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CA 2424400 C 2012/08/21

(11)(21) **2 424 400**

(12) **BREVET CANADIEN**
CANADIAN PATENT

(13) **C**

(22) Date de dépôt/Filing Date: 2003/04/03
(41) Mise à la disp. pub./Open to Public Insp.: 2003/10/05
(45) Date de délivrance/Issue Date: 2012/08/21
(30) Priorité/Priority: 2002/04/05 (EP02076334.8)

(51) Cl.Int./Int.Cl. *C12N 7/00* (2006.01), *A61K 39/12* (2006.01), *C07K 14/08* (2006.01), *C12N 15/40* (2006.01), *C12N 7/02* (2006.01), *C12N 7/04* (2006.01), *C12N 7/08* (2006.01), *G01N 33/543* (2006.01)

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(54) Titre : SITES D'ADAPTATION DU VSDRP
(54) Title: ADAPTATION SITES OF PRRSV

(57) Abrégé/Abstract:

The invention relates to the field of virology , more in particular to the field of vaccine production, more specifically to the in vitro propagation of virus, more specifically to the adaptation of virus to a cell line. The invention provides a method to determine the capability of an arterivirus to replicate in a green monkey cell line, comprising determining the amino acids at positions, which correspond to the amino acid positions 75-107 of GP2a of PRRSV isolate I-1102, more specifically amino acid position 88 and/or amino acid position 95 of said GP2a. The invention further discloses a method to produce arterivirus in green monkey cell line wherein the virulence of said arterivirus is maintained, with increased virus yield. The invention further provides a method to determine the attenuation of an arterivirus, comprising determining the amino acids at positions, which correspond to the amino acid positions 121-148 of GP5 of PRRSV isolate I-1102, more specifically amino acid 136 of said GP5 and/or determining the amino acids at positions, which correspond to the amino acid positions 651-675 and/or amino acid positions 2331-2355 of ORF1ab of PRRSV isolate I-1102, more specifically amino acid 663 and/or 2343 of said ORF1ab. The invention also provides a method to attenuate the virulence of said arterivirus by changing said amino acids.

Abstract

The invention relates to the field of virology , more in particular to the field of vaccine production, more specifically to the *in vitro* propagation of virus, more specifically to the adaptation of virus to a cell line.

The invention provides a method to determine the capability of an arterivirus to replicate in a green monkey cell line, comprising determining the amino acids at positions, which correspond to the amino acid positions 75-107 of GP2a of PRRSV isolate I-1102, more specifically amino acid position 88 and/or amino acid position 95 of said GP2a. The invention further discloses a method to produce arterivirus in green monkey cell line wherein the virulence of said arterivirus is maintained, with increased virus yield.

The invention further provides a method to determine the attenuation of an arterivirus, comprising determining the amino acids at positions, which correspond to the amino acid positions 121-148 of GP5 of PRRSV isolate I-1102, more specifically amino acid 136 of said GP5 and/or determining the amino acids at positions, which correspond to the amino acid positions 651-675 and/or amino acid positions 2331-2355 of ORF1ab of PRRSV isolate I-1102, more specifically amino acid 663 and/or 2343 of said ORF1ab. The invention also provides a method to attenuate the virulence of said arterivirus by changing said amino acids.

Title: Adaptation sites of PRRSV

The invention relates to the field of virology , more in particular to the field of vaccine production, more specifically to the *in vitro* propagation of virus, more specifically to the adaptation of a virus to a cell line and the attenuation of a virus.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small enveloped positive-stranded RNA virus which belongs to the genus arterivirus together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). This genus is the only member in the family of the Arteriviridae. This family together with the Coronaviridae are grouped into the order Nidovirales.

PRRSV was isolated in Europe and in the USA in 1991 (Collins et al., 1992, Wensvoort et al., 1991).

In vivo, PRRSV is able to reproduce primarily in porcine alveolar lung macrophages and blood monocytes (Wensvoort et al., 1991) Macrophages from other tissues, like heart, tonsil, spleen, turbinates, and choroid plexus, are also susceptible for PRRSV infection.

In vitro, the porcine virus can be passaged to primary cultures of porcine alveolar lung macrophages and blood monocytes, and to cultures of the African green monkey kidney cell line MA-104 and the derivative cell lines CL2621 and MARC-145 (Collins et al., 1992, Wensvoort et al., 1991) A few cell lines, like BHK-21 and Vero cells, are available in which the virus cannot penetrate, but once the genome is brought inside the cells artificially, is able to replicate and produce new virus particles (Meulenberg et al., 1998).

PRRSV enters porcine alveolar macrophages and MARC-145 cells via receptor-mediated endocytosis. The uptake of PRRSV by the host cell is a multi-step process, in which one or two viral constituents appear to interact with at least one host proteonic factor before virus-containing clathrin-coated pits are formed (Kreutz, 1998). The entry process of PRRSV into porcine macrophages differs from the binding and internalisation into MARC-145 cells. A membrane protein of 210-kDa has been identified as being a putative receptor for PRRSV on macrophages (Duan et al., 1998). A heparin-like molecule is suggested to be important for the binding of PRRSV to MARC-145 cells (Jusa et al., 1997).

PRRSV has a concise genome structure,(for review: Snijder, and Meulenberg, 1998). The genome of PRRSV, 15.098 nt in length without the poly-A tail, contains 9 open reading frames (ORFs), flanked by a 5' and 3' non-translated region (NTR) of 221, and 114 nucleotides, respectively. The two 5'-terminal ORFs (1a, and 1b) comprises about 75% of the genomic RNA. The structural region of the RNA contains 7 ORFs (2a, 2b, and 3 to7), and occupies the 3'-terminal third of the genome. ORF1a/b encodes the non-structural proteins (nsps) involved in replication of the RNA genome, and the forming of a 3' nested set of subgenomic RNAs (sgRNAs). Only the first two N-terminal proteins, named nsp1α and nsp1β, are studied for PRRSV. The functions of the other nsps are based on predictions, derived from sequence homologies with EAV. Nsp1α and nsp1β are papain-like cysteine proteases, and together with nsp2 and nsp4, a cysteine protease, and a serine protease, respectively, the entire polyprotein ORF1 (a/ab) is assumed to be cleaved into 12 nsps. Nsp9 and 10 are predicted to be the replicase subunits.

The 3'-terminal end of the genome encodes the structural proteins. The ORFs 2a to 6 encodes six structural membrane-associated proteins, the most 3'-terminal ORF encodes the N protein (ORF7). The minor glycoproteins are GP2a (formerly named GP2), GP3, and GP4. The GP2a and the GP4 proteins are class I membrane proteins, and have molecular masses of 27-30 and 31-35 kDa, respectively. Both N-glycosylated proteins are constituents of the virion. The other minor N-glycosylated structural protein of 42-50 kDa is GP3, encoded by ORF3, from which the nature remains unclear till today. The three major structural proteins are GP5, M, and the N protein. GP5, a N-glycosylated protein of 24-26 kDa, is the most variable protein in its sequence. The most conserved protein is the non-glycosylated M protein. This 18-19 kDa integral membrane protein seems to have a function in viral infectivity, forming a heterodimer with GP5, which is present in the virion. The N protein is a small highly basic nucleocapsid protein of 14-15 kDa , which interacts with the viral RNA during assembly (Snijder & Meulenberg, 1998).

Sequence comparisons as well as studies involving the antigenic nature of PRRSV strains revealed two distinct groups, designated as EU (European) and US (North American) strains (Wensvoort et al., 1992, Snijder & Meulenberg, 1998). Another indication for the observed diversity between strains from both continents is the fact that EU field isolates replicate initially easier in alveolar lung macrophages, whereas US isolates are recovered easier in African green monkey kidney cell line MA-104 or derivatives thereof like CL2621 or MARC-145 cells (Bautista et al., 1993). The reason why remains unclear till today.

The candidate protein(s) which will adsorb to the host cell surface of permissive cells are either GP2a, GP2b, GP3, GP4, GP5, the M protein or a heterodimer of GP5 and the M protein.

Usually, when viruses are replicated *in* vitro in cell lines that are derived from a different species, the viruses tend to attenuate in the process of adaptation to the cell line. This characteristic is widely used in vaccine development, and is certainly true for PRRS virus on monkey cell lines (Collins *et al.* 1992).

In a preferred embodiment some changes in PRRS virus genome that occur during the adaptation process are related to adaptation to a cell. This aspect is very useful because PRRS virus can be adapted to replicate in a permissive cell without further loss of virulence.

In another embodiment, changes in other sites of the genome are more related to attenuation. This aspect is very useful because it enables a person skilled in the art to control the level of attenuation of a PRRS virus.

Herein we disclose a method to determine the capability of an arterivirus to replicate in a permissive cell, preferably a green monkey cell, comprising determining the amino acids at positions, which correspond to the amino acid position 75-107 of GP2a of PRRSV isolate I-1102, preferably comprising determining the amino acid at a position, which corresponds to the amino acid position 88 and/or amino acid position 95 of GP2a of PRRSV isolate I-1102. Said knowledge and method will enable any person, skilled in the art, to identify the capability of a PRRS virus to replicate in a permissive cell, preferably a green monkey cell.

Furthermore, we disclose a method to increase the capability of an arterivirus to replicate in a permissive cell, preferably a green monkey cell comprising changing the amino acids at said amino acid positions 75-107, preferably comprising changing the amino acid at said amino acid position 88 from a valine to any other amino acid, preferably a phenylalanine and/or changing the amino acid at said amino acid position 95 from a phenylalanine to any other amino acid, preferably a leucine. This application further discloses that concomitant amino acid changes in the two said sites of GP2a enhance the adaptation of a PRRS virus for a permissive cell, preferably a green monkey cell. Said method is suited for the production of an arterivirus and/or a nucleic acid in a permissive cell and/or cell line wherein the virulence of said arterivirus is maintained and the virus and/or nucleic acid yield from said permissive cell and/or cell line is increased.

In another embodiment, this application teaches a method to determine the attenuation of an Arterivirus, comprising determining the amino acids at positions, which correspond to the amino acid positions 121-148 of GP5 of

PRRSV isolate I-1102, preferably the amino acid position which corresponds to the amino acid position 136 of GP5 of PRRSV isolate I-1102.
This application also teaches a method to control and/or increase the attenuation of an arterivirus comprising changing said amino acids at said amino acid positions 121-148 of GP5 of PRRSV isolate I-1102, preferably by changing said amino acid at amino acid position 136 from a cysteine to any other amino acid, preferably a tyrosine.

In another embodiment, this application teaches a method to determine the attenuation of an Arterivirus, comprising determining the amino acids at positions, which correspond to the amino acid positions 651-675 and/or amino acid positions 2331-2355 of ORF1ab of PRRSV isolate I-1102, preferably comprising determining the amino acid at a position, which corresponds to the amino acid position 663 and/or amino acid position 2343 of ORF1ab of PRRSV isolate I-1102.
This application also teaches a method to control and/or increase the attenuation of an arterivirus comprising changing the amino acids at positions, which correspond to the amino acid positions 651-675 and/or amino acid positions 2331-2355 of ORF1ab of PRRSV isolate I-1102, preferably comprising changing the amino acid at amino acid position 663 from a glutamic acid to any other amino acid, preferably a lysine, and/or changing the amino acid at amino acid position 2343 from a valine to any other amino acid, preferably an alanine. Said methods also enable the production of an attenuated arterivirus.

MATERIALS AND METHODS

Cells and viruses.

Porcine alveolar lung macrophages (PAMs) were maintained in MCA-RPMI-1640 medium containing 5% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. CL2621-cells were propagated in Eagle's minimal essential medium supplemented with Hanks salts (Gibco BRL), 10 % FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 1.5% sodium bicarbonate and 1% L-glutamine. Serial passage of the recombinant PRRS viruses vABV688 and vABV437 was performed as described before (Meulenberg et al, 1998). Challenge virus *LV-Ter Huurne*, a virulent European wild type isolate of PRRSV, was isolated during the 1991 epizootic from a clinical case of PRRS in the Netherlands and propagated on PAMs (Wensvoort et al, 1991). *SDSU#73,* a virulent American wild type isolate of PRRSV (passage 3 on CL2621-cells), was kindly provided by Dr. E. Vaughn (Boehringer Ingelheim, Animal Health, Ames, Iowa), and propagated on MA-104 cells. Virus titres (expressed as 50% tissue culture infective doses [$TCID_{50}$] per ml) were determined on PAMs by end point dilution (Wensvoort et al, 1986) and calculated according to Reed and Muench (Reed et al, 1938).

Inoculation of pigs with PRRSV recombinants and test on their *in vivo* stability.

Three groups of three 8-week-old Dutch Landrace/Yorkshire (LY) SPF pigs, tested free of antibodies against PRRSV, were inoculated on the same day intranasally with 2 ml of a recombinant virus stock of passage 5 each containing a titer of 10^5 $TCID_{50}$/ml. The groups were housed in isolated pens. Experimental procedures and animal management procedures were undertaken according to the Dutch legislation animal experiments. Serum samples were collected at day 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21. At day 21, the pigs were killed.

Immunization of pigs with vABV688 and challenge with *LV-Ter Huurne* and *SDSU#73.*

For each recombinant virus, two groups of five 8-week-old Dutch LY SPF pigs, lacking antibodies against PRRSV, were immunized intramuscularly (half-way between the pinna of the right ear and the cranial ridge of the right shoulder blade) with 2 ml of a virus stock of 10^5 $TCID_{50}$/ml. All groups were housed in isolated pens. Experimental procedures and animal management procedures were undertaken according to the Dutch legislation animal experiments. In order to determine the transmission of recombinant virus from these inoculated pigs, one naive sentinel pig was introduced into each group of inoculated pigs 24 hours post-vaccination and killed 28 days thereafter. At day 28 post-vaccination, two animals were separated from one group of each mutant and challenged intranasally with 2 ml 10^5 $TCID_{50}$/ml *LV-Ter Huurne*. Similarly, two animals were separated from the other group and challenged with 2 ml 10^5 $TCID_{50}$/ml *SDSU#73*. The two challenged animals joined the other three vaccinates after 24 hours. At 28 days after challenge, all pigs were killed.

To confirm the efficacy of challenge, two non-inoculated animals were either inoculated intranasally with 2 ml 10^5 $TCID_{50}$/ml *LV-Ter Huurne* or *SDSU#73*. These challenged animals joined three sentinel animals after 24 hours, and were monitored for two weeks starting at the moment of challenge. Serum samples were collected for all animals three times a week, starting on day 0. During the experiment, the animals were observed daily for signs of disease, i.e. fever (a rectal body temperature higher than 39,7°C), diarrhea and respiratory distress.

Analysis of the genetic stability of the recombinant viruses.

The genetic stability of the recombinant viruses in pigs was tested by inoculation of PAMs with serum from these pigs taken at the last virus-positive day, followed by sequence analysis of the viral RNA. In short, as soon as cytopathogenic effect (cpe) was detected, the culture supernatant was harvested and the viral RNA was isolated as described before [16]. The RNA was reverse transcribed with primer LV76 (5'-TCTAGGAATTCTAGACGATCG$(T)_{40}$-3'; antisense; nucleotide (nt) 15088). The region flanking the introduced mutations was amplified by PCR using primers

LV9 (5'-CTGCCGCCCGGGCAAGTGCC-3'; sense; nt 11746) and LV22 (5'-CATAATAACCCTCAAGTTG-3', antisense; nt 12715) for vABV688. Nt numbers are based on the sequence of the LV isolate, as deposited in GenBank (Accession number M96262). The amplified fragments were analyzed in 2% agarose gels, the PCR fragments were excised from the gel and purified with SpinX* columns (Costar). Sequence analysis of the fragments was performed using the antisense primer of the PCR, except for vABV688, for which primer LV24 (5'-AATCGGATCCTCAGGAAGCGTGCACACTGATGA-3'; antisense; nt 12419) was used. The PRISM* Ready Dye Deoxy Terminator cycle sequencing kit and the ABI PRISM 310* Genetic Analyzer (Perkin Elmer) were used to determine the sequences.

Virus isolation.

10^5 PAMs/ $96mm^2$ were seeded in 96-wells plates and after one day, 50 µl of a 10-fold and 100-fold serial dilution of each serum sample was used to infect PAMs. After 48 hours, 25 µl of the culture supernatant was transferred to new PAMs seeded 16-24 hours prior to incubation. After 24 hours, the medium was discarded; the cells were washed with 0.05M NaCl, dried and frozen for IPMA (Wensvoort, 1986).

Virus titration.

Virus titres were determined by end-point dilution on PAMs (Wensvoort et al, 1986). Samples containing virus titres below the detection level (1,8) were considered negative. From the area under the curve of titre against time the integrated virus titres after vaccination were determined. Statistical analysis was performed by the one-sided paired student's t-test. Results were considered statistically significant when the P-value was ≤ 0.05.

*Trade-mark

Immunoperoxidase monolayer assay (IPMA).

Immunostaining of PAMs was performed according to the method described (Wensvoort et al, 1986). The expression of both the EU- and US-PRRSV N protein was detected with monoclonal antibody (MAb) 122.

Detection of antibodies.

The presence of antibodies against PRRSV in pig sera was determined by ELISA (IDEXX*, Westbrook, Mn, US).

EXAMPLE 1

Identification of adaptation sites.

To identify important genomic regions for adaptation, the field isolate Lelystad virus was passaged for 6 times on CL2621 cells, followed by plaque purification for 3 times on the same cells. This virus was designated LV4.2.1. We determined the entire sequence of the genome of the cell line-adapted strain LV4.2.1. RNA isolation, RT-PCR, and PCR was performed, yielding overlapping cDNA fragments of about 1.5 kb in length. All fragments were cloned into pGEM-T vector of PROMEGA* using the TA-cloning strategy. When accomplishing the whole sequence, every nucleotide was determined at least twice. To exclude mutations introduced by PCR-mismatches a third or even a fourth cDNA-construct was made. When compared to the field strain Lelystad virus we identified 27 nucleotide differences in the coding region and none in the 5' and 3' non-translated region, resulting after translation in 8 amino acid differences (Table 1).

*Trade-mark

Table 1, Amino acid differences between LV4.2.1, the field virus Lelystad virus strain *Ter Huurne*, the infectious cDNA clone pABV437, and the US prototype field virus strain ATCC VR2332

	ORF1A polyprotein			GP2A			GP2B	GP5
	aa 663	aa 1084	aa 2343	aa 29	aa 88	aa 95	aa 27	aa 136
LV4.2.1	Lys	Leu	Ala	Ser	Phe	Leu	Val	Tyr
Ter Huurne	Glu	Pro	Val	Pro	Val	Phe	Ala	Cys
pABV437	Glu	Pro	Val	Ser	Val	Phe	Val	Cys
ATCC-VR2332	Gln	Thr	Val	Leu	Thr	Leu	Ile	Trp

We concluded that the observed difference in growth properties between LV4.2.1 and the field virus strain Lelystad virus was due to a different amino acid sequence in a non-structural or in a structural protein or in a combination of two or more amino acid changes.

Construction of full length cDNA with either non-structural or structural gene mutations.

A set of full length constructs (pABV647, 688. 689, and 690) was constructed, based on pABV437 in which mutations were introduced. *In vitro* transcribed full length RNAs were transfected into the non-permissive BHK-21 cells using lipofectin as a cationic reagent. After 24h the supernatants (p0) were transferred to MARC-145 cells and after 24 to 48h an immunostaining was performed using MAb 122.17 directed against the N protein to detect the effect of the introduced mutations. Recombinant viruses containing responsible substitutions for adaptation showed cell line adapted phenotypes on MARC-145 cells similar to that of the positive control LV4.2.1. For control, PAMs were infected with the same amount of p0 to show infection on the primary target cell of PRRSV. The supernatant p0 of pABV437, and LV4.2.1 virus served as positive controls in infection experiments.

We constructed two full length cDNA clones in which either mutations in the non-structural or in the structural region were introduced, pABV647 and pABV690, respectively (figure 1B). *In vitro* analysis of these two constructs revealed that the RNA-transcript of pABV690 resulted in a phenotype like LV4.2.1. We conclude that the mutations responsible for adaptation are located in the structural part of the genome. The next step was to determine whether the mutations in GP2a or in GP5 were of any effect to the observed cell line-adaptation. pABV688 contains the two amino acid differences in GP2a and pABV689 contains the only amino acid change in GP5 (figure 1B). After performing the screening assay, pABV688 with the two mutations in GP2a resulted in a cell line-adapted phenotype like LV4.2.1 and the GP5 recombinant pABV689 did not. We conclude that adaptation to the cell line is in majority caused by the two mutations in GP2a.

Full length genome constructs with mutations in GP2a.

Two amino acid differences in GP2a were studied. Two new constructs were generated, pABV772, which contains a phenylalanine at amino acid 88 and pABV773, which contains a leucine at position 95 in GP2a (figure 1B). *In vitro*, both substitutions were better adapted to growth in MARC-145 cells than the positive control pABV437, but caused less cell pathologic effects (CPE) than vABV688. A multi-step and a one-step growth curve was performed to investigate the effect of the introduced amino acid residues on the infectivity process. The p0-medium of the recombinant viruses derived from pABV437/688/772/773 were harvested and transferred to PAMs and passaged for three times (p3), parallel with LV4.2.1, to increase the amount of virus. For further growth characterisation, we confirmed the presence of the introduced mutations by sequence analysis and determined the TCID50/ml (data not shown). Multi-step growth curves on both PAMs and MARC-145 were performed using a multiplicity of infection (m.o.i). of 0.05 with vABV437/688/772/773 and LV4.2.1. An amount of 10^6 cells was used in growth curves per 2 cm^2 . At different time points, virus was harvested and stored until the virus titre in TCID50/ml was determined. Introduction of a phenylalanine and a leucine in GP2a at amino acid 88 and 95 respectively had a positive effect on the growth characteristics of a European strain of PRRSV on MARC-145 cells (Fig. 2A). No differences were observed when performing multi-step growth curves on PAMs (Fig. 2B). To secure the observed results, a total amount of 10^6 MARC-145 cells per 2 cm^2 were infected with a m.o.i. of 0.1 with supernatant p3 of vABV437/688/772/773 and LV4.2.1. After 12h an immunostaining was performed using a PRRSV-specific MAb (122.17) against the nucleocapsid protein, and the total amount of positive cells were counted per 2 cm^2 (Fig. 2C).

In conclusion both amino acid residues at position 88 and 95 in the minor glycoprotein GP2a of PRRSV are important for adaptation to MARC-145 cells.

When defining the arteriviral reproduction process in permissive cells seven steps can be distinguished. Attachment of the virus to the cell surface of PAMs or MA-104 (or derivatives thereof) (A), and penetration of the virus through the cell membrane (B) are the first two processes, also called the viral entry. The arterivirus needs to be uncoated in the cytoplasm (C), before replication, transcription and translation (D) can occur. RNA encapsidation and assembly (E), and (F) release of mature virions into the extracellular space are the processes before spread of the virus can take place (G). Using a 12h-time point in the one-step growth curves and knowing that the viral reproduction cycle of PRRSV takes about 10 hours, the observed difference in the amount of positive stained cells is caused by an increased number of cells being infected successfully (A-D). The observed difference is not due to a difference in encapsidation of the RNA or release of the newly produced virions or spread of the virus through a monolayer of MARC-145 cells (E-G). Since a role of glycoproteinGP2a in release of viral RNA into the cytoplasm, in uncoating (C), or in transcription and translation (D) is most unlikely, we conclude that both residues or a domain in which both are present are important for the entry process, i.e. attachment and/or penetration of the virus (A and B).

EXAMPLE 2

Animal experiments with cell line-adapted recombinant PRRSV vABV688.

In recombinant vABV688, amino acids 88 and 95 of the minor envelope glycoprotein GP2a were mutated, resulting in improved growth on MARC-145 cells. This characteristic facilitates the production of virus. In cell culture, these recombinant viruses were shown to be genetically stable, and grow to virus titers, which are sufficient to perform animal experiments. Here, we studied the properties of these PRRSV recombinants with regard to safety and protective efficacy in animal experiments. Their properties were compared with those of virus derived from an infectious cDNA copy, vABV437. This virus is identical to wild type virus, except for a *Pac*I-restriction site, and is therefore assumed to have similar properties as wild type virus. First, the *in vivo* genetic stability of vABV688 was determined in 8-week-old pigs. Subsequently, the immunogenicity, attenuation, and efficacy of these viruses were tested in a homologous and heterologous immunization-challenge experiment in young pigs.

Genetic stability of the PRRSV recombinant vABV688 in 8-week-old pigs.

In the first experiment, we determined the genetic stability of the PRRSV recombinants *in vivo*. Therefore, sequence analysis of viral RNA isolated from serum of pigs inoculated with the recombinant viruses at the last virus-positive day was performed. Of vABV688-inoculated pigs, this was for all inoculated pigs from 14 days post inoculation (dpi). Sequence analysis of the fragments obtained by RT-PCR showed that the introduced mutations were still present, and that no additional changes were introduced in said domain of GP2a, indicating that the recombinant viruses were genetically stable *in vivo*.

Seroconversion of virus inoculated pigs and sentinel pigs.

In the second experiment, we determined whether pigs inoculated with the PRRSV recombinants, and subsequently also the sentinel pigs introduced into the inoculated groups, had seroconverted. Therefore, the presence of PRRSV antibodies was measured in IDEXX ELISA. Antibodies were detected in pigs inoculated with recombinant virus (i.e., ELISA sample-to-positive (S/P) ratio > 0.4), which indicated proper exposure of the viruses to the animals. Virus transmission is one of the characteristics of virulence.

The sentinels had seroconverted at day 14 (vABV688), indicating transmission of virus from the inoculated animals to these sentinels and thus conservation of the virulence.

Duration and height of viraemia after inoculation.

To determine the presence of virus in the serum of inoculated animals, virus isolation was performed for all sera collected. From the virus positive sera, virus titers were determined (Fig. 3). All viruses induced viraemia in the pigs, ranging from 2 dpi till 25 dpi.

Viraemia after transmission of virus to sentinel pigs.

To determine spread of the recombinant virus to non inoculated pigs, one sentinel animal was introduced into each inoculated group at 24 hours after inoculation. The virus titration of all virus positive sera indicated that the maximum virus titres did not differ between inoculated pigs and sentinels (data not shown).

Challenge of virus-inoculated animals with homologous and heterologous PRRSV and transmission of challenge virus.

To test whether inoculation with the recombinant viruses protects pigs against wild type PRRSV, the inoculated pigs were challenged with EU- and US-PRRSV. Pigs inoculated with vABV437 remained virus negative after challenge with LV-*Ter Huurne*. Only one animal of the vABV688-inoculated pigs became virus positive for one day. After challenge with *SDSU#73*, all inoculated animals became viraemic. All inoculated, unchallenged animals remained virus negative after they were united with their group members that were challenged with LV-*Ter Huurne*. In pigs inoculated with vABV688, viraemia was induced after they were united with *SDSU#73*-challenged pigs. In pigs used as challenge control, viraemia was induced in all LV-*Ter Huurne* and *SDSU#73*-challenged pigs as well as in all sentinel animals (data not shown).

Clinical observations.

After inoculation of the viruses, no severe clinical signs of the disease were monitored. Only moderate rectal temperature increases were noted in all inoculated groups (data not shown). After challenge with LV-*Ter Huurne* in vABV688-inoculated animals and all *SDSU#73*-challenged animals, no temperature increases were measured.

EXAMPLE 3

Attenuation of PRRS mutant virus strains with mutations in GP5 and/or ORF1ab in addition to the mutations in GP2a (LV4.2.1). Comparison of virulence of 3 PRRS virus strains in an animal model.

The clinical signs of PRRS may differ among strains after exposure of pigs to

PRRSV: the US PRRSV strains often cause respiratory and reproductive problems, whereas EU PRRSV strains predominantly cause reproductive problems (Steverink *et al.*1999). Respiratory signs and the extent of lung involvement may vary per strain and are not consistently being reproduced under experimental conditions. An infection model should include parameters to study and quantify the virulence of the virus based on frequency and severity of clinical signs, viral parameters (viraemia, virus excretion and virus transmission) and seroconversion. Furthermore, duration, height, and frequency of viraemia and viral excretion after vaccination could also be important parameters to study vaccine safety,. The same parameters after PRRSV challenge of vaccinated animals are important for vaccination efficacy, since reducing virus shedding and virus transmission among pigs is an important target aiming at the control of PRRS. Therefore pigs of 6 till 8 weeks old and 6 months old were infected with 3 different PRRSV strains, i.e. LV ter Huurne, SDSU#73 and LV4.2.1.

The animals were conventional Landrace pigs, obtained from a PRRSV-free farm in Denmark and born from PRRSV-unvaccinated sows. The pigs were free from antibodies against PRRSV as measured by an ELISA (IDEXX). Viraemia was first detected in all pigs at 3 dpi except for the 6 month-old group infected with LV4.2.1 where it was first detected at 7 dpi and in one animal of this group not at all. In 6-8 week-old pigs viraemia was detected until 42 dpi with a maximum frequency of positive animals of 88% and a virus titre ranging at 3 or 4, 7, 10 or 11 and 14 dpi from 2.1-2.6. In 6 month-old pigs viraemia was detected until 28 dpi with a maximum frequency of 65% positive animals per time-point and virus titre ranging from 0.7-1. After 24 dpi, both LV *ter Huurne* and SDSU#73 were intermittently detected in the serum of pigs. LV4.2.1-infected pigs showed a shorter duration of viraemia, it was last detected at 21 dpi. The frequency of positive pigs at 7 and 10 dpi was significantly lower, had a maximum of 41% ($p \leq 0.05$), and a lower ^{10}log virus titre as compared to LV *ter Huurne* and SDSU#73. Kinetics of viraemia was significantly different at 14 dpi for LV *ter Huurne*-infected pigs and SDSU#73-infected pigs.

More pigs of 6-8 week-old excrete virus (maximum of positive animals per time-point was 50%), than pigs of 6 month-old (maximum of 37%). Furthermore, 6-8 week-old pigs showed a higher virus ^{10}log titre (between 0.2 and 1.9) in tonsillar swabs than 6 month-old pigs (ranging from 0.0 to 0.3) at day 3 or 4, 7, 10 or 11 and 14. Per time-point, more pigs infected with LV *ter Huurne* excrete virus (maximum 100%) as compared to LV4.2.1 (maximum 17%) and SDSU#73 (maximum 50%). In addition, all pigs infected with LV ter Huurne excreted virus, but not all pigs infected with LV4.2.1 or SDSU#73 excreted virus. The ^{10}log virus titre in tonsillar swabs was higher for *LV ter Huurne*-infected pigs ranging from 0.0 to 3.0, while the ^{10}log titre of the LV4.2.1 infected group was 0.0 and the ^{10}log titre of the SDSU#73 infected pigs was ranging from 0.0 to 0.5. At 14 dpi, pigs were still excreting virus.

LV *ter Huurne* and SDSU#73 appear to be the most virulent as a result of the occurrence of a high level of virus-positive pigs and a long duration of viraemia of both virus strains, the instigation of most severe clinical signs of SDSU#73, and a high level of virus excretion for LV *ter Huurne*. LV4.2.1 was less virulent, since it showed less clinical signs and a low viraemia and virus excretion. The reduced virulence of LV 4.2.1 was confirmed by impairment in its ability to cause reproductive problems for gestation sows as compared to LV *ter Huurne* (Steverink *et al.* 1999). Sows infected with LV *ter Huurne* and LV4.2.1 resulted in the same number of piglets, but the piglets of the sows infected with the higher virulent strain were weaker. In addition, the group infected with the higher virulent virus LV *ter Huurne*, showed a higher frequency of virus positive piglets until 28 days post farrowing (Steverink *et al* 1999). Apparently, the lower virulence of LV4.2.1 is not related to macrophage infection, since the growth curve of LV4.2.1 *in vitro* on macrophages is comparable to the growth curve of LV *ter Huurne*.

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Fig. 1:
Schematic representation of amino acid differences between the LV4.2.1 strain, which is adapted to MA-104 cells and pABV437, which is the infectious cDNA clone of the field strain Ter Huurne (A). Overview of generated full length constructs based on pABV437, to observe the responsible mutation(s) for the adaptation phenotype (B). Mentioned numbers in the bar corresponds to the 9 open reading frames.

Fig. 2A: Multi-step growth curves on MARC-145 cells. Virus titre in TCID50/ml.

Fig. 2B: Multi-step growth curves on PAMs. Virus titre in TCID50/ml.

Fig. 2C: Immunostaining of infected cells using a PRRSV-specific Mab (122.17) against the nucleocapsid protein. The total amount of virus positive cells was counted per 2 cm^2.

Fig. 3: Mean virus titre per group of pigs after inoculation with vABV688, vABV707, vABV746 and vABV437 as determined by end point dilution. Significance of the differences between the virus groups ($p \leq 0.05$) was determined using the Chi test.

SEQUENCE LISTING

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(v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC/DOS/MS-DOS
- (D) SOFTWARE: PatentIn version 3.1

(vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: 2,424,400
- (B) FILING DATE: 2003-04-03

(vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: EP 02076334.8
- (B) FILING DATE: 2002-04-05

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 61
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) ORGANISM: Artificial Sequence

(ix) FEATURE:
(A) OTHER INFORMATION: primer LV76

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTAGGAATT CTAGACGATC GTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT 60
T 61

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) ORGANISM: Artificial Sequence

(ix) FEATURE:
(A) OTHER INFORMATION: primer LV9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTGCCGCCCG GGCAAGTGCC 20

(2) INFORMATION FOR SEQ ID NO: 3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) ORGANISM: Artificial Sequence

(ix) FEATURE:
(A) OTHER INFORMATION: primer LV22

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) ORGANISM: Artificial Sequence

(ix) FEATURE:
- (A) OTHER INFORMATION: primer LV24

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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AATCGGATCC TCAGGAAGCG TGCACACTGA TGA                                   33
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CLAIMS:

1. A method to increase the capability of a European field porcine reproductive and respiratory syndrome (PRRS) virus to replicate in a green monkey cell, comprising changing the amino acid at amino acid position 88 of GP2a having SEQ ID NO: 5 from a valine to a phenylalanine, or changing the amino acid at amino acid position 95 from a phenylalanine to a leucine.

2. A method according to claim 1, wherein the virulence of the virus is maintained.

3. A method according to claim 1 or claim 2, to increase virus yield from said cell.

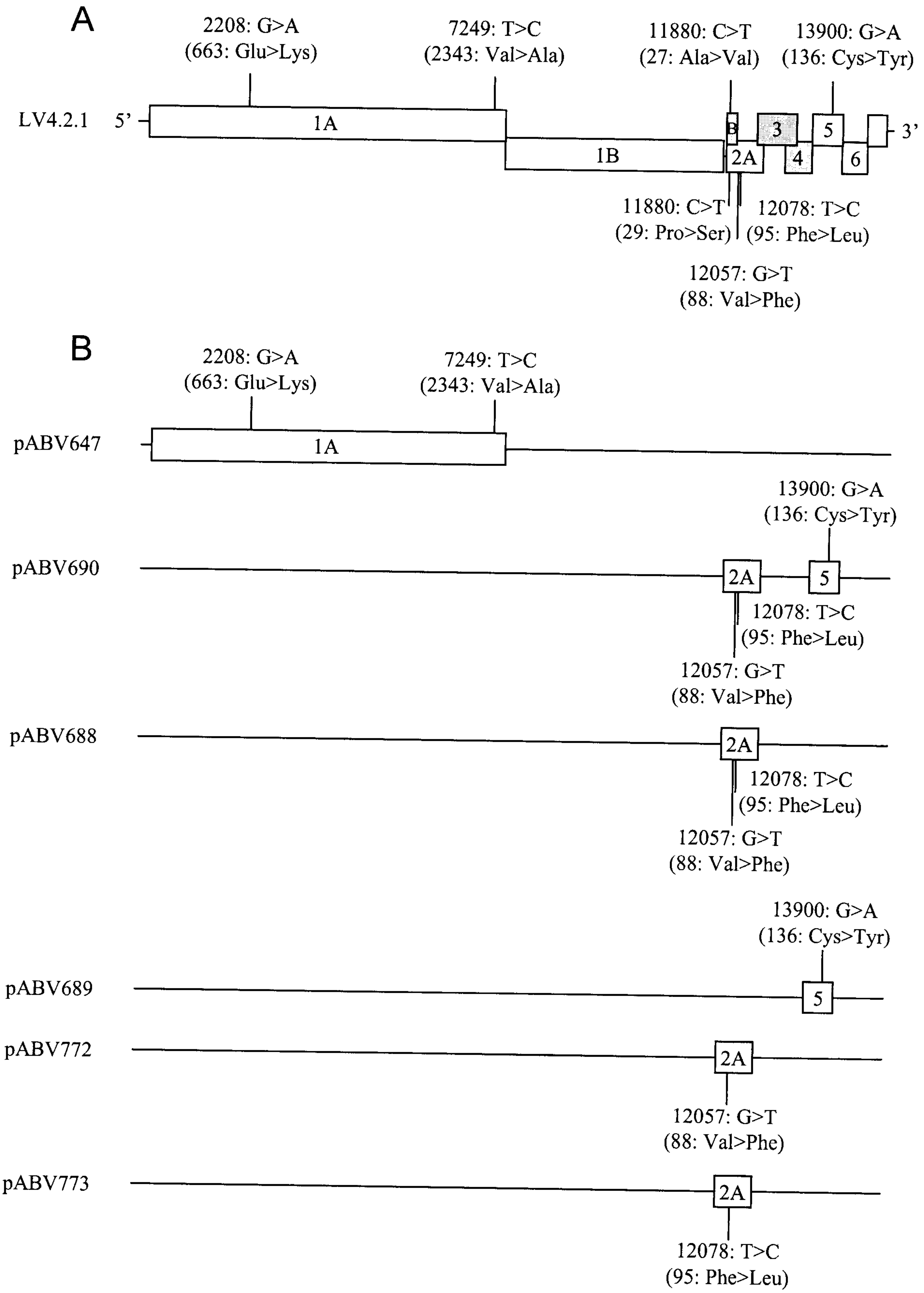
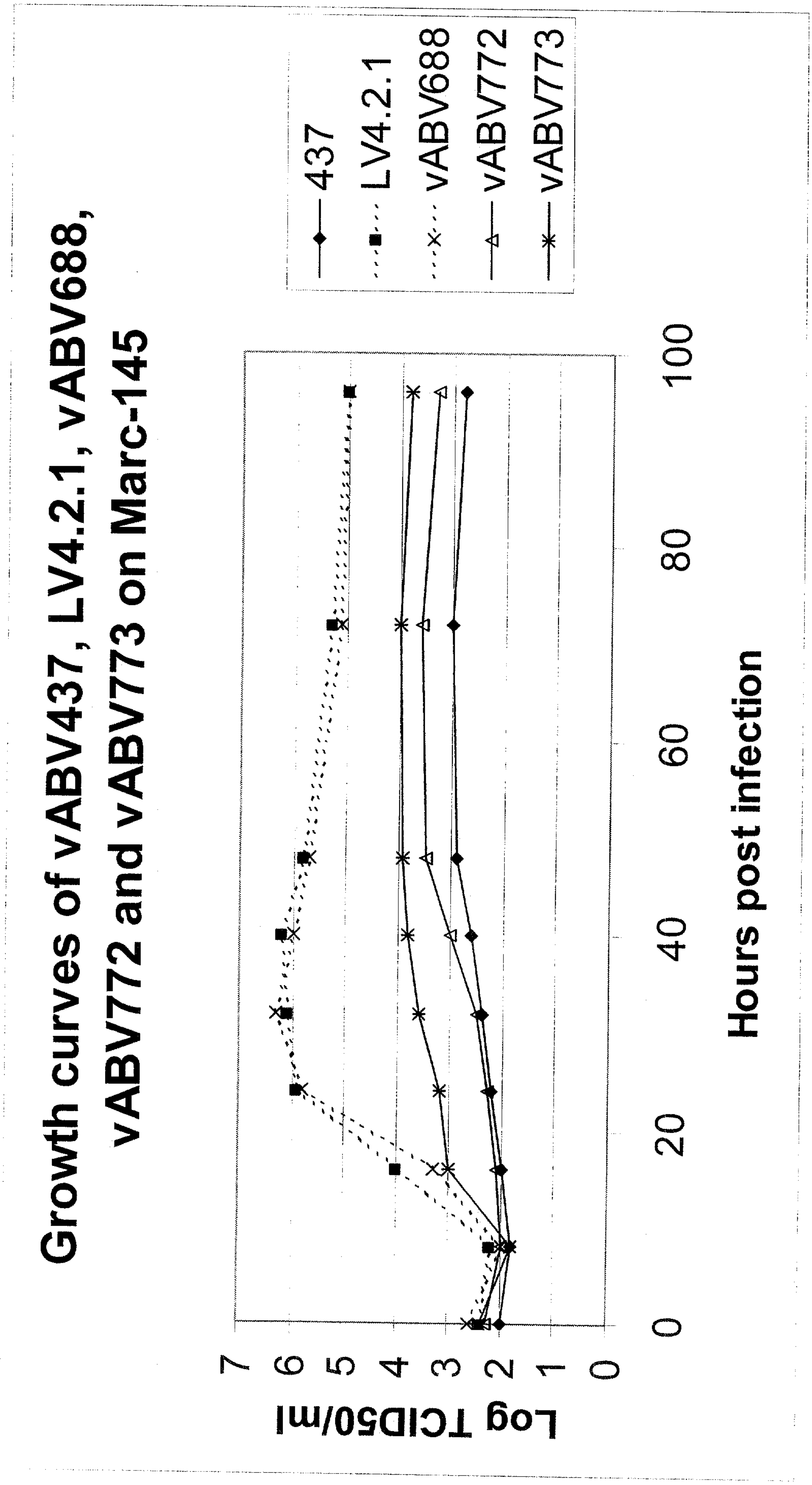

A
2208: G>A
(663: Glu>Lys)
7249: T>C
(2343: Val>Ala)
11880: C>T
(27: Ala>Val)
13900: G>A
(136: Cys>Tyr)
LV4.2.1
5'
1A
1B
B
2A
3
4
5
6
3'
11880: C>T
(29: Pro>Ser)
12078: T>C
(95: Phe>Leu)
12057: G>T
(88: Val>Phe)
B
2208: G>A
(663: Glu>Lys)
7249: T>C
(2343: Val>Ala)
pABV647
1A
13900: G>A
(136: Cys>Tyr)
pABV690
2A
5
12078: T>C
(95: Phe>Leu)
12057: G>T
(88: Val>Phe)
pABV688
2A
12078: T>C
(95: Phe>Leu)
12057: G>T
(88: Val>Phe)
13900: G>A
(136: Cys>Tyr)
pABV689
5
pABV772
2A
12057: G>T
(88: Val>Phe)
pABV773
2A
12078: T>C
(95: Phe>Leu)

Fig. 1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Log TCID50/ml
0
1
2
3
4
5
6
7
0
20
40
60
80
100
Hours post infection
437
LV4.2.1
vABV688
vABV772
vABV773

Fig. 2A

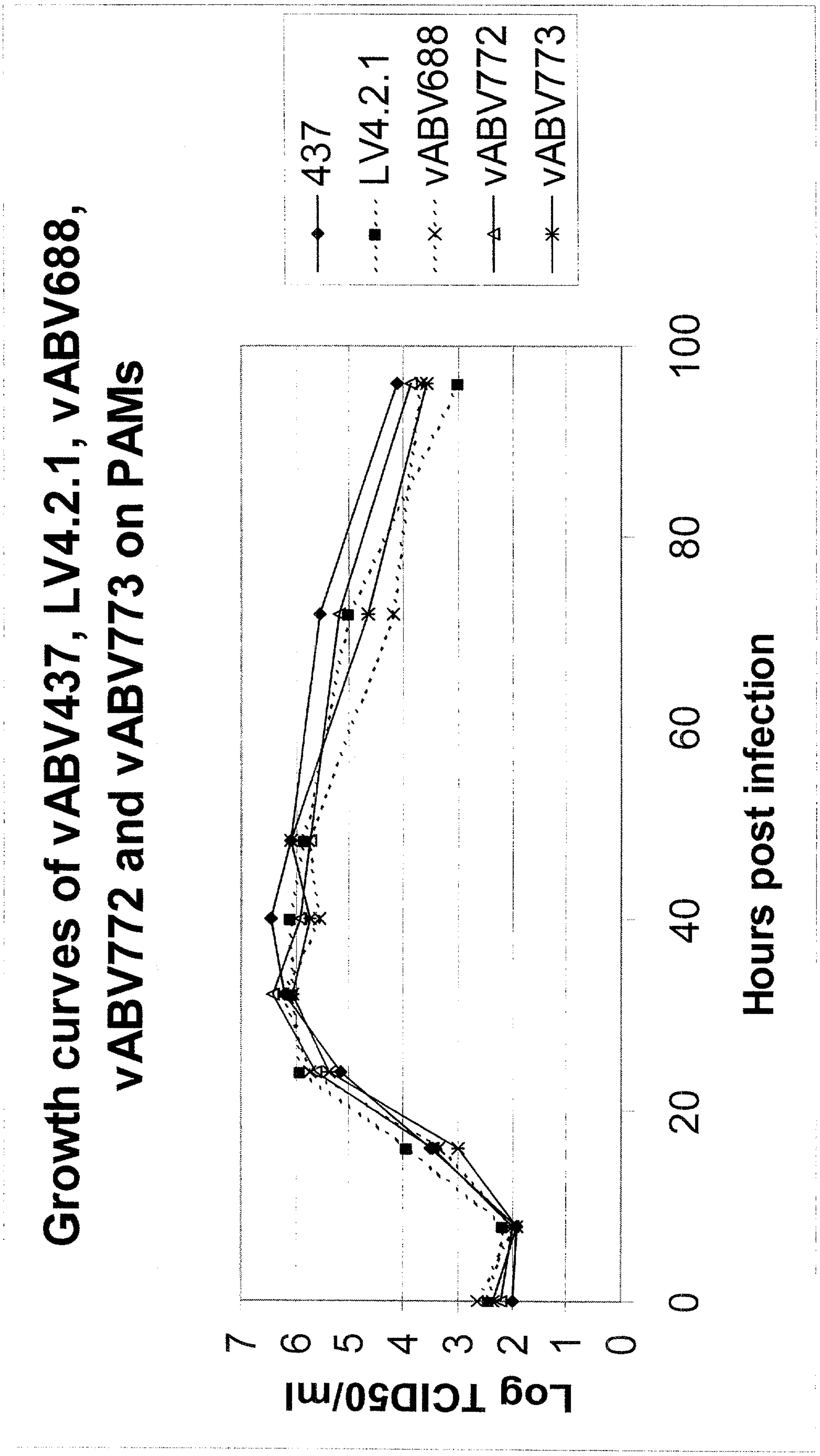
Growth curves of vABV437, LV4.2.1, vABV688, vABV772 and vABV773 on PAMs
Log TCID50/ml
7
6
5
4
3
2
1
0
0
20
40
60
80
100
Hours post infection
437
LV4.2.1
vABV688
vABV772
vABV773

Fig. 2B

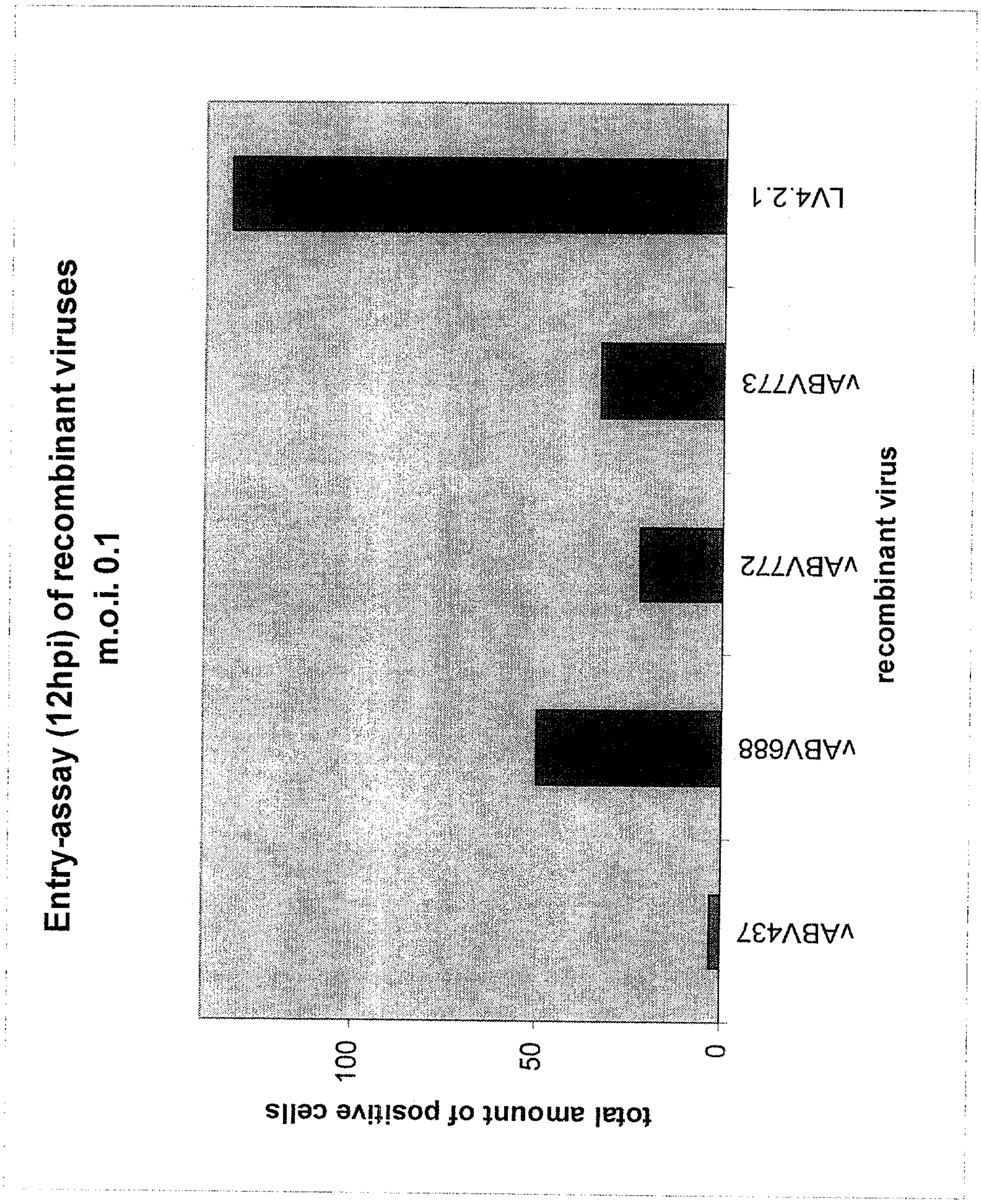
Entry-assay (12hpi) of recombinant viruses
m.o.i. 0.1
total amount of positive cells
0
50
100
vABV437
vABV688
vABV772
vABV773
LV4.2.1
recombinant virus

Fig. 2C

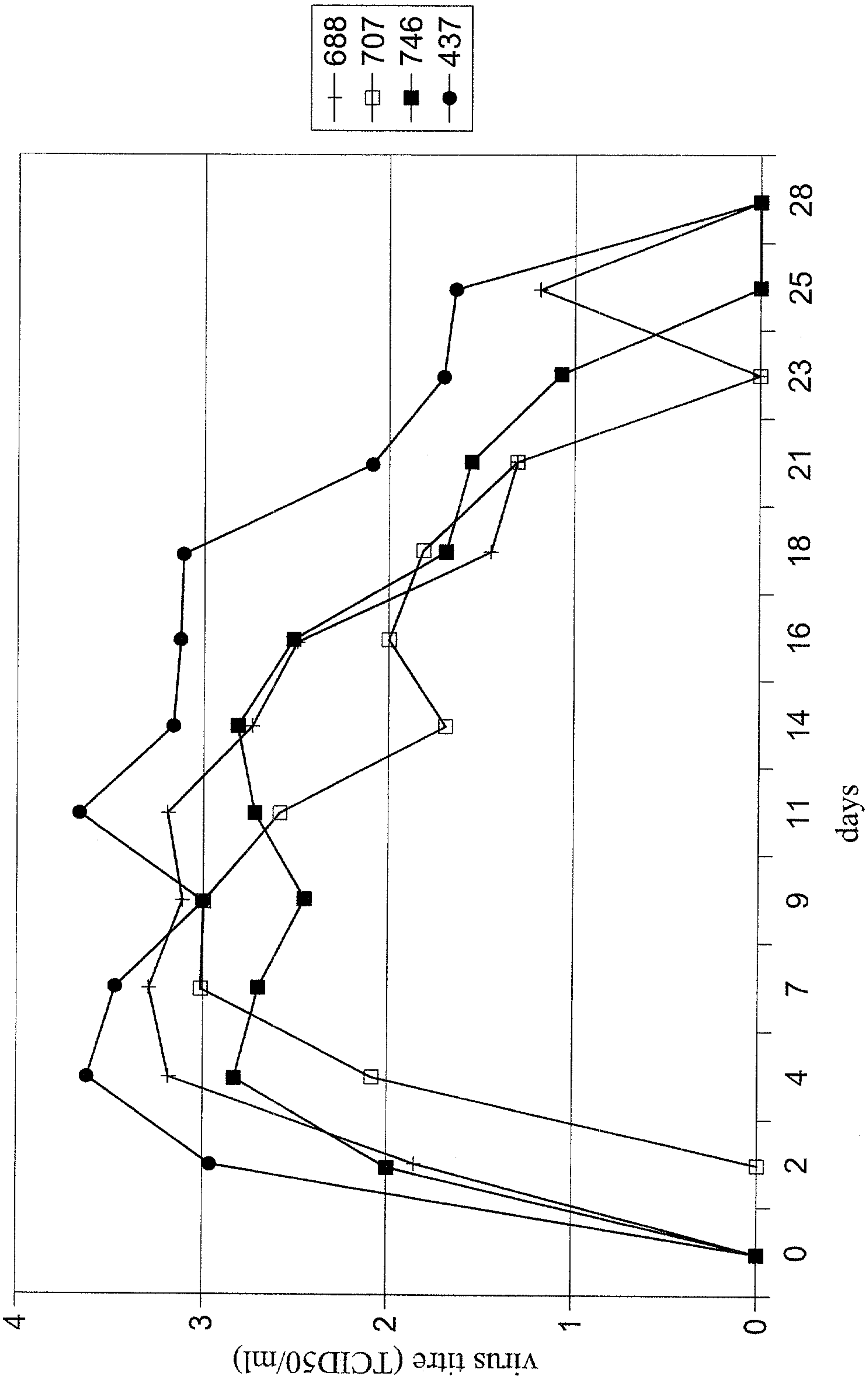
688
707
746
437
virus titre (TCID50/ml)
0
1
2
3
4
days
0
2
4
7
9
11
14
16
18
21
23
25
28

Fig. 3