 genogroups a and b, with only one copy of the capsid gene were constructed and transfected in PK15 cells. IPMA revealed that 15 to 20% of the PK15 cells transfected were positive 24h after transfection for PCV-2 antigens in the nucleus of the cells.

Two PCV-2 capsid mutants, PCV-2a (PCV-2b motif) and PCV-2b (PCV-2a motif) mutants, were produced by exchanging in the parental clones the capsid motif specific of the genogroup. After transfection of both mutants, 10 to 15% of the cells expressed viral antigens. However the staining differed in IPMA-positive cells transfected with each mutants. PCV-2 antigens were detected in the cytoplasm and the nucleus with the other PCV-2a (PCV-2b motif) DNA while they were in the nucleus with the PCV-2b (PCV-2a motif) DNA. In all the tranfection tests, neither the cells transfected with pcDNA3.1 nor those transfected with pcDNA3.1-GFP were detected positive by IPMA.

Both PCV-2 parental clones are able to produce viral proteins 24h after transfection as well as both capsid mutant clones. However the localization of the detection of viral proteins vary with one mutant, the PCV-2a (PCV-2b motif).

The ability of parental clones to generate infectious virus was assessed. The supernatants of transfected cells were collected 24h after transfection and put on PK15 cells. If PCV-2 antigens are detected by IPMA in some of these cells, it means that the cells were infected by infectious viral particles present in the supernatant.

The supernatants from mock-transfected cells revealed no detection of PCV-2 antigens (Fig.1A). Several cells infected with supernatants of both PCV-2 parental clones showed PCV-2 antigens in their nucleus (Fig.1B and 1C). Thus, both parental PCV-2a and PCV-2b clones are infectious *in vitro* and able to produce viral infectious particles.

Supernatants recovered from PK15 cells transfected with PCV-2a (PCV-2b motif) mutant allowed the infection of fresh PK15 cells as IPMA-positive cells were observed (Fig.1D). Nevertheless, the number of infected cells was lower than the number of cells infected with both PCV-2 parental clones. More infected cells were noted with the supernatants collected after transfection of the PCV-2b(PCV-2a motif) mutant (Fig1E) at the same level as with the PCV-2a parental clone. Thus, the two PCV-2 mutants generate infectious particles *in vitro* but at a lesser rate for the PCV-2a (PCV-2b motif) mutant compared its PCV-2a clone parent.

Clinical and post-mortem findings

None of the control or inoculated pigs showed signs of clinical PMWS. Nevertheless, several pigs presented hyperthermia (rectal temperature> 40°C) in the immunostimulated group 2 and in all the inoculated groups mostly between 8 and 14 dpi. The hyperthermia could be due to the immunostimulation used in the trial. However, the mean of rectal temperatures was significantly higher in the PCV-2b (PCV-2a motif) group 6 ($p<0.05$) than in all the other groups during the second and third week post-inoculation (data not shown). Compared to the non-immunostimulated control piglets (group 1), the daily weight gain decreased in all the other groups in the second and third weeks post-inoculation but with no differences between the immunostimulated groups (data not shown).

Macroscopic lesions were not observed in the control group 1 (Table III).

Mild to moderate hypertrophy of tracheo-bronchial and inguinal lymph nodes were noted in all the immunostimulated groups. In spite of this, the number of pigs displaying these gross lesions differed in the different groups (Table III). Three out of 4 immunostimulated control pigs showed a mild to moderate increase of size of tracheo-bronchial lymph nodes (TB LN) with a mean score of 0.625. Comparable results were obtained with the pigs inoculated with both mutants. Four out of eight pigs had these lesions in the PCV-2a (PCV-2b motif) group (score: 0.625 on average) and

6/8 in the PCV-2b (PCV-2a motif) group (score: 0.875 on average). In the groups that received the parental clone, hypertrophy of TB LN affected all the animals with an increase of the severity of the lesions compared to the other groups ($p<0.05$). Only the PCV-2 parental clones induced significant gross lesions in TB LN.
Histo-pathological analyses revealed no microscopic lesions in the control animals.
Microscopic lesions in the immunostimulated animals consisted of mild hepatitis, moderate to severe interstitial pneumonia and mild to moderate lymphocyte depletion with histiocytic infiltration (data not shown). The differences in severity of the lesions observed through histology were not significant between all immunostimulated groups.
The presence of viral antigens was assessed by IHC in the organs of all animals. Viral antigens were not detected in all animals of the two control groups 1 and 2 as well as in the animals inoculated with one mutant, the PCV-2a (PCV-2b motif) clone. On the opposite, the majority of the pigs that received the parental clones and the second mutant, PCV-2b (PCV-2a motif), were positive by IHC.

Serological results and PCV-2 genomic loads in the serum of the pigs
The eight control pigs, immunostimulated (group 2) or not (group 1) did not present PCV-2 antibodies until the end of the experiment (Table IV). The animals inoculated with both parental PCV-2a (group 3) and PCV-2b (group 4) were first seropositive at 21 dpi (Table IV). All the piglets seroconverted between 28 and 35 dpi in the PCV-2a group 3 and one week earlier in the PCV-2b group 4. In the PCV-2b (PCV-2a motif)-transfected group 6, the same seroconversion profile is observed as in the parental PCV-2b-transfected group with 6/8 seropositive animals at 21 dpi and 8/8 between 21 and 28 dpi. On the contrary, all the animals that received the other PCV-2a (PCV-2b motif) mutant DNA remained PCV-2 seronegative throughout the *in vivo* study.

Viral genomic loads were assessed in sera each week by quantitative real-time PCR (Table V). No viral genomes could be detected in the control groups 1 and 2. The first detection of viral genomes in the PCV-2a group 3 was found in 5/8 animals at 14 dpi, one week earlier than the first seroconversions, with a mean of the genomic loads in this PCV-2a group of $10^{4.8}$ copies/ml (Table V). Only 3 of these 5 pigs showed viral genomes from 14 dpi until the end of the experiment (mean of the group equal to $10^{3.9}$ copies/ml at 35 dpi). The piglets inoculated with PCV-2b clone (group 4) were all PCR positive in the serum with $10^{6.8}$ copies/ml on average as soon as 14 dpi and until 35 dpi. In the PCV-2b (PCV-2a motif) mutant group, the maximum of PCR positive serum was noticed at 14 dpi with a mean of $10^{4.1}$ copies/ml. However, the presence of viral genome was transient in this group. In contrast, no viral genomes was amplified in the serum in all pigs that received the PCV-2a (PCV-2b motif) mutant. These results are in line with the absence of seroconversion in these animals. Significant differences were evidenced between groups (Table V). Altogether, the PCR quantitative results were not different in PCV-2a group 3 and the PCV-2b (PCV-2a motif) group 6 but statistically differed ($p<0.05$) from all the other groups. Moreover, the PCv-2b group 4 was also different of the PCV-2a (PCV-2b) group 6.

PCV-2 genomic loads and infectious viral titer in the organs of pigs
The presence of viral genomes (Fig.2-I) and infectious viral particles (Fig.2-II) was determined in the tracheo-bronchial lymph nodes and tonsils at the end of the trial (between 35 and 40 dpi) by quantitative PCR and IPMA respectively. The organs of all control piglets were negative with both techniques.
Viral genomes were detected in all pigs of the PCV-2a group 3 and the mean of the genomic load was $10^{8.3}$ copies/g of tissue (Fig.2-I). The other parental group PCV-2b showed higher loads than in the PCV-2a group ($p<0.05$) with $10^{10.6}$ copies/g of tissue on average. The animals inoculated with the PCV-2b (PCV-2a motif) had similar results as in the PCV-2a group with a mean of $10^{7.8}$ copies/g of tissue. The lowest average of the genomic load between all the groups ($p<0.05$) was observed in the PCV-2a (PCV-2b) mutant group 5 with $10^{6.1}$ copies/ g of tissue. Six out of the eight

piglets presented viral genomes in their organs demonstrating that the plasmid DNA was injected in those animals.
Infectious particles were detected in all pigs of the PCV-2a, PCV-2b and PCV-2b (PCV-2a motif) groups at equivalent levels with respectively $10^{4.4}$, $10^{4.6}$ and $10^{4.3}$ $TCID_{50}$/g of tissue on average (Fig.2-II). The differences noted for the genomic loads between parental group were not still observed. On the contrary, the infectious titers of the PCV-2a (PCV-2b motif) are in agreement with the viral genomic load in the organs. The mean of the infectious titers was $10^{2.7}$ $TCID_{50}$/g of tissue and significantly different from the other groups ($p<0.05$). Infectious particles were detected in the organs indicating that infections also occurred *in vivo* with both parental clones and both mutant clones.

Discussion and Conclusion

Several studies reported that PCV-2b genogroup was reported in North America only after the PMWS outbreak of 2005-2006 (5, 10). Each genogroup presents a particular signature in the capsid protein, localized between amino acids 86 and 91. This study aimed to assess if PCV-2 isolates representative of each PCV-2 genogroup differ in virulence and if the capsid genogroup motif was involved in virulence.
Two isolates representative of the main PCV-2 genogroups a and b were selected and two clones displaying 1.5 copies of PCV-2a and PCV-2b genomes were constructed. The capsid motif specific of each genogroup was inverted between both clones providing PCV-2a (PCV-2b motif) and PCV-2b (PCV-2a motif) mutants. The *in vitro* characterization of both PCV-2a and PCV-2b parental clones and of both PCV-2 capsid motif mutants revealed that they could produce viral antigens after transfection and also infectious particles. We demonstrated that all the constructed clones were infectious *in vitro* like several PCV-2 clones and PCV-1 PCV-2 chimeric clones (8, 9, 12, 18, 22).
In vivo, the PCV-2b parental clone induced more severe gross lesions than the other PCV-2a clone and a slightly earlier seroconversion. The genomic loads were significantly higher in serum and in the lymphoid organs of PCV-2b clone than those of PCV-2a, while no difference were evidenced for the infectious titers in the organs. The pathogenicity of both virus were evaluated on several experiments. Such differences were also noted on pigs inoculated at the age of 8 months without immunostimulation (11). However, PMWS was reproduced on SPF piglet with the PCV-2b virus alone (1). A study reported that a PCV-2b infectious clone was infectious *in vivo* (18) and was able to trigger clinical PMWS in SPF piglet when PCV-2b clone and immunostimulation were combined (12). In this latter study, we reported for the first time the reproduction of clinical PMWS in an SPF immunostimulated piglet transfected by the intramuscular route with a pure inoculum of tandem-cloned PCV-2b DNA. The PCV2 genomic load found in the lymphoid tissues of the PMWS-affected piglet reached an average of 10^{14} genome copies/g of tissue at 32 dpi. In our present study, the maximum genomic load in organs was 10^{11} genome copies/g of tissue at 35 dpi in a pig inoculated with the PCV-2b clone which is the PMWS diagnosis threshold of 10^{11} genome copies/g of tissue proposed by Blanchard et al. (2).
PCV-2a clone was not previously tested for its virulence "*per se*". Nevertheless the PCV-2a virus was found to cause reproduction disorders when inoculated by the intra-uterine route (23) but failed to induce PMWS in piglets (24). Thus the two parental clones showed differences in virulence in experimental conditions like the infectious virus, the PCV-2b isolate showing a higher pathogenicity. On the other hand, this difference in virulence could be observed between isolates of the same genogroup (21). All these results suggest that a difference of virulence exists between PCV-2 isolate independently of the PCV-2 genogroup.
In this work, the two mutant clones were also characterized *in vivo* to check if the capsid motif specific of the genogroup could alter the virulence compared to their parent clones. All the parameters examined to define the virulence (macroscopic lesions, genomic loads in serum and in organs, infectious titers in organs) were weakened in pigs inoculated with the PCV-2b (PCV-2a motif) mutant in comparison to the pigs inoculated with the PCV-2b clone. This mutant showed

features close to the PCV-2a clone and was infectious *in vivo* and allowed the development of a PCV-2 immunogenic response. Similarly, the PCV-2b (PCV-2a motif) mutant presented a decrease in virulence relatively to the PCV-2a clone, its parent. But the introduction of the capsid motif specific of the PCV-2b genogroup potentially more virulent did not confer these high virulence properties of the genogroup to the recipient virus. No seroconversion, no genome copies in the serum and no viral antigens in the lesions were detected in the group inoculated with this PCV-2b (PCV-2a motif) mutant. Only slight genomic loads and infectious titers were revealed in some organs but not in all pigs. This data proved that the PCV-2a (PCV-2b motif) was also infectious *in vivo* but the produced virus may have difficulties to infect cells or to replicate. Recent studies reported antigenic differences of the capsid protein among several PCV-2 isolates of the PCV-2a and PCV-2b genogroups (16, 26). It has been suggested that the capsid motif specific of the genogroup could be involved in antigenic difference, and this can be supported by our results.
In conclusion, this study described a difference of virulence between two isolates of PCV-2a and PCV-2b genogroup. Moreover, the capsid motif specific of PCV-2 genogroup is a molecular marker of virulence and could be essential to produce well-structured infectious viral particles. Altogether this work could open on new perspectives when designing new PCV-2 vaccines.

This study will be presented at the 12th French virology congress (Journées francophones de Virologie, 18-19 March 2010, Paris, France), at the 4th annual meeting of Epizone, an EU network on epizootic diseases (7-10 June 2010, St Malo, France) and at the 21st International Pig Veterinary Society congress (18-21 July 2010, Vancouver, Canada).

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Table I: Primers used in this study for the construction of the parental infectious clones

Oligonucleotide name	Oligonucleotide sequence	Application
PCV-2a I for	CCATGCCCTGAATTTCCATA	Amplification of 1 PCV-2a genome copy
PCV-2a I rev	CCGTGGATTGTTCTGTAGCA	
PCV-2a II for	AAGTATTACCAGCGCACTTC	Amplification of the Rep gene of PCV-2a
PCV-2a II rev	ACCATTACGAAGTGATAAAA	
PCV-2b I for	ACAACGGAGTGACCTGTCTA	Amplification of 1.5 PCV-2b genome copy
PCV-2b I rev	ACCATTACGAAGTGATAAAA	

Table II: Primers used in this study for the construction of the mutant clones

Mutant name	Oligonucleotide sense	Oligonucleotide antisense
PCV-2a (half of PCV-2b motif) (TK/86/88/89/91/SP)	CCCCGGGAGGGGGGAGCAACCCAATCTCTATACCCTTT	AAAGGGTATAGAGATTGGGTTGCTCCCCCCTCCCGGGG
PCV-2a (PCV-2b motif) (TKII/86/88/89/91/SPRV)	GGGAGGGGGGAGCAACCCAAGGTCTGTACCCTTTGAATAC	GTATTCAAAGGGTACAGACCTTGGGTTGCTCCCCCCTCCC
PCV-2b (half of PCV-2a motif) (SP/86/88/TK)	CTTCCCCCAGGAGGGGGCACGAACAAGCGCTCTGTGCCCTTTGAA	TTCAAAGGGCACAGAGCGCTTGTTCGTGCCCCCTCCTGGGGGAAG
PCV-2b (PCV-2a motif) (SPRV/86/88/89/91/TKII)	CCCAGGAGGGGGCACGAACAAGATCTCTATACCCTTTGAATACTACAGAATAA	TTATTCTGTAGTATTCAAAGGGTATAGAGATCTTGTTCGTGCCCCCTCCTGGG

Table III: Macroscopic lesions observed in tracheo-bronchial lymph nodes

Group number	Inoculation	Number of pigs with hypertrophy of tracheo-bronchial lymph nodes (mean of the macrolesion score)
1	PBS	0/4 (0) [a]
2	PBS + immunostimulation	3/4 (0.625) [a]
3	PCV-2a DNA+ immunostimulation	8/8 (1.375) [b]
4	PCV-2b DNA + immunostimulation	8/8 (1.5) [b]
5	PCV-2a (PCV-2b motif) DNA + immunostimulation	4/8 (0.625) [a]
6	PCV-2b (PCV-2a motif) DNA + immunostimulation	6/8 (0.875) [ab]

[a, b] indicates significant differences ($p<0.1$) among groups

Table IV: Seroconversion to PCV-2 assessed by ELISA

Group number	Inoculation	Number of positive pigs/total pigs for the detection of PCV-2 antibodies					
		0 dpi	7 dpi	14 dpi	21 dpi	28 dpi	35 dpi
1	PBS	0/4	0/4	0/4	0/4	0/4	0/4
2	PBS + immunostimulation	0/4	0/4	0/4	0/4	0/4	0/4
3	PCV-2a DNA+ immunostimulation	0/8	0/8	0/8	5/8	7/8	8/8
4	PCV-2b DNA + immunostimulation	0/8	0/8	0/8	6/7	7/7	7/7
5	PCV-2a (PCV-2b motif) DNA + immunostimulation	0/8	0/8	0/8	0/8	0/8	0/8
6	PCV-2b (PCV-2a motif) DNA + immunostimulation	0/8	0/8	0/8	7/8	8/8	8/8

Table V: Viral genomic load in serum determined by PCV-2 quantitative PCR throughout the experiment

Group number	Inoculation	Number of positive pigs/total pigs (mean of the genomic load expressed in log_{10})						Prob a-bility
		0 dpi	7 dpi	14 dpi	21 dpi	28 dpi	35 dpi	
1	PBS	0/4	0/4	0/4	0/4	0/4	0/4	a
2	PBS + immuno-stimulation	0/4	0/4	0/4	0/4	0/4	0/4	a
3	PCV-2a DNA+ immuno-stimulation	0/8	0/8	5/8 (4.8)	4/8 (4.3)	3/8 (3.8)	3/8 (3.9)	b
4	PCV-2b DNA + immuno-stimulation	0/8	0/8	8/8 (6.8)	7/7 (5.8)	7/7 (5.9)	7/7 (6.9)	c
5	PCV-2a (PCV-2b motif) DNA + immuno-stimulation	0/8	0/8	0/8	0/8	0/8	0/8	a
6	PCV-2b (PCV-2a motif) DNA + immuno-stimulation	0/8	0/8	5/8 (4.1)	2/8 (3.1)	0/8	1/8 (4.2)	b

[a, b, c] indicates significant differences ($p<0.05$) among groups

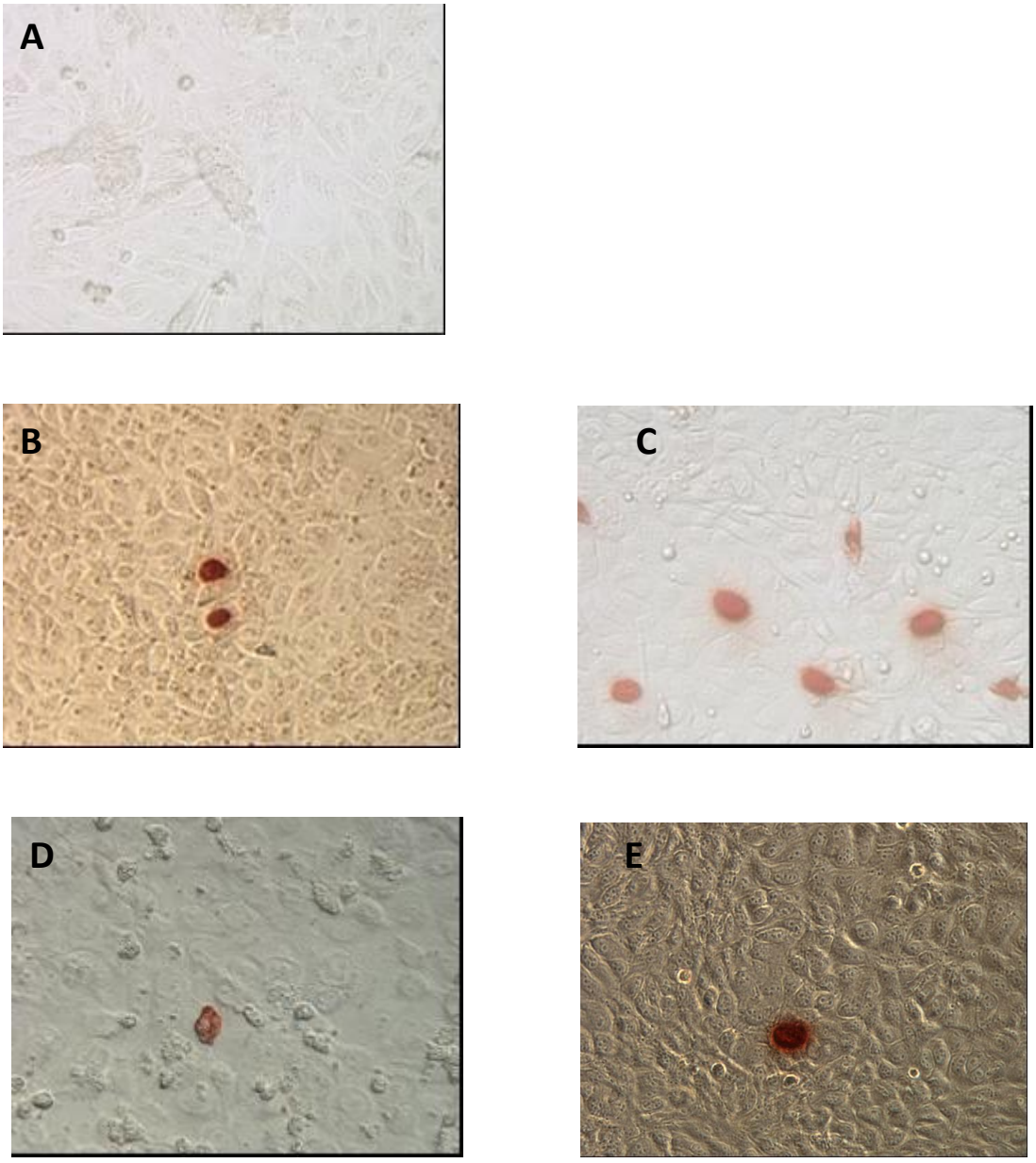

Figure 1: Assessment of viral infectivity after infection of PK15 cells with the supernatants of cells tranfected with the two parental clones: PCV-2a (B) and PCV-2b (C), and with the two mutant clones: PCV-2a (PCV-2b motif) (D) and PCV-2b (PCV-2a motif) (E) (x40 microscope objective).

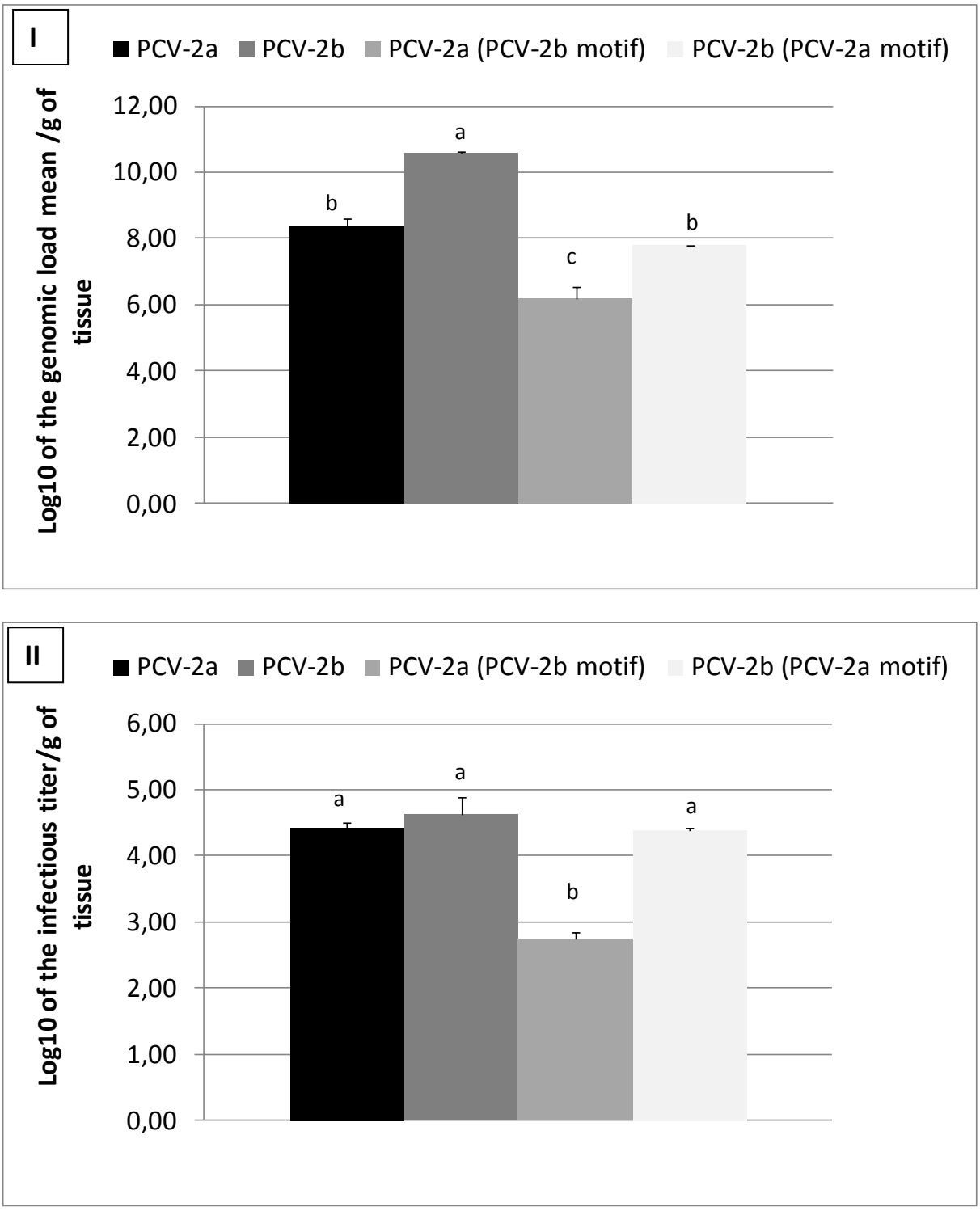

Figure 2: Means of the PCV-2 genomic loads (I) and infectious titers (II) for each inoculated groups, expressed in log $_{10}$, determined per gram of tissue (Tonsils, tracheo-bronchial and inguinal lymph nodes) at 35 dpi.
a, b, c indicates significant differences ($p<0.05$) among groups