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SHORT PAPER

Development of a PCR system for porcine cytomegalovirus detection and determination of the putative partial sequence of its DNA polymerase gene

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SUMMARY

After PCR amplification with conservative cytomegalovirus primers, a 520 nucleotide putative partial sequence of the DNA polymerase gene of porcine cytomegalovirus (PCMV) was determined. Sequence comparison revealed homology to DNA polymerase genes from various beta herpes viruses and a dendrogram was constructed depicting the relationship of PCMV to other members of the *Herpesviridae* family. The dendrogram indicates that PCMV is indeed a beta herpes virus that is more closely related to human herpes virus types 6 and 7 than to type 5.

To address the difficulties encountered during conventional PCMV detection and characterization a set of nested PCR primers were constructed which generated DNA fragments of 415 and 257 bp from the DNA polymerase gene. The nested PCR system proved specific for PCMV and provided a novel means for the detection of this poorly characterized herpes virus in pig populations, vaccines and in organs used in xenotransplantation.

Porcine cytomegalovirus (PCMV) is a poorly characterized beta herpes virus. No sequence information for the virus is available and its classification as a cytomegalovirus is based on its replication in cell culture. PCMV can cause generalized infection in new-born piglets with high mortality. It can also cause *in utero* infections in sows leading to loss of foetuses and birth of weak piglets as well as rhinitis, pneumonia and poor growth in piglets and weaned pigs. Infection in adult pigs is silent [1].

The virus probably has a world wide distribution and the prevalence has been shown to be very high on a herd level [2, 3].

In view of the present interest in xenotransplantation, PCMV may present a risk to humans. The risk is associated with receipt of an infected organ, causing infection of the host and possibly also with further spread of the virus in the human population. The high prevalence of PCMV underlines this risk. The risk for contamination of vaccine preparations based on swine cells or other swine derived components should also be considered. In conclusion a highly sensitive test for PCMV is needed.

Current tests for indirect detection of PCMV are generally based on immunofluorescence [4]. For detection of antiviral antibodies ELISA tests have been developed [5, 6], but these assays suffer from limited sensitivity and specificity. To our knowledge, no test for PCMV detection based on the polymerase chain (PCR) reaction has previously been described.

The aim of the present work was first, to develop a PCR assay that may ultimately prove to be more sensitive and specific than current tests for PCMV and secondly, to provide a limited characterization of the PCMV genome to enable us to gain some insight into the evolutionary relationships between PCMV and other herpes viruses.

Amplification of PCMV DNA by PCR was undertaken as a first step. A supernatant from porcine alveolar macrophages (PAMs) infected with PCMV strain B6 (kindly provided by N. Edington, Royal

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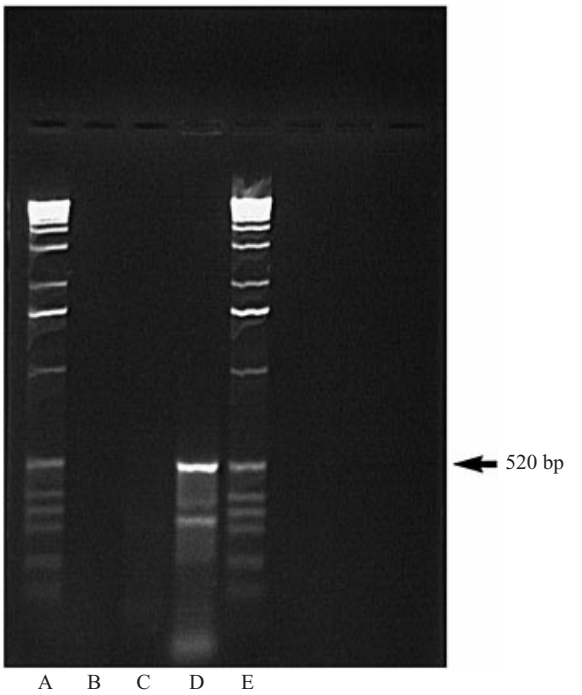

Fig. 1. PCR amplification of PCMV strain B6 with primers P1 and P2. Lanes A and E, molecular size marker, Life Technologies, 1 kb DNA ladder; lane B, no template control; Lane C, negative control; lane D, positive control. Amplification products and molecular size marker run on 2% agarose gel and stained with ethidium bromide. The arrow indicates a 520 bp DNA fragment in lane D, amplified from PCMV strain B6.

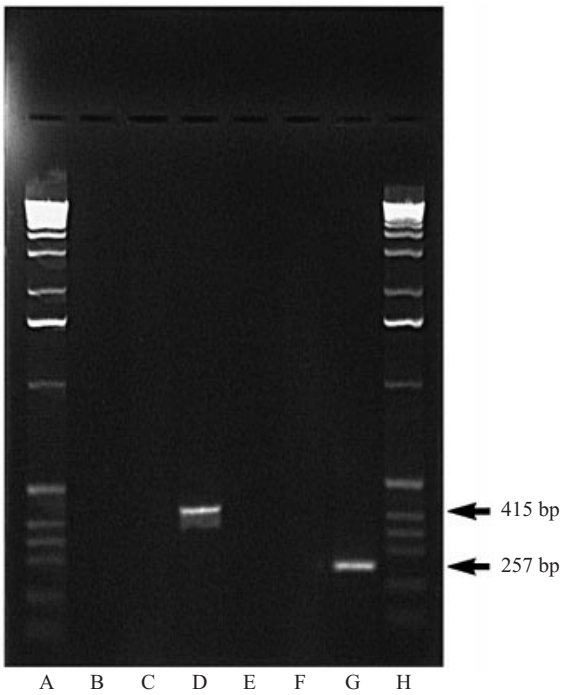

Fig. 2. PCR amplification of PCMV strain B6 with putatively specific primers 2 and 4. Lanes B, C and D, and with primers 1 and 3, lanes E, F and G. Lanes A and H, molecular size marker, Life Technologies, 1 kb DNA ladder. Lanes B and E, no template control; lanes C and F, negative control; lanes D and G, positive control. Amplification products and molecular size marker run on 2% agarose gel and stained with ethidium bromide. The arrows indicate a 415 bp DNA fragment in lane D and a 257 bp DNA fragment in lane G, amplified from PCMV, strain B6.

Veterinary College, London, UK) and purified DNA from cytomegalovirus free PAMs were used as positive and negative targets respectively. A no-template control tube was also included. The PCR primers were selected from the nucleotide sequence of the human CMV DNA polymerase gene determined by Rozenburg and Lebon [6]. The same primer sequences have also been used by Beuken and colleagues [7] to amplify the analogous region of rat CMV DNA. The sequences of the primers were: 5′ CGA CTT TGC CAG CCT GTA CC 3′ (P1) and 5′ AGT CCG TGT CCC CGT AGA TG 3′ (P2). The PCR was carried out in 50 μl volumes in Perkin–Elmer PCR buffer II, 3·5 mM magnesium chloride, 200 μM dNTP and 10 μM of each primer. A 'hot start' programme for thermocycling was used starting with 10 min denaturation at 95 °C.

The temperature was then held at 80 °C and 2·5 units Taq polymerase (Amplitaq, Perkin–Elmer) were added to each tube. The programme proceeded with 40 cycles of 1 min, denaturation, at 95 °C, 1 min, annealing, at 45 °C and 2 min, extension, at 72 °C. The programme ended with a final extension for 7 min at 72 °C.

The PCR product was analysed by horizontal electrophoresis on a 2% agarose gel, and visualized on a UV-transilluminator (254 nm) following staining with ethidium bromide.

From this initial PCR an amplification product of just over 500 bp was observed (Fig. 1). The PCR-product was used, directly without further treatment, for ligation with linearized pCR 2.1 vector (Invitrogen). The ligated plasmid construct was used to transform Inv aF′ cells (Invitrogen). Purified plasmid DNA preparates from selected *E. coli* clones were analysed for recombinant plasmids by digestion with *Eco*RI (Promega) and subsequent agarose gel electrophoresis.

In total, plasmid DNA from six recombinant clones was sequenced using the ABI prism kit and an automated sequencer (Applied Biosystems 377). Sequencing of recombinant plasmids resulted in a 520 bp sequence.

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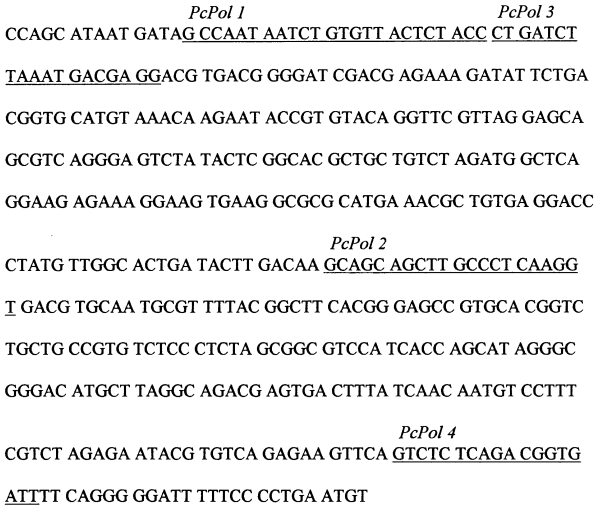

Fig. 3. Nucleotide sequence of the amplicon in 5′ to 3′ direction not including primer sequences selected from the human cytomegalovirus DNA polymerase gene determined by Rozenburg and Lebon [6]. Primers selected from the consensus sequence are underlined. Primers PcPol 1, PcPol 2, PcPol 3 and PcPol 4 are labelled.

A consensus sequence of the inserted DNA was determined from the six clones and used to design putatively PCMV-specific PCR primers.

Two pairs of nested primers were chosen from the consensus sequence.

These were: GCC AAT AAT CTG TGT TAC TCT ACC (PcPol 1), CTG ATC TTA AAT GAC GAG G (PcPol3), ACC TTG AGG GCA AGC TGC TGC (PcPol 2) and AAT CAC CGT CTG AGA GAC (PcPol 4). These primers have been used as separate pairs (Fig. 2) or nested (data not shown), in order to amplify the PCMV DNA template.

One of the putatively PCMV-specific primer pairs was used for PCR amplification of PCMV strain B6 and the amplification product (approx. 400 bp) was used for a second ligation and transformation. Transformed *E. coli* clones were selected and analysed for recombinant plasmids. Purified plasmid preparations from two different clones were sequenced in both directions as described above. Sequencing of recombinant plasmids resulted in a 415 bp sequence.

A consensus sequence (Fig. 3) was determined from the original six clones and the two clones derived from the putatively PCMV-specific primers. This consensus sequence was compared with virus subsection of the EMBL database using the FASTA program [8].

The PCMV sequences revealed the highest degree of similarity to the DNA polymerase gene sequences of the rat cytomegalovirus, MuHV-2 (62% similarity in 360 nt overlap); murine cytomegalovirus, MCMV-1 (63% similarity in 297 nt overlap); guinea-pig cytomegalovirus CaHV-2 (59% similarity in 394 nt overlap); and human cytomegalovirus, HHV-5 (58% similarity in 406 nt overlap) for the positive strand and human herpes virus 7, HHV-7 (61% similarity in 470 nt overlap); and human herpes virus 6, HHV-6 (58% similarity in 471 nt overlap) for the negative strand.

The position of the sequenced fragment in the putative PCMV DNA polymerase gene can be approximated to the region between nucleotides 65453 and 65930 of the HSV-1 genome (EMBL X14112).

The PCMV consensus sequence, and the sequence of the HSV UL30 homologoues of the alpha herpes viruses human herpes virus 1 (HHV-1, EMBL X044771), varicella zoster virus (HHV-3, EMBL X04370), pseudorabies virus (PRV, EMBL L24487) and equine herpes virus 1 (EHV-1, M86664); the beta herpes viruses HHV-5 (EMBL M14709), HHV-6 (EMBL M63804), HHV-7 ([9], EMBL U43400), MuHV-2 (EMBL U50550), CaHV-2 (EMBL L25706) and MCMV-1 (EMBL M73549); and the gamma herpes viruses Epstein–Barr virus (HHV-4, EMBL V01555), equine herpes virus 2 ([10], EMBL U20824) and herpes virus saimirii 2 (SaHV-2, EMBL M31122) were also aligned using the PILEUP program from the GCG software package [11]. Phylogenetic analysis was carried out on this alignment using the DNADIST, implicating the Kimura 2-parameter model, and the NEIGHBOR programes of the PHYLIP package [12]. The resultant phylogenetic tree suggests that PCMV is closely related to the beta herpes viruses, especially to HHV-6 and HHV-7, while the alpha and gamma herpes virus were grouped separately (see Fig. 4).

The present investigation indicate that the putatively PCMV-specific primers chosen from the consensus sequence may be useful for the PCR detection of PCMV in various specimens and could prove to be more sensitive than the Fluorescent Antibody Test (FAT). However, it may be necessary to select new, less conserved primers to ensure higher specificity for PCMV. A comparison of the sensitivity of the PCR and FAT is currently underway. This new study should indicate the value of the PCR as a tool for investigating the causes of porcine reproductive problems and for screening vaccine preparations.

In view of the current development in xenotransplantation techniques, a PCR test for PCMV detection may prove to be a valuable tool for the screening of

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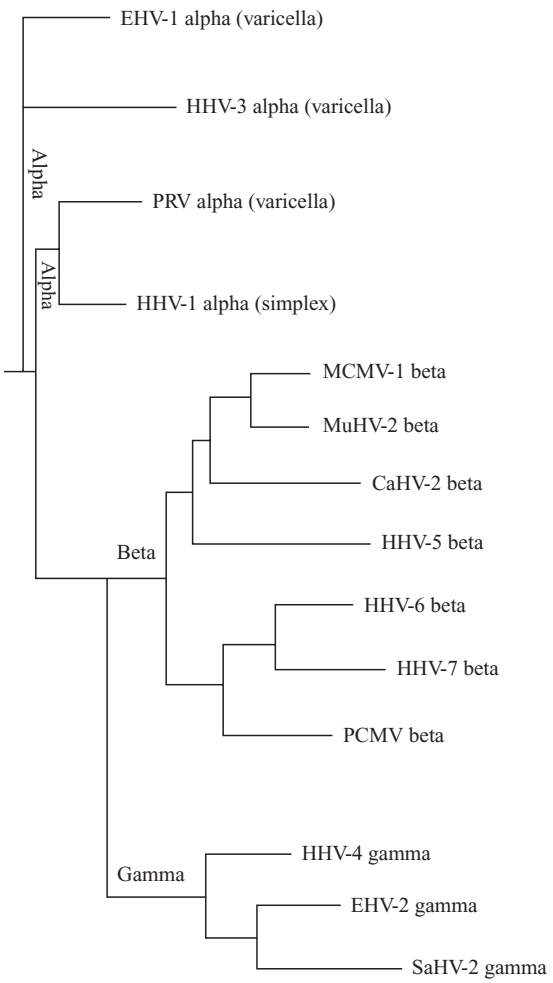

Fig. 4. Dendrogram depicting relationship among selected alpha, beta and gamma herpes viruses, prepared from nucleotide sequences of HSV UL30 homologues (from EMBL database) and the PCMV consensus sequence. Branch lengths are proportional to the distance between the respective sequences. The dendrogram was obtained using the DNADIST and the NEIGHBOR programs of the PHYLIP package [12].

transplanted organs, to protect recipients and to prevent the possible establishment of variants in the human population. Given the well conserved sequence of the DNA polymerase gene, the primers P1 and P2 used for the initial cloning or one of the primer pairs derived from well conserved regions of the PCMV sequence could also prove to be useful as pan-herpes primers.

This work has benefited from the use of the SEQNET facility.

The nucleotide sequence has been deposited in the EMBL database under the accession number AJ222640.

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