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Application of Bovine Viral Diarrhoea Virus as an Internal Control in Nucleic Acid Amplification Tests for Hepatitis C Virus RNA in Plasma-Derived Products

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Plasma-derived products are produced from plasma via fractionation and chromatography techniques, but can also be produced by other methods. In the performance of nucleic acid amplification tests (NAT) with plasma-derived products, it is necessary to include an internal control for the monitoring of all procedures. In order to avoid false negative results, we confirmed the usefulness of the bovine viral diarrhoea virus (BVDV) for use as an internal control in the detection of hepatitis C virus (HCV) RNA in plasma-derived products. These products, which were spiked with BVDV, were extracted and then NAT was performed. Specificity and sensitivity were determined via the adjustment of primer concentrations and annealing temperatures. BVDV detection allows for validation in the extraction, reverse transcription, and amplification techniques used for HCV detection in plasma-derived products.

Keywords: hepatitis C virus, nucleic acid amplification test, plasma-derived products, internal control

The hepatitis C virus (HCV) is a small, enveloped virus, consisting of a positive-stranded RNA genome that occurs in the form of a single strand, with non-coding regions located at the 5′ and 3′ termini. HCV appears to be related to both the *pestiviruses* and *flaviviruses*. HCV is an important human pathogen which has been demonstrated to cause acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Ryu and Lee, 2004). HCV is also transmitted via blood products (WHO and the Viral Hepatitis Prevention Board, 1999).

Reports of HCV infection subsequent to intravenous infusions of immunoglobulin products have accelerated the implementation of donation screening tests, and have also resulted in the rapid introduction of virus-inactivation/removal steps in the processes by which plasma-derived products are manufactured (Chandra *et al.*, 1999; Cristiano *et al.*, 1997; Echevarria *et al.*, 1996).

The screening of donated plasma by blood banks is now mandatory, as is the confirmation of the safety of the pooled plasma for fractionation by the manufacturers. The manufacturers are also compelled to include virus clearance steps in the production of plasma-derived products. In addition, some manufacturers voluntarily perform NAT on their final products, in order to demonstrate that their products are non-reactive to viruses. In order to avoid false negative results in the test of these plasma-derived products, it is important that an internal control be established (Cristiano *et al.*, 1997).

Several types of internal controls exist. However, DNA internal control does not ensure reverse transcription, the step that is most vulnerable to the possible effects of inhibitors carried over during the sample extraction procedure. RNA transcripts are also ineffective with regard to the validation of the extraction step for the virus. In order to circumvent the limitations of these methods, Cleland employed the bovine viral diarrhoea virus (BVDV) as an internal control in plasma (Cleland *et al.*, 1999). BVDV has been employed extensively as a model for HCV in the virus validation studies. BVDV, a member of the genus *Pestivirus*, has been described as being closely related to HCV, on the basis of the results of genome analysis (Choo *et al.*, 1991). Due to the similarity of its physicochemical properties to those of HCV, BVDV appears to be a good candidate for an internal control in the confirmation of NAT procedures. In this

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study, we confirmed the availability and efficacy of BVDV as an internal control for the detection of HCV RNA, and applied this internal control to other matrices, plasma-derived products.

Materials and Methods

HCV RNA samples

We employed the HCV RNA international standard (IS) provided by the National Institute for Biological Standards and Controls (Code No.96/790, NIBSC, UK) as the positive control in every run, and also used the IS as a spiking agent for the plasma-derived products.

BVDV preparation

The BVDV NADL strain and the Madin-Darby bovine kidney (MDBK) cell line were used in our study. The cells were grown in minimum essential medium (MEM, GibcoBRL., USA), containing 1.0 mM of sodium pyruvate, 1.5 g/l of sodium bicarbonate, 50 µg/ml of gentamycin, and 10% heat-inactivated horse serum. MDBK cells were cultured in 75 cm^2 cell culture flasks, infected with the virus, and eventually were induced to the cytopathic effect (CPE). After the CPE was induced, the MDBK cells were subjected to three cycles of freezing to –70°C and thawing to room temperature. The cell-free BVDV supernatant was then harvested from the culture. The virus was aliquoted in volumes of 10 µl, and stored at –70°C prior to extraction. We obtained 1.5 x 10^7 copies/ml of BVDV via a limiting dilution assay (Cleland *et al.*, 1999). Amplifiable BVDV RNA sequences (37.5 copies) were added per reaction for subsequent use as an internal control.

Plasma-derived products

We tested a series of plasma-derived products, including albumin, intravenous immunoglobulin (IVIG), intramuscular immunoglobulin (IMIG), coagulation factor VIII, coagulation factor IX, and antithrombin III, all of which had been obtained from the Green Cross PD Co., Korea. The lyophilized products were then reconstituted with DEPC-treated water, and all of the plasma-derived products were stored at 4°C prior to use, with the exception of albumin, which was stored at room temperature. In order to detect the plasma-derived products, we serially diluted 5,000 IU/ml of IS with each of the products, to 500 IU/ml, 250 IU/ml, 100 IU/ml, and 50 IU/ml.

RT-PCR

RNA was isolated from a 280 µl sample with 5 µl (37.5 copies) of spiked BVDV, using a QIAamp viral RNA mini-kit (Qiagen, Germany). The QIAamp viral RNA extraction kit was used in accordance with the manufacturer's instructions. The RNA was then reverse transcribed and amplified, using the primer sets shown in Table 1. Reverse transcription (RT) was then performed using the GeneAmp PCR 9700 system (Perkin Elmer Co. USA.) with the RNA PCR kit, version 2.1 (Takara Shuzo Co., Ltd. Japan). cDNA synthesis was conducted in 20 µl reaction volumes, which included 10 µl of RNA at 50°C for 30 min using PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), with 5 mM $MgCl_2$, 1 mM dNTPs, outer antisense primer for 0.5 µM of HCV and for 0.5 µM of BVDV, 1 U/µl RNase, and 0.25 U/µL Avian Myeloblastosis virus (AMV) reverse transcriptase (Promega, USA).

For the first round of PCR, which was conducted with 20 µl of cDNA products, 2.5 mM $MgCl_2$, 0.2 µM of outer primers for HCV and 0.1 µM for BVDV, and 2.5 U of Takara Taq polymerase were utilized. For the second round of PCR, including 1 µl of the first round of PCR products, we used 1.5 mM $MgCl_2$,

Table 1. Nucleotide sequences of primers used for amplification of BVDV and HCV

Virus	Outer/inner	Nucleotide positions	Polarity	Sequences	References
HCV	outer	83-102	+	CCATGGCGTTAGTATGAGTG	Petrik *et al.*, 1997
		339-321	–	TGCACGGTCTACGAGACCT	
	inner	98-118	+	AGTGTTGTGCAGCCTCCAGG	
		313-295	–	CACTCGCAAGCACCCTATC	
BVDV	outer	85-106	+	CGAAGGCCGAAAAGAGGCTAGC	Cleland *et al.*, 1999
		645-626	–	GGCCCYGGYTTCAGGTAGAT	
	inner	107-128	+	CATGCCCTTAGTAGGACTAGCA	
		603-582	–	TTACCCGACCTGCAGTCACCTC	

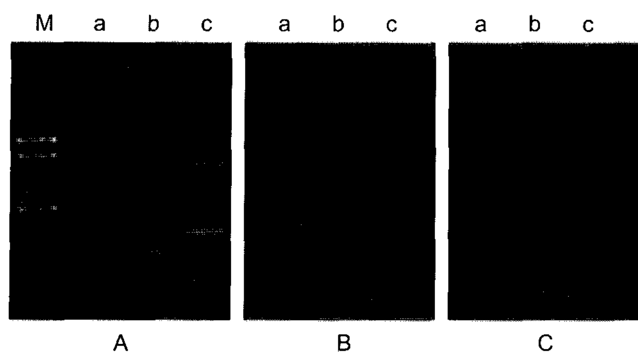

Fig. 1. Resistance of *D. concentrica* KFRI 40-1, *T. versicolor* KFRI 20251 and *P. chysosporium* KFRI 20742 to various styrene concentrations depending on incubation days.

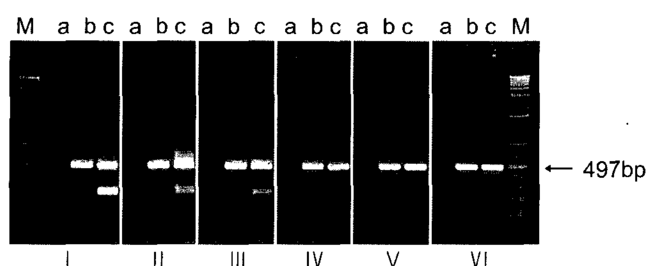

Fig. 2. Specificity profile for various annealing temperatures of HCV and BVDV primers against BVDV template in 20% human serum albumin. M, size standards, 1 kb plus ladder; a, HCV primer only; b, BVDV primer only; c, BVDV primer and HCV primer. The profile shows the annealing temperatures in order from left to right. I, 48°C; II, 51°C; III, 55°C; IV, 58°C; V, 61°C; VI, 65°C.

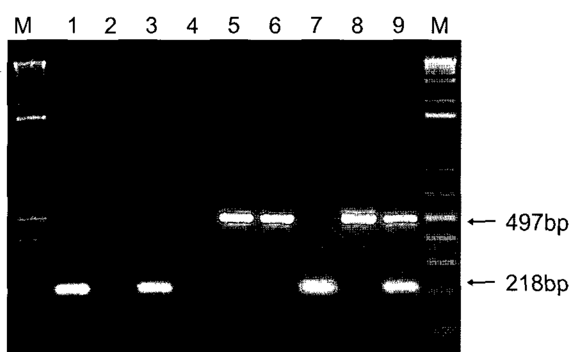

Fig. 3. Specificity of the method with internal control using BVDV at different primer combinations in 20% human serum albumin. Spiked only with HCV in lanes 1- 3, spiked only with BVDV in lanes4-6, spiked with both HCV and BVDV in lanes 7-9. PCR was performed with primers (HCV primer : 1, 4, 7 lane : BVDV primer : 2, 5, 8 lane : both HCV and BVDV primers : 3, 6, 9 lane). BVDV and HCV bands shown at 497 bp and 218 bp, respectively.

0.2 μM of inner primers for HCV and 0.1 μM for BVDV, and 2.5 U of Takara Taq polymerase. Amplification conditions were as previously described, except that the annealing temperature was adjusted to 65°C (Yoo *et al.*, 2003).

Results

Specificity of BVDV and HCV

We used primers encompassing the 5' non-coding region for HCV. For the detection of BVDV, we used primers encompassing a region spanning the 5'NCR and the amino terminus of the protease gene, N^{pro} (Table 1). The primers used for HCV and BVDV exhibited no sequence similarity, as was confirmed via screening with the Basic Local Alignment Search Tool (BLAST). However, we did detect 2 occasions of non-specific bands: first, in the case of the HCV template versus the BVDV primer, and second, in the case of the BVDV template versus the HCV and BVDV primers. In the first case, we attenuated the concentration of the BVDV primer, from 0.6 μM to 0.2 μM (Fig. 1). This was detected in the smear band, at a concentration of 0.4 μM. However, we detected no such band at a concentration of 0.2 μM. In the second case, we attempted to determine the specificity via a method that involved the titration of annealing temperature (Fig. 2). At 48°C, we noted a clear, non-specific band at approximately 200 bp. The annealing temperature was determined to vary inversely with the density of the band. At 65°C, we detected no non-specific band maintaining the sensitivity.

Primer ratio affects sensitivity

In the absence of the BVDV template, HCV was detected up to a concentration of 100 IU/ml, using 0.2 μM HCV and BVDV primers, respectively. However, HCV was not detected even at a concentration of 500 IU/ml after the addition of BVDV. Meanwhile, HCV sensitivity was restored via the dilution of the BVDV primers to a concentration of 0.1μM, but further dilutions of the primer resulted in reduced BVDV amplification efficiency, rendering it no longer useful as an internal control (data not shown). This result was consistent with the findings reported by Cleland (Cleland *et al.,* 1999). The optimum primer concentration for BVDV was 0.1 μM, and its for HCV was 0.2 μM. As a result, the PCR condition was optimized, allowing us to maintain sensitivity and specificity without interfering with any other template and primer sets. Fig. 3 shows BVDV and HCV bands at 497 bp and 218 bp, respectively (lane 9).

Performance in the plasma-derived products

Table 2 summarizes the HCV RNA detection efficiency of this method in the plasma-derived products. When the BVDV was spiked with albumin, im-

Table 2. The method for HCV RNA detection with spiked BVDV internal control in various plasma-derived products

Plasma-derived product		500 IU/ml	250 IU/ml	100 IU/ml	50 IU/ml
		No. positive/no. tested			
Albumin	S	3/3	3/3	3/3	1/3
	IC	3/3	3/3	3/3	3/3
IVIG	S	4/4	4/4	4/4	1/4
	IC	4/4	4/4	4/4	4/4
IMIG	S	3/3	3/3	3/3	1/3
	IC	3/3	3/3	3/3	3/3
Coagulation factor VIII	S	4/4	4/4	3/4	1/4
	IC	4/4	4/4	4/4	3/4
Coagulation factor IX	S	3/3	3/3	2/3	0/3
	IC	3/3	3/3	3/3	3/3
Antithrombin III	S	3/3	3/3	3/3	0/3
	IC	3/3	3/3	3/3	3/3

S, sample; IC,: internal control; IVIG, intravenous immunoglobulin; IMIG, intramuscular immunoglobulin

munoglobulins, and coagulation factors, this method was rendered more precise, allowing for detection at concentrations as low as 100 IU/ml. This also ensured a high degree of confidence in all of our positive results for BVDV. Thus, we confirmed the efficacy of BVDV as an internal control for the plasma-derived products.

Discussion

When using BVDV as an internal control, the prevention of primer/template interference is crucial. BVDV spiking in the HCV RNA detection technique functions as a sort of multiplex PCR. The presence of more than one primer pair within the multiplex PCR results in an increased chance of obtaining non-specific amplification products, primarily due to the formation of primer dimers (Brownie *et al.,* 1997). These non-specific products may also be amplified, thereby consuming reaction components and resulting in impaired annealing and amplification rates. Non-specific interaction is also to be avoided in the optimization of multiplex PCR (Elnifro *et al.,* 2000). It is necessary, in the design of primer parameters such as primer homology, to take into careful consideration such factors as the target nucleic acid sequences, their length, the GC content, and their concentrations (Suber *et al.,* 1995; Kim *et al.,* 2005). Ideally, all of the primer pairs in a multiplex PCR scheme should enable similar amplification efficiencies for their respective targets. This can be accomplished by using primers with similar optimum annealing temperatures, which exhibit no significant homology with one another (Henegariu *et al.,* 1997).

With regard to the non-specific band detected in the case of the HCV templates versus the BVDV primer, we surmised that the BVDV primer concentration had been too high to react with the non-specific band. We overcame this effect by controlling the concentration of the BVDV primer. The non-specific band detected in the case of the BVDV template versus the HCV and BVDV primers was attributed to PCR bias. When we performed PCR with the BVDV template and the HCV primer, we detected no such band, and this was attributed to the absence, in this case, of non-complementary sequences. However, when we performed PCR with the BVDV template versus the HCV and BVDV primers, we believe that PCR bias occurred due to differences in avidity for hybridization, interference from another primer, etc. According to the oligonucleotide dissociation temperature curve, the kinetics of binding between a complementary single oligodeoxynucleotide, such as a primer or a probe, and the targeted DNA may vary widely, depending principally on temperature (Ishii and Fukui, 2001). Therefore, we determined the specificity by raising the annealing temperature. These results verified that, when performing multiplex PCR, it is critical to monitor both primer concentration and annealing temperature.

The performance of NAT in the detection of HCV RNA with BVDV is a procedure which is somewhat different from multiplex PCR. Therefore, we focused on HCV sensitivity, while attempting to maintain specificity. This objective was accomplished via the control of the concentration of the BVDV primer.

If the plasma-derived products are suspect with regard to HCV infection outbreaks, several measures could be taken, including GMP investigation for the virus clearance process, epidemic surveys for the patients, and the determination of the presence or absence of the virus in the products (Murozuka *et al.,* 1999). When we performed HCV RNA detection with the products, some extraction methods proved ineffective, as some of the products were highly concentrated proteins, which may have attenuated extraction efficiency (Yoo *et al.,* 2002). In this respect, it was necessary to include a internal control, in order to validate all of the constituent procedures of the technique, involving extraction. Cleland was the first to suggest that BVDV might prove an effective internal control in screening tests with donated plasma. We evaluated the efficacy of BVDV, and determined it to be a good candidate for use as an internal con-

trol in other matrices or plasma-derived products.

In summary, this method should prove useful in the manufacture of plasma-derived products, and has shown capable of confirming the absence of HCV RNA in final products. The basic technique might also be applicable to quantitative PCR for the validation of virus clearance in plasma-derived products.

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