

Use of the Polymerase Chain Reaction with a Murine Model of Picornavirus-Induced Myocarditis

I. LEPARC,¹ F. FUCHS,^{2*} H. KOPECKA,³ AND M. AYMARD²

Laboratoire de Virologie¹ and Agence du Médicament—Unité de Virologie,² 8, Avenue Rockefeller, 69373 Lyon Cedex 08, and Institut Pasteur, 25, rue du Docteur Roux, 75015 Paris,³ France

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Enteroviruses are common pathogens responsible for a wide spectrum of systemic infections. Conventional diagnosis of these infections relies on the isolation of viruses in cell culture and their identification by seroneutralization with polyclonal or monoclonal antibodies. Among enteroviruses, coxsackieviruses B have been involved as causative agents for viral myocarditis. Most of the time, in the case of cardiac pathologies, viral isolation is negative. Molecular biology techniques appear to be an alternative to conventional diagnosis and could supply evidence for the direct implication of enteroviruses in these severe pathologies. In this paper, we describe a murine experimental model of infection with the presumed highly cardiopathogenic coxsackievirus B type 3. A kinetics of infection was observed for a period of 31 days, and the classical virological markers (viral isolation from feces and heart biopsies, seroconversion) were monitored and compared by means of molecular techniques (molecular hybridization, polymerase chain reaction [PCR]). In this 31-day period, the detection of coxsackievirus B type 3 RNA in the heart was possible only by using two successive seminested PCRs. After 9 to 11 days of active viral replication, when all other virological markers were negative, positive PCR signals were obtained, which supports the hypothesis of a shift to persistent enteroviral infection.

Enteroviruses have been considered to be a possible cause of idiopathic cardiomyopathy (10, 19). Idiopathic dilated cardiomyopathy is the most frequent form of these pathologies, and, at the present time, different authors suppose that these pathologies have a viral origin (1, 27).

According to Eggers and Mertens (6), an etiologic relationship is to be considered proven according to the following three criteria on the pathology of the disease: isolation of virus from the heart, isolation of virus from other organs along with the corresponding serodiagnosis, and, finally, association of viral infection with cardiac pathology.

Molecular biology techniques have been applied to the diagnosis of the cardiomyopathies which tend to involve enteroviruses and particularly those that involve the coxsackieviruses B (12). Different teams sought evidence of these viruses in idiopathic dilated cardiomyopathy; some have obtained evidence of enterovirus in idiopathic dilated cardiomyopathy by slot blot and in situ hybridization (2–4, 10–12).

Recently, other groups examined heart biopsies by polymerase chain reaction (PCR [9, 26]). Zoll et al. (29) demonstrated the possibility of enteroviral RNA detection by using PCR, and this could be related to the concept of persistent infection.

Other authors suggest that both the normal and abnormal heart may represent a site of latent or low-grade persistent enteroviral infection and therefore that a positive PCR result is not specifically associated with idiopathic dilated cardiomyopathy (25).

Being particularly interested in the study of the persistence of enteroviruses in heart diseases, but knowing that human heart biopsies are too valuable, we undertook to develop a murine experimental model of coxsackievirus B type 3 (CBV3)-induced myocardial pathology and compared the conventional isolation methods with our PCR technique.

MATERIALS AND METHODS

Viruses. CBV3 Nancy strain and poliovirus type 1, 2, and 3 strains were provided by the National Reference Center for Enteroviruses in Lyon, France. Virus stocks were grown in BGM K cells. Cell lines were propagated in Eagle minimal essential medium (Wittaker Laboratory, Les Ullis, France) supplemented with 7% fetal calf serum (Wittaker Laboratory)–1% (vol/vol) amino acids, glutamine, and antibiotics.

Viral stocks were purified by isopycnic centrifugation in a CsCl density gradient for 18 h at 4°C (15).

Mice. CD1 female mice 4 to 5 weeks old were purchased from Charles River (Charles River, Les Oncins, France). Mice were inoculated intraperitoneally with 10⁶ 50% tissue culture infective doses (TCID₅₀s) in 0.3 ml of the Nancy strain of CBV3.

Processing of heart tissue for assays of virus content. Of 60 mice, 30 were inoculated with CBV3 and 30 were used as controls. Mice (two inoculated and two control animals) were sacrificed every other day during the first and second weeks postinfection (p.i.) and twice a week until day 31 thereafter. Hearts were immediately aseptically removed after death and were cut into three parts: the left third for virus isolation, the right third for molecular biology investigations, and the apical part for further histological analyses. Heart samples were kept in liquid nitrogen until used.

When mice were sacrificed, blood and fecal samples were collected for serological and viral isolation examinations.

Virus isolation. Heart biopsies and fecal samples were diluted in sterile Eagle's minimal essential medium treated with antibiotics for 1 h at room temperature and were clarified by centrifugation (2,500 × g for 1 h at 4°C). These crude extracts were inoculated to BGM K cells, and the cytopathic effect appeared 48 to 72 h after inoculation.

Infectivity assays. Titers of the viral reference strains were determined in microtitration plates and expressed as TCID₅₀s of virus per 0.05 ml (17).

Titration of antibodies. Mouse neutralizing antibody assays were performed by a micromethod with BGM K cells

* Corresponding author.

TABLE 1. Primers for PCR

Oligonucleotide	Sequence	Nucleotide localization
Primer 2	5'-CAAGCACTTCTGTTCGCCGG-3'	164-184 (5'NC)
Primer 3	5'-ATTGTCACATAAGCAGCCA-3'	584-603 (5'NC)
Primer F2	5'-CTTGGCGGTTACGAC-3'	516-530 (5'NC)
5'NC probe		221-670 (5'NC)
Primer A	5'-CAGGGCCAGTGGGAAGA-3'	2448-2464 (VP1)
Primer B	5'-GGCGTTTCCTCGGTCCAAA-3'	2972-2953 (VP1)
Primer C	5'-GACCTTGAATGGTAGTTCTT-3'	2629-2610 (VP1)

(24). Equal volumes of diluted serum containing 100 TCID₅₀S of CBV3 virus were incubated for 2 h at 37°C before the cell suspension was added. After 5 days of incubation, titers were expressed as the highest dilutions of serum in a volume of 0.05 ml that inhibited the cytopathic effect of 100 TCID₅₀S of virus.

Antibodies were also checked by an enzyme-linked immunosorbent assay (ELISA), as previously described (15). Plates were pretreated with Polybrene (30 µg/ml in phosphate-buffered saline [PBS]; Aldrich Chemie, Chesnes, France) and coated with 2 µg of purified viral antigen per well diluted in bicarbonate buffer (pH 9.6) and were allowed to stand for 1 night at room temperature. After a blocking step with 100 µl of a 10% nonfat dry milk-PBS solution, the serum dilutions were incubated for 1 h at 37°C. Peroxidase-labeled sheep anti-mouse immunoglobulin G (IgG) or IgM conjugate was then added for 1 (IgG) or 2 (IgM) h at 37°C. The colorimetric reaction was developed by adding a solution of enzyme substrate (ABTS; Amersham), and the chromogen was stopped by addition of azide-citric acid. Plates were read at A₄₀₅.

RNA extraction. Cell lysates (100 µl) were digested with proteinase K (100 µg/ml [Boehringer Mannheim]) for 1 h at 37°C, and then a phenol chloroform extraction step was performed, followed by ethanol precipitation (23).

The heart tissues were digested overnight with sodium dodecyl sulfate (SDS)-10% proteinase K-1 mg of Tris-EDTA buffer per ml.

RNA was extracted by a standard method described in reference 23.

Viral cDNA synthesis and PCR gene amplification. (i) **Primers and riboprobe.** Primer sequences were selected from highly conserved parts of the 5' noncoding (5'NC) region and have been described elsewhere (29). In parallel, we selected primers in the VP1 region of the published sequence of CBV3 (14). The primers consisted of 15 to 20 bases with 100% homology with the known viral RNA sequences (Table 1).

With primer combination 2 and 3 (2+3), the PCR resulted in a 435-bp product, and with primer combination 2+F2, a 362-bp product was obtained. With primer combination A+B, the PCR band obtained was 524 bp, and with the A+C combination a 181-bp product was obtained.

A riboprobe of negative polarity corresponding to the 5'NC region (nucleotides 221 to 670) was used (7).

(ii) **First-strand cDNA synthesis.** We synthesized the cDNA with an oligo(dT15) primer (Boehringer Mannheim, Grenoble, France). Briefly, a 20-µl reaction mixture contained 4 µl of reverse transcription buffer (250 mM Tris-HCl [pH 8.4], 50 mM MgCl₂, 350 mM KCl, 15 mM dithiothreitol), 40 U of RNase inhibitor (Boehringer Mannheim), 0.25 mM (each) deoxynucleoside triphosphate (Boehringer Mannheim), 50 pM oligo(dT15), 4 µl of H₂O-pyrocubic acid

diethyl ester (DEPC), 10 U of avian myeloblastosis virus reverse transcriptase (Promega), and RNA isolated from the viral strains or heart biopsies. These mixtures were incubated at 42°C for 30 min and then at 95°C for 5 min. The samples were then ready for PCR gene amplification.

(iii) **First PCR gene amplification.** Five microliters of cDNA was added to the PCR mixture containing 10 µl of 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, 0.01% (wt/vol) gelatin buffer, 0.25 mM (each) deoxynucleoside triphosphate, 0.1 µM (each) primer (2+3 primer pair for 5'NC and A+B for VP1 region), and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). RNA-cDNA hybrids were denatured at 94°C for 5 min. The amplification was performed during 30 cycles consisting of denaturation for 30 s at 94°C, primer annealing for 45 s at 50°C, and elongation for 1 min at 72°C.

The reactions were analyzed by electrophoresis in 1% agarose gels and dot blot hybridization (see Southern blot analysis).

(iv) **Seminested PCR amplification.** One microliter of the amplified products was added to 99 µl of the previously described PCR mixture including the 2+F2 primer pair or the A+C pair for the VP1 region. Amplification cycling was performed as described above.

PCRs were analyzed by electrophoresis in 1% agarose gels or dot hybridization.

Southern blot analysis. The agarose gels were denatured for 10 min in a 1.5 M NaCl-1.5 M NaOH denaturation solution and then were neutralized for 10 min in a 1 M Tris-1.5 M NaCl solution. The PCR products were then transferred to a positively charged nylon membrane (Boehringer Mannheim) by using a Transvac apparatus (Hofer) and a 2× SSC diffusion buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Prehybridization and hybridization assays were performed as previously described (7). The filters were baked at 120°C for 30 min and prehybridized at 42°C for 1 h. The hybridization solution was deionized 50% formamide-5× SSC-0.1% *N*-lauroylsarcosine-0.02% SDS-5% blocking reagent (supplied by Boehringer Mannheim). The digoxigenin-labeled probe corresponding to the 5'NC was added at a 300-ng/ml concentration (13).

Hybridization was carried out overnight at 42°C. The filters were then washed twice in 2× SSC-0.1% SDS for 5 min each at room temperature and twice in 0.1× SSC-0.1% SDS for 15 min each at 42°C. The digoxigenin-labeled hybrids were detected by ELISA with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase. After hybridization, the digoxigenin-RNA probes were revealed by means of an enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) and nitroblue tetrazolium (supplied by Boehringer Mannheim), producing a purple precipitate. More recently, we used a chemiluminescent detection technique. Alkaline phosphatase dephosphorylates a chemiluminescent substrate, AMPPD (Boehringer Mannheim), which upon decomposition emits light visualized on X-ray films.

RESULTS

Sensitivity of the PCR procedure. The sensitivity of our PCR procedure was assessed by amplifying quantitative amounts of RNA. For this purpose, RNA was extracted from the reference CBV3 strain (initial titer, 10⁷ TCID₅₀S/50 µl), and serial RNA dilutions ranging from 0.04 µg to 40 fg were prepared and amplified with the 2+3 primer pair. PCR

TABLE 2. Sensitivity of the seminested PCR assay in the 5'NC region^a

Assay	Assay sensitivity at RNA dilution of:						
	40 ng	4 ng	0.4 ng	40 pg	4 pg	0.4 pg	40 fg
PCR	+	+	+				
PCR + hybridization	+	+	+	+	+	+	
Seminested PCR	+	+	+	+	+	+	
Seminested PCR + hybridization	+	+	+	+	+	+	+

^a Tenfold dilutions of CBV3 RNA were prepared, and then the cDNA and PCR assays were performed. Hybridization of the PCR products was performed with the 5'NC riboprobe.

products were visualized directly and further transferred onto nylon membranes and hybridized with the 5'NC digoxigenin-labeled probe.

In a second step, the PCR products were reamplified with the 2+F2 primer pair and directly visualized, further transferred to membranes, and then hybridized. The results are summarized in Table 2 and illustrated in Fig. 1 and 2.

The same experiments were repeated to quantify the PCR assay in the VP1 region. A first PCR was performed with the A+B primer pair, and serial RNA dilutions were prepared from a new RNA extract (13 ng to 1.3 fg). The seminested PCR with the A+C primers resulted in a 181-bp fragment. Our results showed that 130 fg could be detected in the first PCR assay and 1.3 fg could be detected in the seminested PCR.

Induction of myocarditis in CD1 mice. Mice inoculated with 10⁶ TCID₅₀s of CBV3 readily displayed evidence of myocarditis by day 7 or day 8; the dilatation and the inflammatory aspect of freshly autopsied specimens were observed and compared with those of control samples. The lesions were identical in appearance to published photographs of myocardial lesions induced by CBV3 in other

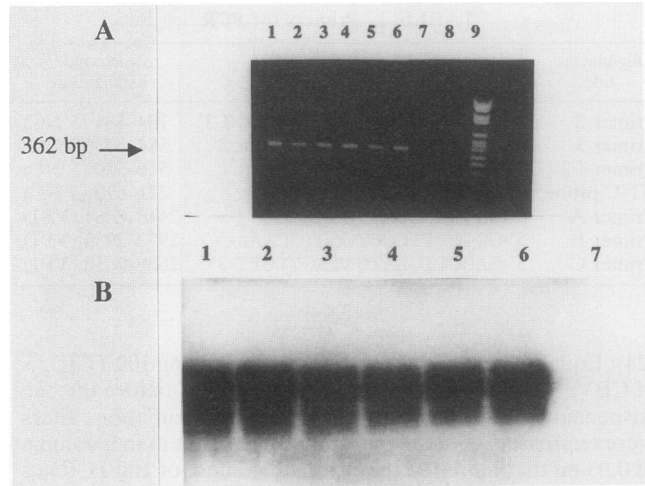


FIG. 2. Seminested PCR amplification (5'NC region) of PCR products derived from the first amplification (2+F2 primer pair). PCR products were visualized by agarose gel analysis (A) and Southern blot hybridization (B) as described in Materials and Methods. (A) Lanes 1 to 7 correspond to PCR products (362 bp) derived from amplification of 10-fold dilutions of viral RNA ranging from 40 ng to 40 fg (10⁶ TCID₅₀s to 1 TCID₅₀); lane 8 corresponds to the control and lane 9 corresponds to the molecular weight marker (marker VI; Boehringer Mannheim). (B) Lanes 1 to 6 correspond to dilutions ranging from 4 ng to 40 fg, and lane 7 corresponds to the control.

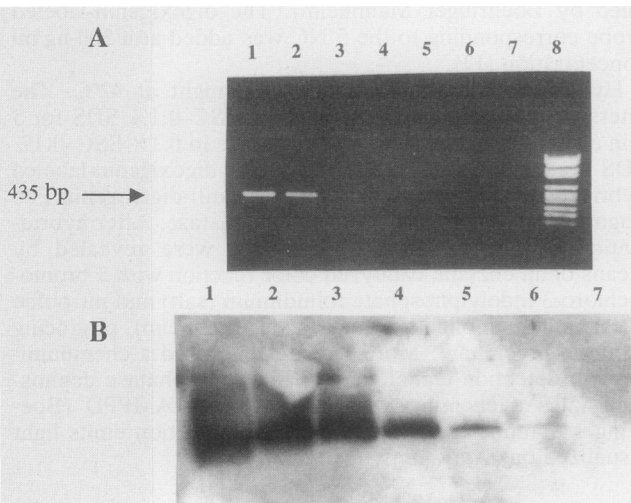


FIG. 1. PCR amplification in the 5'NC region of RNA extracted from CBV3-infected cell culture (2+3 primer pair). PCR products were visualized by agarose gel analysis (A) and Southern blot hybridization (B) as described in Materials and Methods. Lanes 1 to 7 correspond to PCR products (435 bp) derived from amplification of 10-fold dilutions of viral RNA ranging from 40 ng to 40 fg (10⁶ TCID₅₀s to 1 TCID₅₀); lane 8 corresponds to the molecular weight marker (marker VI; Boehringer Mannheim).

mouse models (18, 28). However, by day 14 p.i., the mortality due to myocarditis had decreased and less inflammation of the heart was observed.

Blood and fecal samples were collected every other day for monitoring the infection; the results are summarized in Fig. 3. Mouse droppings were also treated and inoculated to the BGM K cells, and as shown in Fig. 3, viral excretion was observed from days 2 to 9 p.i. Samples from control mice were collected identically, and all were negative for seroconversion and viral excretion. After heart biopsies were inoc-

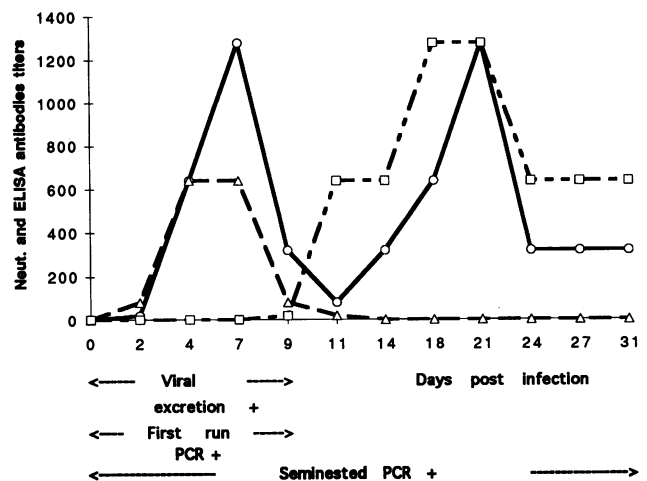


FIG. 3. Follow-up of CBV3 infection in CD1 mice. Blood and fecal samples were collected from days zero to 31 p.i. and were further investigated for serological and excretion studies. ○, neutralizing antibodies; △, ELISA IgM; □, ELISA IgG.

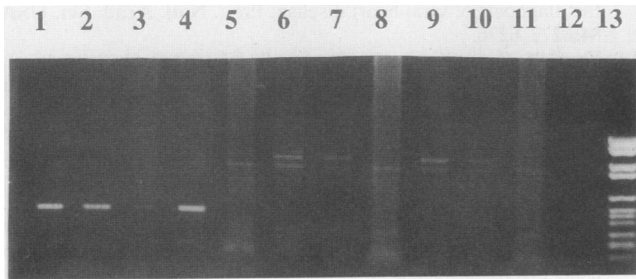


FIG. 4. Gel electrophoresis analysis of PCR products obtained after a first-run amplification (2+3 primer pair) in the 5'NC region of RNA extracted from murine heart biopsies. Lanes 1 to 11 correspond to days 2, 4, 7, 9, 11, 14, 18, 21, 24, 28, and 31 p.i. with CBV3. Lane 12 corresponds to the negative control (distilled water), and lane 13 corresponds to the molecular weight marker (marker VI; Boehringer Mannheim).

ulated to the BGM K cell culture, CBV3 was isolated only on days 2 and 4 p.i. and virus was unculturable during the kinetics of infection for up to 31 days p.i.

Detection of the CBV3 genome in heart biopsies. After SDS and proteinase K digestion, RNA was extracted, cDNA was synthesized, and PCR was performed as described above (see Materials and Methods). Controls were included in each reaction.

The results of 5'NC PCR amplification are shown in Fig. 4 and 5. In the first PCR run, viral RNA was visualized from day 2 p.i. up to day 9 p.i. Seminested amplification was then performed on the first PCR products. As shown in Fig. 5, the 362-bp band was obtained until day 31 p.i. Control animal samples were negative by both PCR procedures.

According to the same protocol, amplification of the heart biopsies was also performed in the VP1 region. The first PCR assay resulted in the detection of the 524-bp band up to day 21 p.i. The seminested PCR assay resulted in the detection of the expected 181-bp band until day 31 p.i.

DISCUSSION

In a previous study, we described the use of enterovirus riboprobes for the detection of enteroviruses in clinical samples. More recently, we and others have developed a PCR procedure for enteroviruses (29). In order to increase

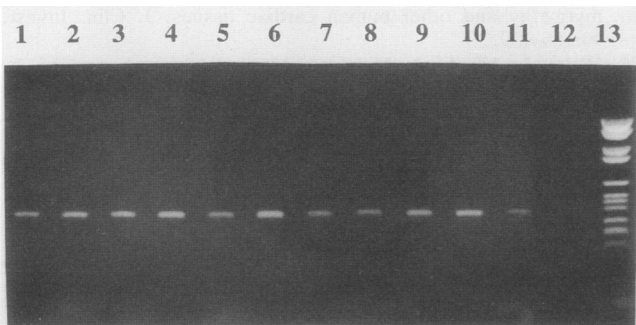


FIG. 5. Gel electrophoresis analysis of PCR products obtained after a seminested amplification (2+F2 primer pair) in the 5'NC region of RNA extracted from murine heart biopsies. Lanes 1 to 11 correspond to days 2, 4, 7, 9, 11, 14, 18, 21, 24, 28, and 31 p.i. with CBV3. Lane 12 corresponds to the negative control (distilled water), and lane 13 corresponds to the molecular weight marker (marker VI; Boehringer Mannheim).

the sensitivity of our PCR assay, we have developed a seminested PCR assay that includes the replacement of one of the primers in the 5'NC or VP1 region.

In our first experiments, we synthesized the cDNA by using the primer corresponding to the 5'NC region. Recently, we decided to use the universal primer dT15, which results in the yield of an equivalent level of cDNA compared with primer 3 but allows the detection of the total intact genome (13a). Indeed, the visualization of the 5'NC amplified band at the opposite side of the 3' poly(A) extremity proves the synthesis of a total cDNA. Therefore, the positive PCR detection involves the presence of an intact potentially infectious CBV3 genome.

In this paper, we have assessed the sensitivity and the specificity of our PCR procedure, but we are aware of the limited accuracy of these evaluations—it is impossible to define precisely the efficacy of the different enzymes involved in the PCR process. Moreover, after a few PCR cycles, the amplification of nonspecific products initially present at low concentrations is observed (8). Therefore, the use of a second primer results in increased specificity; it is also possible to change both primers in a nested PCR assay, as described for cytomegalovirus (5).

According to the estimations of Rotbart, indicating a 100:1 particle/infectivity ratio, our two successive amplifications allow the detection of femtograms of viral RNA corresponding to 1 TCID₅₀ (initial titer, 10⁶ TCID₅₀/5 μl) and thus to 100 RNA molecules (21). Because of the low resolution threshold of ethidium bromide (1 ng) in electrophoresis (23), the hybridization of PCR products with the 5'NC specific riboprobe results in increased sensitivity and control of the specificity of the PCR band. Rotbart described both a limit of sensitivity which was approximately 10 pg of RNA when tested in saline and a lower level of sensitivity when the PCR assay was performed in body fluids (22).

The choice of our primers in the VP1 region appears suitable for VP1 amplification. The sensitivity of both PCR assays was approximately 100 times as high as that of the 5'NC assay: 0.13 versus 40 pg in the first run and 1.3 pg versus 0.4 pg in the seminested PCR.

With our murine model, we followed up the classical virological markers. Viral excretion occurred from days 2 to 9. Viral isolation in the heart was observed only on days 2 and 4 p.i., and despite successive cell passages, no virus could be later isolated. Seroconversion occurs rapidly on the third day p.i. according to the literature (20). The curves of neutralizing and ELISA IgM and IgG antibodies were identical. Interestingly, we observed an apparent disappearance of the immune response corresponding to the end of viral excretion. This could correspond to the aggregation of immune complexes which may be directly or indirectly related to persistence of infection.

The detection of CBV3 viral genomes after the first PCR assay was observed between days 2 and 9 p.i., corresponding to the excretion period due to active viral replication. The seminested PCR assay allows the detection of viral genomes up to day 31 p.i. All control mouse samples were negative by both PCR assays.

We chose primers in the 5'NC and VP1 regions with the intention of subsequently sequencing the PCR products. Indeed, the VP1 region appeared interesting because (i) it shows variability, (ii) it induces the production of neutralizing antibodies, and (iii) it corresponds to the binding site to cell receptors.

These preliminary results show the presence of the viral CBV3 genome in the heart for a period extending to 31 days.

At present, the sequencing of the PCR products amplified in both the 5'NC and VP1 regions is being developed in our laboratory in an attempt to detect possible genomic modifications related to the emergence of viral persistence. We therefore supported the hypothesis of genomic modification of CBV3 during experimental murine infection resulting in viral persistence.

It has been described elsewhere that variants of CBV4 with different tissue tropisms can be isolated from the same subject and are antigenically distinguishable from each other with monoclonal, but not polyclonal, antisera (16). The combination of both the natural high variability of the coxsackieviruses B and several factors including immune responses and genetic factors of the host could result in the emergence of variants.

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