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Production of positive controls for calicivirus-specific PCR using recombinant baculovirus technology

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Recent advances in our knowledge of the genetic structure of human caliciviruses (HuCVs) and small round-structured viruses (SRSVs) have led to the development of polymerase chain reaction (PCR)-based molecular tests specific for these viruses. These methods have been developed to detect a number of human pathogenic viruses in environmental samples including water, sewage and shellfish. HuCVs and SRSVs are not culturable, and no animal model is currently available. Therefore there is no convenient method of preparing viruses for study or for reagent production. One problem facing those attempting to use PCR-based methods for the detection of HuCVs and SRSVs is the lack of a suitable positive control substrate. This is particularly important when screening complex samples in which the levels of inhibitors present may significantly interfere with amplificiation.

Regions within the RNA polymerase regions of two genetically distinct human caliciviruses have been amplified and used to produce recombinant baculoviruses which express RNA corresponding to the calicivirus polymerase. This RNA is being investigated as a positive control substrate for PCR testing, using current diagnostic primer sets. Recombinant baculovirus technology will enable efficient and cost-effective production of large quantities of positive control RNA with a specific known genotype. We consider the development of these systems as essential for successful screening and monitoring applications

Introduction

Human caliciviruses (HuCVs) and small round-structured viruses (SRVs) within the family Caliciviridae are among the most important nonbacterial causative agents of gastroenteritis. Infection with these agents is via the faecal–oral route and both waterborne and foodborne outbreaks have been reported (Kaplan *et al.* 1982; Alexander *et al.* 1986; White *et al.* 1986; Herwaldt *et al.* 1994). Infection has been linked with consumption of sewage-contaminated bivalve molluscs (Grohmann *et al.* 1980; Gill *et al.* 1983) and even to commercial ice made from a contaminated water supply (Cannon *et al.* 1991). Attempts to propagate these viruses in a wide range of tissue culture systems have been unsuccessful (Chiba *et al.* 1979; Cubitt *et al.* 1979). No standard laboratory animals are susceptible to infection although Norwalk virus was shown to produce asymptomatic infection in a chimpanzee model (Wyatt *et al.* 1978). This is similar to the situation seen for hepatitis E virus – another member of the Caliciviridae – but in marked contrast to the many caliciviruses infecting animals, such as Feline calicivirus and San Miguel sealion virus which can be replicated readily in tissue culture (Carter *et al.* 1991). The only source of HuCVs and SRSVs for study has been stool samples collected as clinical specimens or from deliberately infected human volunteers (Jiang *et al.* 1990). This has hampered studies as viruses are usually present at low concentrations in these samples, making the collection of a large pool of an individual virus strain difficult and time-consuming. Advances in our knowledge of the genomic structure of these viruses have enabled a number of molecular methods to be developed to permit their identification in a range of samples.

Recently, a number of research groups have reported use of the polymerase chain reaction (PCR) for the detection of SRSVs and HuCVs in clinical specimens (De Leon *et al.* 1992; Jiang *et al.* 1992). Surveillence methods using PCR are currently being developed for a variety of human pathogenic viruses in commercial shellfish. These include poliovirus, hepatitis A virus, HuCVs and SRSVs (Zhou *et al.* 1991; Guyader *et al.* 1994; Atmar *et al.* 1995; Lees *et al.* 1995). They are of considerable public health importance since viruses do not appear to be efficiently removed from shellfish during commercial depuration procedures (Enriquez *et al.* 1992). One of the main problems associated with the use of PCR-based detection methods on shellfish tissue is the presence of potent PCR inhibitors within the molluscan tissue (Atmar *et al.* 1993; Lees *et al.* 1994). Although virus purification and nucleic acid extraction methods have been developed to minimise such inhibitors (Lees *et al.* 1994), inhibition is still a problem with field samples. The degree of inhibition may vary between shellfish species and within shellfish samples taken from different sites. Positive controls for the PCR which can show the levels of inhibitors present in individual field samples are vitally important if negative results are to be meaningful, since very low virus levels may still be sufficient for human infection (Dolin *et al.* 1971; Spratt *et al.* 1978). It is not feasible to use virus as a positive control due to problems of obtaining sufficient quantities. Currently a number of different primer pairs are used for calicivirus and SRSV detection by PCR. Due to the high level of sequence diversity seen between different strains of these viruses, even within the more conserved regions of the genome, no currently available primers are capable of detecting all known viruses within these groups. Since sensitivity of the PCR depends in part on the degree of sequence difference between the amplification primers and the substrate, a consistent positive control would have to consist of a single virus strain. HuCVs and SRSVs are RNA viruses and hence PCR procedure is preceded by a RNA reverse transcription step. Ideally, the positive control substrate would consist of RNA rather than DNA, in order to identify any problems associated with RNA degradation and/or the reverse transcription step.

Here we describe the construction of a pair of recombinant baculoviruses, each encoding an RNA polymerase segment of a human calicivirus. When used to infect *Spodoptera frugiperda* insect cells, these viruses produce large quantities of polymerase RNA which can then be purified and used as a consistent, quantifiable RNA-PCR substrate. This approach has already been shown to produce a positive control system for hepatitis A-specific PCR (Lees, Henshilwood & Butcher 1995).

Design of PCR primers for amplification of HuCV and SRSV RNA polymerase regions

Human calicivirus and SRSV sequences from Genebank and EMBL databases were aligned using the PILEUP and PRETTY programmes from the GCG package (version 8, Genetics Computer Group, University of Wisconsin 1994). RNA polymerase motifs highly conserved between RNA viruses were identified within all strains studied, as previously noted (Jiang *et al.* 1993; Wang *et al.* 1994). The relative positioning of these conserved motifs is shown in Figure 1.

Binding sites of published diagnostic primers (Moe *et al.* 1994; Green *et al.* 1995) are situated in the area downstream of the LKDEL motif, finishing within the YGDDE motif. OVERLAP is the short region of 16 to 20 nucleotides which encodes both ORF1 and ORF2 amino acids, being read in two overlapping, different reading frames (Lambden *et al.* 1993). The length of this overlap varies in different strains (Liu *et al.* 1995).

All currently known diagnostic PCR primers bind within the RNA polymerase region, and several of the reverse primers that are used bind within the YGDDE conserved polymerase motif. Since the sequences of the available clinical isolates are unknown, primers were designed within regions of apparently conserved sequence, producing an amplicon large enough to contain all the binding sites of known diagnostic primers. Forward primers were designed around the area of the LKDEL polymerase motif. This is encoded by nucleotides 4320 to 4335

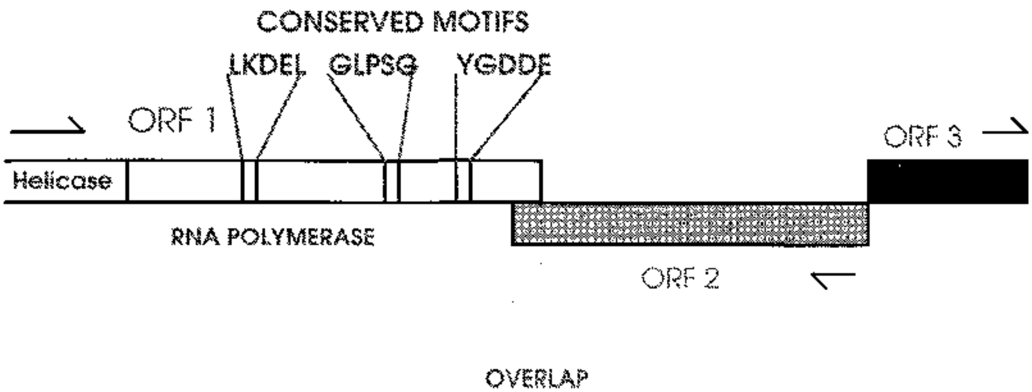

Figure 1. Conserved areas within the calicivirus genome of interest for primer design.

(nucleotide numbering corresponds to L07418 sequence). The reverse primers were designed around the overlap region (see Fig. 1). The range of sequences available in this area is shown in Table 1, and the alignment of sequences around this area is shown in Figure 2. Primers were designed within these regions (Table 2) using the OLIGO™ primer design software (version 4, Medprobe). The primers were designed to produce an amplicon of *ca.* 1030 base pairs.

	1		22
TV24	g - - - - - a - - -	- - - - - - g - - t	- -
SRSV-OTH-25/89/J	g - - - - - a - - -	- - - - - - g - - t	- -
HAWAII	g - - - - - a - - -	- - - - - - g - - t	- -
BRISTOL	g - - - - - a - - -	- - - - - - g - - t	. .
SOUTHAMPTON	a - - - - - t - - -	- - - - - - t - - g	. .
SRSV-KY-89/89/J	a - - - - - t - - -	- - - - - - t - - g	. .
DSV395	a - - - - - t - - -	- - - - - - t - - g	. .
NORWALK	a - - - - - t - - -	- - - - - - t - - g	- -
Consensus	-AATGA-GAT	GGCGTC-AA-	GA
	******************		

Figure 2. ORF1/ORF2 overlap region sequence in different isolates.
****** denotes the short overlap area of the Norwalk-like group (Lie *et al.* 1995).

Table 1. *Previously published sequences used to design PCR primers within the ORF1/ORF2 overlap region (see Fig. 1).*

Strain	Accession number	Reference
TV24	U02030	Lew *et al.* 1994c
SRSV-OTH-25/89/J	L23830	Wang *et al.* 1994
SRSV-KY-89/89/J	L23828	Wang *et al.* 1994
HAWAII	U07611	Lew *et al.* 1994b
BRISTOL	X76716	Green *et al.* 1994
SOUTHAMPTON	L07428	Lambden *et al.* 1993
DSV395	U04469	Lew *et al.* 1994a
NORWALK	M87661	Jiang *et al.* 1993

Table 2. *Oligonucleotide PCR primers.*

Name	Sequence	Polarity
ORF1F2	GCG CTT AAA GAT GAA CTA GTC AA	+
OVERLAPR	TCC TTA GAC GCC ATC ATC ATT	–
ORF1F3	TCA AGG ATG AGC TGG CCA A	+
ORF1REV	TCA TTC GAC GCC ATC TTC ATT	–

Amplification and cloning of RNA polymerase regions

Stool specimens taken from individuals with gastroenteritis, confirmed by electron microscopy (EM) to contain HuCVs or SRSVs, were kindly provided by Dr Charles Bangham, PHL John Radcliffe Hospital, Oxford. Samples were clarified for EM studies, and stored at 4°C. Specimens were investigated for their ability to be amplified in PCR reactions using different sets of previously described primers binding within the RNA polymerase region of the virus. The primers used were NI/E3 (Green *et al.* 1995), 51/3 (Moe *et al.* 1994) and CALF/CALR (S. Butcher, unpublished). Total RNA was extracted from 50 µl volumes of faecal suspension using the Invisorb™ kit (Invisorb total RNA kit I, Bioline (UK) Ltd, cat. KN6010100) which uses a silica carrier in a modified version of the method reported by Boom *et al.* (1990) RNA was resuspended in 50 µl TE buffer and stored at –20°C until needed. RNA was reverse transcribed with 10 units of AMV reverse transcriptase (Life Sciences Inc) in the presence of 0.5 µM reverse primer, 40 units RNAse inhibitor (Promega), 1.5 mM magnesium chloride, 0.2 mM each dNTP, 20 mM ammonium chloride, 75 mM Tris Cl and 0.01% Tween (w/v) for one hour at 42°C. The sample was then heated at 95°C for 5 min and held on ice for 5 min. PCR reagents were then added to the reverse transcription sample to give the following: 2.5 units Red hot™ DNA polymerase (Applied Biotechnology), 0.5 µM forward primer, 1.5 mM magnesium chloride, 20 mM ammonium sulphate, 75 mM Tris-Cl pH 9, 0.01% (w/v) Tween, 0.2 mM each dNTP. The mixture was then overlaid with 50 µl mineral oil and cycling was performed in a Hybaid TR3 Omnigene PCR machine using a cycle of 94°C for 2 min followed by 40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, with a final 3 min at 72°C. PCR products were analysed by electrophoresis in 2% (w/v) agarose gels containing 0.3 mg/ml ethidium bromide, and bands were visualised by UV irradiation. Bands corresponding to the 1030 bp RNA polymerase fragment were gel purified to remove excess dinucleoside triphosphates, oligonucleotides and nonspecific PCR products, and then cloned using the commercial PCR-cloning vector system Sureclone™ (Pharmacia) following the manufacturer's instructions. Inserts were sequenced to verify identity and orientation. Sequence was obtained from the amplicon derived from clinical specimen 9021, amplified using the PCR primer pair ORF1F2/OVERLAPR. This was compared with other known sequences in the NCBI database using the GCG programme BLAST and found to be most similar to Southampton, Norwalk, SRSV-KY-89/89/J and DSV395 sequences. Sequence was also obtained from the amplicon derived from clinical specimen 1550, amplified with the primer pair ORF1F3/ORF1REV. When this was compared with NCBI database sequences using BLAST, it showed most similarity with Bristol, TV24 and Hawaii sequences. It is significant that Norwalk and DS395 have been assigned to a single genogroup within the human caliciviruses, whereas Hawaii and DS395 have been assigned to a different genogroup (Green *et al.* 1994; Lew *et al.* 1994b).

Clones containing the expected insert were identified for both calicivirus isolates. These clones were then verified to contain an insert which could be amplified by the original PCR primers. The calicivirus RNA polymerase insert was subcloned into the baculovirus transfer vector pAcCL29-8 (Livingstone & Jones 1989), which had previously been linearised by digestion with BamHI and Asp718 and dephosphorylated with shrimp alkaline phosphatase (USB). The resulting insert was again partially sequenced to verify insert identity before being used for cotransfection. *Spodoptera frugiperda* cells were cotransfected with the transfer vector and wild-type baculovirus DNA as previously described (King & Possee 1992). Recombinant viruses were purified by three rounds of plaque purification.

Production of recombinant viral RNA

Spodoptera fugiperda insect cell line SF9 cells were grown in spinner culture and infected at a multiplicity of infection of 5 with recombinant virus. Cells were then cultured for 3 days at

28°C, sedimented by centrifugation and the medium was removed. RNA was extracted from the cell pellet using TRIZOL™ reagent, following the manufacturer's instructions (GIBCO BRL). The final RNA pellet was resuspended in RNAse-free double distilled water and stored frozen at –70°C. Further work will proceed as previously described for a recombinant baculovirus expressing hepatitis A RNA (Lees, Henshilwood & Butcher 1995). The RNA will be titrated in standard PCR reactions together with the diagnostic primers in order to quantify the sensitivity of the reaction when using the recombinant viral RNA as the substrate. This is necessary to determine the optimum amount of RNA to act as a positive control – small enough to be sensitive to inhibitors while still being sufficient to give a clear positive product under normal operating conditions. This RNA is being investigated as a positive control substrate in polymerase reactions using various current diagnostic PCR primers which all bind within the cloned RNA polymerase region. It is anticipated that the use of such positive control reagents will greatly improve the potential of PCR methods for the detection of human caliciviruses and SRSVs, particularly in field samples where significant inhibitors may be present.

Concluding remarks

Sets of PCR primers have been designed to amplify large fragments of the RNA polymerase region. These primer pairs have been used successfully to amplify this region from two genetically distinct clinical isolates. Amplicons have been inserted into baculoviruses and shown to produce RNA corresponding to the original calicivirus RNA polymerase region. RNAs will be tested as positive controls for PCR screening using a range of field samples.

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