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Development of sensitive methods for detection of porcine endogenous retrovirus-C (PERV-C) in the genome of pigs

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Abstract

Porcine endogenous retroviruses (PERV) represent a risk for xenotransplantation using pig cells, tissues or organs. PERV-A and PERV-B are present in the genome of all pigs and both infect human cells in vitro. PERV-C infects only pig cells and it is integrated in the genome of most, but not all pigs. Recombinants between PERV-A and PERV-C were described that infect human cells and replicate at high titres. To avoid such recombinations, PERV-C positive animals should not be used for breeding animals suited for xenotransplantation.

In order to detect PERV-C positive pigs, different methods were developed such as specific PCRs using different primers, a highly sensitive nested PCR and a real-time PCR allowing measurement of proviral copy numbers. The real-time PCR was found to be useful to discriminate between contamination and actual provirus copies. The PCRs were optimized and their sensitivity was determined. Screening can be started with PCR1, if the result is negative, PCR2 to PCR5 or the nested PCR should be used, if the result is positive, the real-time PCR should be used to exclude contaminations. All methods were used to evaluate the prevalence of PERV-C and to identify PERV-C free animals. Due to the risk of contamination with cells from other animals testing should be performed with blood cells, not with ear biopsies.

1. Introduction

Pig to human xenotransplantation is a promising approach to alleviate the shortage of human donor organs (Ekser and Cooper, 2010). Pigs were selected as the most suitable donor animals because of several advantages: (i) similarities in physiology, (ii) they can be modified genetically easily and cloned, (iii) their generation time is relatively short, (iv) their litters are large and (v) their breeding costs are low. Nevertheless there are three hurdles to overcome: immunological rejection, physiological compatibility and microbiological safety (Pierson, 2009). Transgenic and knock-out animals were generated to overcome hyperacute and acute rejection ([Petersen et al., 2009] and [Klymiuk et al., 2010]). In terms of microbiological safety, most of the potentially zoonotic porcine viruses and other microorganisms can be eliminated by qualified pathogen-free breeding ([Tucker et al., 2002] and [Denner, 2008a]). In contrast, porcine endogenous retroviruses (PERV) represent a particular risk. PERV-A and PERV-B are integrated in the genome of all pig strains, they are released from normal pig cells and infect human cells (Patience et al., 1997). Another PERV, PERV-C, infects only pig cells and is not present in all pigs, nevertheless its prevalence appears to be relatively high. In a previous study 176 from 181 (92%) animals were found to harbor PERV-C in their genome (Dieckhoff et al., 2009).

Although PERV-C does not infect human cells and therefore does not represent a direct risk for xenotransplantation, recombinants between PERV-A and PERV-C have been described in pigs, that are able to infect human cells ([Bartosch et al., 2004] and [Denner, 2008b]). These recombinant viruses were characterised by high virus titres compared to the parental PERV-A (Harrison et al., 2004) and they were found to behave as exogenous viruses integrating *de novo* into the genome of

pig cells in some organs, mainly the spleen (Dieckhoff et al., 2007), but they were absent from the germ line. Since PERV-A/C are characterised by high titre replication and increasing titres after passaging on human cells due to genetic alterations in the LTR (Denner et al., 2003), a consensus statement of the International Xenotransplantation Association recommended not to use pigs harbouring PERV-C for xenotransplantation in order to avoid PERV-A/C recombinations (Denner et al., 2009). To select PERV-C free pigs, sensitive methods have to be used. To screen for PERV-C free animals in pig colonies different methods including (i) standard PCR methods using five different primer pairs, (ii) a sensitive nested PCR and (iii) a real-time PCR allowing estimation of the copy number and to detect contaminations are described.

2. Materials and methods

2.1. Animals and DNA isolation

Ear biopsies and blood samples were taken from 38 transgenic and non-transgenic pigs at the Friedrich Löffler Institute, Mariensee, and from German landrace pigs in animal farms near Frankfurt. Transgenic pigs were carrying either human heme oxygenase (hHO-1), human tumour necrosis factor-alpha inducible gene A20 (hA20), human CD59, human decay-accelerating factor (DAF, CD55) or they were alpha 1,3 galactosyltransferase knockout (GalKO) pigs (Petersen et al., 2009). Animals were kept with an Ethical Committee approval and according to international standards for animal welfare.

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using LSM 1077 lymphocyte separation medium (PAA, Pasching, Austria) and DNA was isolated using the DNeasy blood and tissue kit (Qiagen, Hilgen, Germany). DNA isolation from ear biopsies was performed using the same kit and the TissueLyser LT (40 s at 30 Hz) (Qiagen).

2.2. PCR and nested PCR

In addition to the PCR based on primers published by Takeuchi et al. (1998) (designated PCR1) four other PCR setups were developed, based on different primer pairs (PCR2 to PCR5) (Fig. 1, Table 1). Furthermore, a nested PCR was developed, based on flanking primers identical to the primers used in PCR1 and inner primers identical to the primers used in PCR2 (Fig. 1, Table 1). Reactions were performed in a 25 µl volume using the Kapa2G robust PCR Kit (Peqlab, Erlangen, Germany). The mixture consisted of 5 µl of 5x PCR-Buffer A (final concentration 1.5 mM MgCl₂), additional 0.5 µl MgCl₂ (25 mM), 0.5 µl dNTPs (10 mM each dNTP), 1.25 µl primer (10 µM) each, 5 µl 5x Enhancer I, 0.1 µl of Kapa2G polymerase (5 units/µl) and 1–3 µl template (100 ng DNA) filled up to 25 µl with nuclease-free water. For the nested PCR 3 µl of the first step PCR product were used. The flanking PCR was performed under following cycle conditions: 15 min of pre-incubation at 95 °C and 30 cycles in three steps each (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and 72 °C for 5 min. Cycle conditions for the inner PCR were 3 min of pre-incubation at 94 °C and 30 cycles in three steps (94 °C for 30 s, 63 °C for 40 s, 72 °C for 30 s) and 72 °C for 10 min. Both PCRs were performed using 100 ng DNA from the pig cell line PK15 as a competitor.

2.3. Real-time PCR

For quantitation of envC-specific proviral DNA the primers real-time for and real-time rev (Table 1, Fig. 1) were designed. A duplex real-time PCR was performed based on cyclophilin (cyp) as reference gene using the primers cyp real-time for and cyp real-time rev and quantifying the PCR with a cyp probe (Table 1). The 25 µl reaction mixture consisted of 2.5 µl 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂, 0.01% (w/v) gelatin), 3 µl MgCl₂ (25 mM), 0.5 µl dNTP (20 mM), 0.5 µl (10 µM) of each primer, 0.5 µl of both probes (10 µM), 0.1 µl ROX (Invitrogen, Darmstadt, Germany), 0.3 µl AmpliTaq Gold[®] polymerase (Roche, Mannheim, Germany) (5 U/µl), nuclease free water and the template DNA (100 ng). The thermal cycling conditions used were 7 min at 95 °C followed by 50 cycles of 95 °C for 20 s and 58 °C for 30 s in a Stratagene MX4000 machine, the quantitative real-time PCR software was used (Agilent, Waldbronn, Germany). Efficacy was calculated by measuring ten fold serial dilutions of genomic envC positive DNA from 1.1 × 10¹⁰ to 0.2 plasmid copies per reaction.

Amplicons generated by real-time PCR were subcloned into the vector pBluescript II KS (Agilent, Waldbronn, Germany), sequenced and the plasmid was used in the real-time PCR to measure the copy numbers.

2.4. Sequencing

Amplicons generated by PCR3 as indicated in Fig. 1 were sequenced bidirectionally using Sanger's dideoxy method, using 10 ng of amplicon, 5 pmol of primer, 1 μ l of 5 \times Buffer 2 μ l of BigDye (Applied Biosystems), and the corresponding primers.

3. Results

3.1. Characterisation and sensitivity of the PCR and nested PCR

To screen animals for PERV-C provirus integration, a standard PCR (PCR1) was used, employing the differences in the sequences in the receptor-binding site between PERV-A, PERV-B and PERV-C (Takeuchi et al., 1998) (Fig. 1). The sensitivity of the PCR1 was estimated to be 1.1×10^4 copies per genome using the molecular clone PERV-C(1312) (Preuss et al., 2006) (Fig. 2A). To increase the sensitivity of nucleic acid amplifications, a nested PCR was developed, using flanking primers identical to the primers used in PCR1 and two inner primers. The sensitivity of the nested PCR was estimated by serial dilutions to be 1–0.6 copies per genome using molecular clone PERV-C(1312) in the presence of DNA from the pig cell line PK15 (Fig. 2B). To exclude false-negative results due to mutations in the primer binding sites, three additional PERV-C-specific PCRs such as PCR2, using the inner primers of the nested PCR, PCR3, PCR4 and PCR 5 were developed (Table 1, Fig. 1).

When DNA samples from 38 transgenic and non-transgenic pigs were analysed, 35 of them (92%) were positive in PCR1, 2 and 3 (Table 2), indicating that the PCRs gave the same result and that these animals were PERV-C positive. PCR4 was not applied knowing that it gives the same result as PCR1 (Dieckhoff et al., 2009). When DNA from 8 animals were tested using PCR5, the results were the same as obtained with PCR1-3.

When the DNA from animals negative for PERV-C according to PCR1-3 were analysed using the highly sensitive nested PCR, the results remained negative. However, when ear biopsies and blood from the same animal were tested using the nested PCR, in some cases DNA from ear biopsies was positive whereas DNA from PBMCs was negative for PERV-C proviruses. The reason for the false positive results was certainly the presence of cells from neighbour animals on the skin of the animals or contaminations by the instrument used to take the biopsies (this will be confirmed below by real-time PCR). Since the contamination was low and not detected by standard PCR, these results also underline the high sensitivity of the nested PCR.

3.2. Characterisation and sensitivity of the real-time PCR

To quantify the number of integrated PERV-C proviruses, a real-time PCR was established. Primers and a probe were designed in a region specific for PERV-C (Fig. 1, Table 1). To achieve optimal conditions the size of the amplicon was kept small (92 bp). As an internal standard for quantitation and for quality control of the template DNA a part of the house keeping gene cyclophilin was co-amplified. There was no interference between both real-time PCRs as confirmed by an analysis of serial dilutions of genomic DNA from one pig, which was PERV-C positive. The PCR showed an efficacy of 93.7% for cyclophilin and 99.3% for envPERV-C (Fig. 3). The detection limit of the real-time PCR was 100 copies as determined by a plasmid standard and a linear co-amplification was obtained between 1 pg to 100 ng of genomic template DNA.

3.3. Screening for PERV-C negative animals

In total, 38 transgenic and non-transgenic landrace pigs from different sources were analysed using standard PCR, nested PCR and the real-time PCR. As shown above, 35 of 38 animals were tested positive by PCR1, PCR2 and PCR3 (Table 2). When the DNA from the PERV-C positive animals was analysed by real-time PCR, the signals for PERV-C and cyclophilin (cyp) appeared at the same cycle threshold value (ct-value approximately 25, Fig. 3). Real-time PCR was also used to analyse DNA from ear biopsies and DNA from PBMCs which gave varying results in the nested PCR. Both DNA samples originated from the same animal (see above). In case of DNA originating from ear biopsies the ct-value of the PERV-C signal was much higher compared to the ct-value of cyclophilin (between 35 and 40 compared to 25, Fig. 3), indicating that there is less than one copy per genome. At the same time real-time PCR of the DNA from PBMCs confirmed the absence of PERV-C. This indicates that PERV-C is not endogenous in these animals and therefore represents a contamination, possibly by cells from neighbour PERV-C positive pigs or by a contamination due to the instrument used for taking biopsies. This means, the real-time method may also be used to discriminate between contaminations with DNA or cells from PERV-C positive animals on the one hand and presence of an integrated endogenous provirus on the other hand. Calculation of copy numbers revealed 1 to 7 PERV-C copies per genome in PERV-C positive animals and less than 1 copy in 300 genomes in the case of contaminations.

3.4. Confirmation by sequencing

Genomic DNA from PERV-C positive animals (three transgenic animals and two farm pigs) were amplified using PCR3 and the amplification products were sequenced. As a result, the sequences were found identical to the sequences shown in Fig. 1, indicating that the proviral PERV-C sequences were identical at least in this region.

4. Discussion

Different methods were developed to screen for the presence of PERV-C proviruses in the genome of farm pigs and of transgenic pigs bred for xenotransplantation. These methods include five standard PCR using different primer pairs, a nested PCR and a real-time PCR. Using these methods, 35 of 38 (92%) non-transgenic and transgenic animals were found to carry PERV-C in their genome, confirming a previous study in which 176 of 181 animals (92%) were PERV-C positive (Dieckhoff et al., 2009). The transgenic animals studied here expressed different genes that should reduce hyperacute and vascular rejection such as hHO-1, hA20, CD59, DAF or represent GalKO knockout animals (Table 2). Although the number of PERV-C negative pigs is low, these animals can be used as source for genetically modified animals designed for xenotransplantation.

Since it was recommended not to use animals harbouring PERV-C in their genome in order to avoid PERV-A/C recombinations (Denner et al., 2009), these methods are instrumental for the detection of PERV-C free animals. PERV-A/C were until now detected only in miniature pigs and in these animals only in certain organs, e.g., in the spleen, but not in other organs and never in the germ line ([Bartosch et al., 2004], [Harrison et al., 2004] and [Dieckhoff et al., 2007]). PERV-A/C were also not found in the New Zealand designated pathogen-free herd (Garkavenko et al., 2008). However, there are new reports on PERV-A/C in commercially used pigs with an increased number in clinically affected animals (Pal et al., 2011). In addition, there are also other reasons not to use PERV-C positive animals. First, the ability of PERV-C to infect cells that do not harbor the specific receptor by receptor-independent infection (Lavillette and Kabat, 2004), and second, mutations in the envelope protein of PERV-C may change the tropism towards human cells (Gemeniano et al., 2006). These data indicate, that breeding out PERV-C will reduce the risk of PERV transmission in xenotransplantation.

The methods are sensitive to detect up to 1.1×10^3 copies per genome in the standard PCR1, 1 copies per genome in the nested PCR and 100 molecules in the real-time PCR. The use of such sensitive methods may always be associated with the risk of contaminations and false positive results. Accordingly, a high risk of contamination was observed, when ear biopsies were used, either due to cells originating from other animals on the ear of the tested animal or on the instrument used for taking

the biopsy. In general, a high sensitivity and at the same time a high specificity are important for the detection of retroviral infections. Recent reports showing infection with a murine retrovirus, XMRV (xenotropic murine leukemia virus-related virus), in patients with prostate carcinoma and chronic fatigue syndrome (CFS) as well as in healthy individuals in some laboratories in the USA, but not in Europe (for review see Denner, 2010) are an example underlining this requirement. At present there is overwhelming evidence that the detection of XMRV or other murine viruses in human tissues represents the result of laboratory contaminations by murine DNA or cells (Smith, 2010). Therefore, PCRs designed to detect retrovirus infections or proviruses of endogenous retroviruses should be performed under appropriate conditions and all necessary precautions should be undertaken.

Based on these results, for an effective screening for PERV-C in pigs the use of blood samples (not ear biopsies, to avoid contaminations with cells from PERV-C positive animals) is recommended. The screening should start with PCR analyses such as PCR1. If the result is negative, mutations in the primer binding may be the reason and in this case PCR2 to PCR5 should be used. If PCR1 gives a positive result, a real-time PCR has to be performed to test for contamination in order to avoid false-positive results. If PCR1 to PCR5 give a negative result, a nested PCR or the real-time PCR should be performed to detect animals with low copy numbers of PERV-C.

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Tables and Figures

Table 1. Primers and probes used for amplification in the nested PCR and real-time PCR.

Primer/probe	Sequence 5'-3'	Location	Accession nos.
PCR1 = nested outer for	CTGACCTGGATTAGAACTGG (Takeuchi et al. (1998))	6606...6625	AM229312
PCR1 = nested outer rev	ATGTTAGAGGATGGTCCTGG (Takeuchi et al. (1998))	6867...6886	AM229312
PCR2 = nested inner for	GTGCTCTCCTTCAGACCTAGATTAC	6635...6659	AM229312
PCR2 = nested inner rev	CGTCAAGACCGTATTTGGTCC	6822...6842	AM229312
PCR3 for	CTGACCTGGATTAGAACTGG	6606...6625	AM229312
PCR3 rev	AAGTTTTGCCCCCATTTTGTAGT	6924...6944	AM229312
PCR4 = envC.2 for	GATTAGAAGCTGGAAGCCCAAGTGCTCT	6614...6641	AM229312
PCR4 = envC.2 rev	TCTGATCCAGAAGTTATGTTAGAGGATGGT	6872...6901	AM229312
PCR5 for	CTATTCGCCTCAAATAAACCAG	6778...6800	AM229312
PCR5 rev	CATGTGCATTGGTCTCTATG	7086...7105	AM229312
pCyp real-time for	TGCTTTCACAGAATAATTCCAGGATTTA	Duvigneau et al. (2005)	
pCyp real-time rev	GACTTGCCACCAGTGCCATTA	Duvigneau et al. (2005)	
pCyp probe	Cy5-TGCCAGGGTGGTGACTTCACACGCC-BHQ2	Duvigneau et al. (2005)	
envC real-time for	CCCCAACCCAAGGACCAG	6853...6870	AM229312
envC real-time rev	AAGTTTTGCCCCCATTTTGTAGT	6924...6944	AM229312
envC real-time probe	FAM-CTCTAACATAACTTCTGGATCAGACCC-BHQ1	6878...6904	AM229312

Table 2. Incidence of PERV provirus in nontransgenic and transgenic pigs.

Number of animals	Pig strain	Origin of the animals	Transgene, knockout	PCR 1	PCR 2	PCR 3	% PERV-C positive
16	German landrace	Farm Frankfurt	none	14/16	14/16	14/16	87.5
15	German landrace	FLI Mariensee	HO-1, A20, CD59, DAF	14/15	14/15	14/15	93.3
7	German landrace × Duroc × Minipig	FLI Mariensee	GalKO	7/7	7/7	7/7	100

For details of the strains, the origin of the animals, the transgenes or GalKO, and the different PCR methods (PCR 1-PCR3) see Section 2 and Fig. 1.

Figure 1. (A) Schematic presentation of the location of the primers for the PCRs and the probe for the real-time PCR. The length of the amplicons in bp and the approximate localisation of the virus receptor domain A (VRA) and B (VRB) and the proline rich region (PRR) in the receptor binding site of the surface envelope protein of PERV-C are indicated. (B) Sequence comparison of part of the surface envelope protein of PERV-C (*envC*), PERV-A (*envA*) and PERV-B (*envB*). The sequences of the outer primers (PCR1) and inner primers (PCR2) of the nested PCR, primers of PCR 3, 4 and 5 as well as primers and probes of the real-time PCR are indicated.

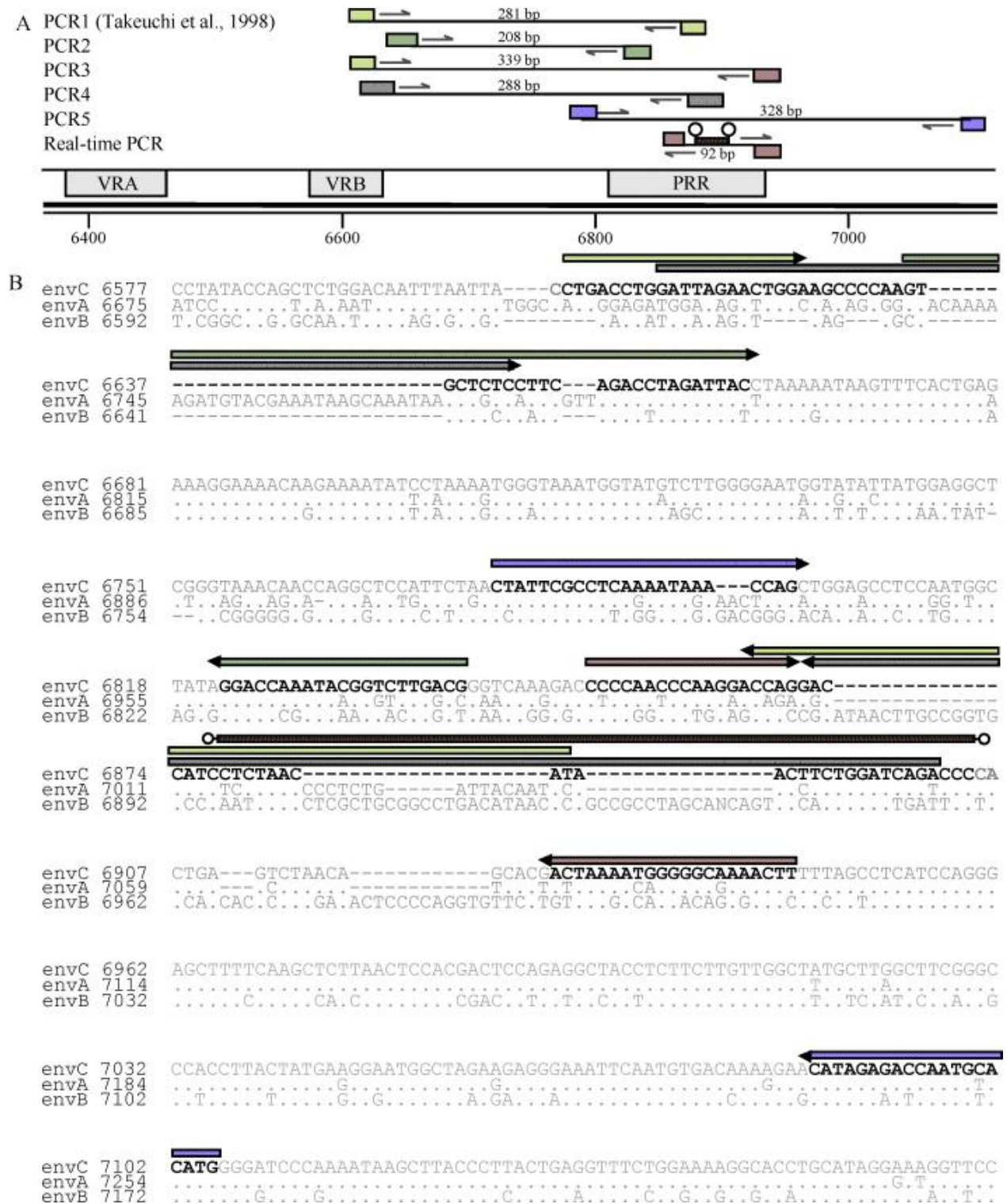


Figure 2. Determination of PERV-C copies in plasmid molecules. The sensitivity of plasmid molecules of molecular clone PERV-C(1312) (Preuss et al., 2006) was estimated. (A) Standard PCR (PCR1); (B) nested PCR using outer primers (PCR1) and inner primers (PCR2). Each sample contains 100 ng DNA of pig cell line PK15 as competitor. Negative control means water plus PK15 DNA; M, molecular marker.

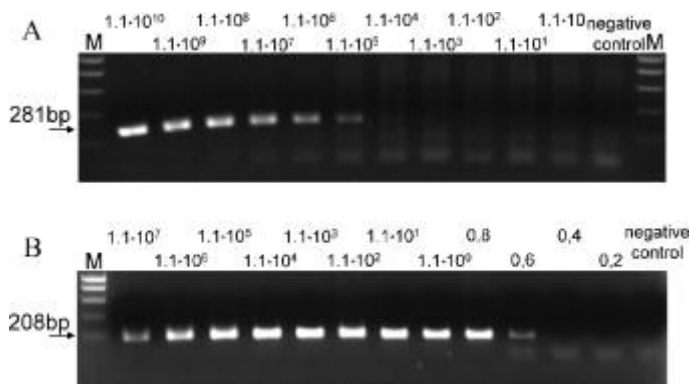


Figure 3. (A) Estimation of the efficacy of the duplex real-time PCR. Dilutions of the PERV-C env clone (99.3% efficacy) and of a cyclophiline plasmid (93.7% efficacy) were measured. (B) and (C) Results of the real-time PCR amplification of cyclophiline (B) and PERV-C env (C). All control PCR (B, cyclophiline, Cy5-signal) showed a ct-value of approximately 29, in the PERV-C measurement (C, FAM-signal) most samples showed endogenous PERV with a ct-value of 29, some samples showed contaminations with higher ct-values.

