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Genetic analysis of a ganciclovir-resistant human cytomegalovirus mutant

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Abstract

We isolated a ganciclovir (GCV)-resistant human cytomegalovirus (HCMV) from a laboratory strain, AD169, and analysed the mutant. Attempts were also made to identify directly the mutated gene. The 50% inhibitory concentration (IC_{50}) of GCV for the mutant strain was five times higher than that of the wild-type strain. The mutant strain showed similar sensitivity to phosphonoacetic acid and cidofovir as the wild-type strain. These data suggest mutation in the UL97 gene encoding for the phosphotransferase that phosphorylates GCV. Molecular analysis of the mutant strain revealed that a single base substitution of adenine by cytosine occurred at the 1796 nucleotide position of the UL97 gene region, resulting in the substitution of lysine by threonine at codon 599 in the UL97 gene product. Marker transfer experiment confirmed that this mutation conferred HCMV resistance to GCV. The mutation at codon 599 was easily identified by means of *Rsa*I digestion of the selected PCR product.

Keywords: Ganciclovir; Cidofovir; Phosphonoacetic acid; UL97 mutation

1. Introduction

Human cytomegalovirus (HCMV) is a typical opportunistic pathogen which causes severe and often life-threatening diseases in immunocompromised hosts, such as recipients of bone marrow or

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solid organ transplants, and patients with acquired immune deficiency syndrome (AIDS) (Drew, 1988; Erice et al., 1989, 1997). Antivirals currently available for the management of HCMV diseases include ganciclovir (GCV), foscarnet (phosphonoformic acid, PFA) and cidofovir ((*S*)-1-(3-hydroxy-2-phosphononylmethoxypropyl)cytosine, CDV). GCV is a widely used anti-HCMV drug in clinical practice, not only for the induction and maintenance therapy but also for the prophylaxis in high-risk groups for HCMV diseases. PFA is usually used for rescue therapy in patients with no response to GCV treatment (Jacobson et al., 1991). CDV is the most recently approved anti-HCMV drug for treatment of HCMV retinitis in AIDS patients (Lalezari et al., 1997). The prolonged maintenance therapy with anti-HCMV drugs, which is often required in immunocompromised patients, favors the emergence of drug-resistant HCMV strains, as has been demonstrated particularly with GCV.

The viral DNA polymerase is the ultimate target for the currently available anti-HCMV drugs. GCV, a nucleoside analogue, requires monophosphorylation by a virus-encoded UL97 phosphotransferase (Litter et al., 1992; Sullivan et al., 1992). The subsequent phosphorylations are carried out by cellular enzymes (Biron et al., 1985). GCV-triphosphate is the active form which is a substrate and inhibitor for viral DNA polymerase. Therefore, drug-resistant mutants can originate from mutations in either the UL97 phosphotransferase gene, or DNA polymerase gene, or both. CDV, a nucleoside monophosphate analogue, does not require the activation by virus-encoded enzyme to become a substrate or inhibitor of the viral DNA polymerase (Cihlar and Chen, 1997; Xiong et al., 1997). PFA and phosphonoacetic acid (PAA), pyrophosphate analogues, are noncompetitive inhibitors of the viral DNA polymerase. Therefore, resistant mutants to CDV, PFA, or PAA can result from mutations in the viral DNA polymerase gene (UL54). The GCV-resistant mutants derived from the laboratory strain AD169 have mutations in both the DNA polymerase gene (UL54) and phosphotransferase gene (UL97) (Lurain et al., 1992; Sullivan et al., 1993). The GCV-resistant clinical isolates have mutations in the UL97 gene, or UL54 gene (Harada et al., 1997), or both (Erice et al., 1997; Smith et al., 1997). GCV-resistant mutations only in the UL54 gene occur very rarely, in comparison with UL97 mutations and mutations in both UL97 and UL54 (Harada et al., 1997). The majority of clinical UL97 mutations, which still remain sensitive to CDV and PFA, contain a single amino acid substitution (Wolf et al., 1995; Baldanti et al., 1996; Chou et al., 1997), although some contain a deletion.

In this study, we isolated a GCV-resistant HCMV mutant from the laboratory strain AD169 and determined its susceptibility to various antiviral agents. Attempts were made to identify the mutation. Molecular analysis of this mutant revealed a novel mutation in UL97 gene, resulting in an amino acid substitution at codon 599.

2. Materials and methods

2.1. Cell and virus

Human embryonic lung (HEL) fibroblasts, between the 18 and 23 passages, were grown in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (Flow Laboratories Inc., Irvine, VA), kanamycin (60 μg/ml), and 0.10% $NaHCO_3$. Maintenance medium for the cells contained 5%, instead of 10%, fetal bovine serum. HEL cells were used for isolation, propagation and titration of HCMVs. MRC-5 cells were also used for transfection. The laboratory strain AD169 was used for the isolation of GCV-resistant virus.

2.2. Isolation of GCV-resistant mutant

The plaque-purified AD169 strain of HCMV was passaged in the presence of various concentrations (1–10 μM) of GCV. The HCMV which was passaged in the presence of GCV and yielded less than 1/10 of the control virus replicated in the absence of GCV and was used for the next cycle of selection. This cycle was repeated over a period

of 14 months with a final concentration of 10 μM GCV. Stock samples of the GCV-resistant mutant were plaque purified twice and then filtrated through the membrane filter with a pore size of 450 nm to remove multicapsid and aggregated virions. Finally, stock samples were again plaque purified twice.

2.3. Antiviral agents

GCV was generously gifted by Dr K.L. Powell (The Wellcome Research Lab., Beckenham, UK) and CDV was kindly provided by Professor E. De Clercq, Leuven University, Belgium. PAA was purchased from Sigma, St. Louis, MO.

2.4. Susceptibility test of HCMV strains against antiviral agents

The susceptibility of HCMV strains to antiviral agents was determined by plaque-reduction assay. HEL monolayers in 35 mm plastic dishes (IWAKI Glass Co., Funahashi, Japan) were inoculated with approximately 100 plaque-forming units (PFU) of each strain of HCMV. After virus adsorption at room temperature for 60 min, the monolayers were overlaid with maintenance medium containing 2.25% methylcellulose, 0.24% $NaHCO_3$, and different concentrations of each antiviral agent. Duplicate dishes were used for each concentration of the antiviral agent. The dishes were incubated at 37°C for 10 days in a humidified atmosphere containing 5% CO_2. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the agent required to effect a 50% decrease in plaque numbers compared to those in the control.

2.5. Preparation of DNA

Viral DNA was prepared according to the Hirt's procedure (Hirt, 1967). The Hirt's supernatant was treated with proteinase K (50 μg/ml) at 56°C for 2 h, followed by phenol:chloroform extraction and ethanol precipitation. Finally, DNA was dissolved in 10 mM Tris–HCl and 1 mM EDTA (TE) buffer. For marker transfer experiments, wild-type virions of the AD169 strain were prepared as described by Ihara et al. (1994). The viral DNA was treated as mentioned above.

2.6. Amplification of viral DNA fragments by polymerase chain reaction (PCR)

The primers were designed to amplify the two overlapping fragments of the UL97 coding region. Each primer was labelled with biotin at the 5′ end for non-radioisotope single strand conformation polymorphism (SSCP) analysis and sequencing. The sequences of the primer pairs were as follows; 5′-TACGGCGTTATTGCATGT-3′ (VS977) and 5′-ATTCGTGCAGCATGGTCT-3′ (VS976) for fragment I (nucleotide No. 1634–1990), and 5′-ATCACCAGTGTCGTGATAGC-3′ and 5′-CGACATGCAATATCGAAGTA-3′ for fragment II (nucleotide No. 1340–1654). The schematic diagram indicating the target region for the primers is shown in Fig. 1. The PCR reaction mixture was consisted of viral DNA as template, appropriate primer pairs (1 μM each), deoxyribonucleoside triphosphates (200 μM each), and Taq polymerase (Takara Shuzo Co., Kyoto, Japan) in a total volume of 100 μl 1 × PCR buffer. The fragment was amplified by 35 cycles of denaturation at 94°C for 2 min, annealing at 37°C for 2 min, and extension at 72°C for 3 min.

2.7. Single strand conformation polymorphism analysis and sequencing

For SSCP analysis, the PCR product was diluted to 1/10 or 1/100 with a loading buffer consisting of 95% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. After heating at 90°C for 2 min, 1 μl of the mixture was subjected to gel electrophoresis in 5% polyacrylamide gel containing 5% glycerol as described by Orita et al. (1989). After electrophoretic separation, DNA was transferred to positively charged nylon membrane by capillary blotting and radiated by imaging high-chemiluminescent detection kit according to instructions of the manufacturer (TOYOBO Co., Ltd., Osaka, Japan). For sequencing, the PCR product was purified from agarose gel using QIAX II Extraction Kits (QIAGEN GmbH and QIAGEN Inc.,

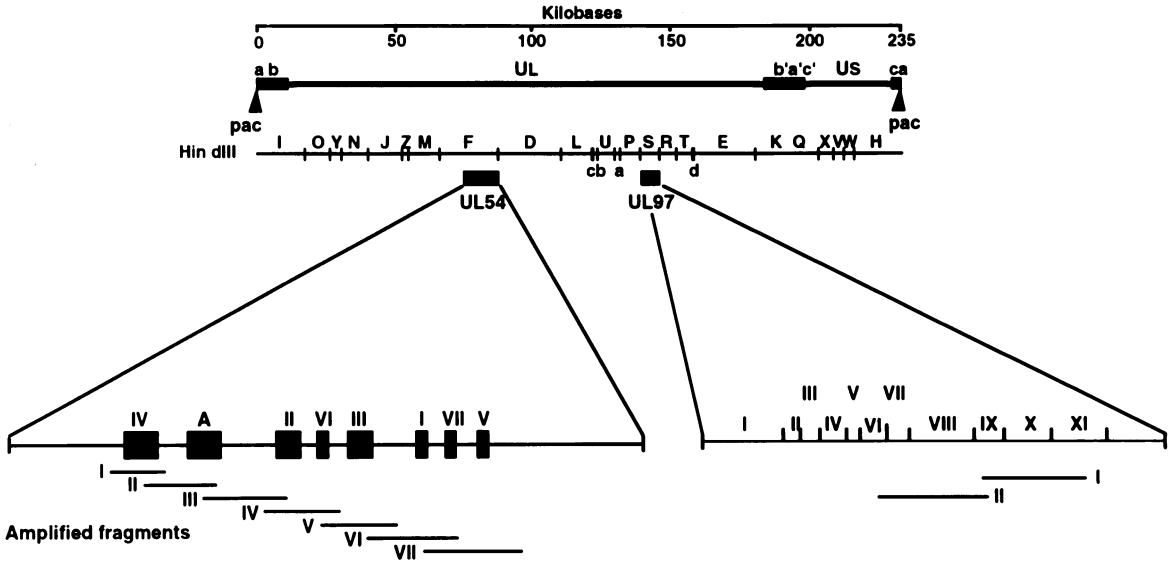

Fig. 1. Schematic representation of the HCMV (AD169) gene showing the location of the UL54 and UL97 genes and the approximate positions of the primers used in PCR. The top line is a size scale in kilobase pairs. The second line shows the unique sequences flanked by inverted repeats (boxed areas) and packaging signals (pac) are indicated near the termini. UL, unique long sequence; US, unique short sequence. The third line shows the restriction enzyme profile of the HCMV DNA cleaved with *Hin*dIII. The location of UL54 and UL97 genes are shown below the *Hin*dIII map. The bottom line illustrates the schematic diagram of UL54 and UL97 gene regions showing the location of the amplified fragments in relation to the conserved and catalytic domains, respectively.

Hilden, Germany). Sequencing of the PCR product was carried out by dideoxy termination method using Δ*T*th DNA polymerase Sequencing High-Cycle kit (TOYOBO Co., Ltd., Osaka, Japan). The cycling parameters were as follows: denaturation at 94°C for 30 s, annealing at 37°C for 60 s, extension at 72°C for 90 s, and 35 cycles. The sequencing products were subjected to electrophoresis in 8% polyacrylamide gel containing 8.3 M urea. After electrophoretic separation, the DNA was transferred and radiated as mentioned above. Sequencing experiments were carried out twice.

2.8. Restriction fragment length polymorphism (RFLP) analysis

The PCR product was digested with *Rsa*I (Nippon Gene Co., Toyama, Japan) and subjected to 2% agarose gel electrophoresis. The electrophoretic pattern was photographed under UV-light.

2.9. Marker transfer experiments

The DNA fragment containing mutation was amplified by PCR and directly cloned into pCR®2.1 Vector using the Original TA Cloning® Kit according to the instructions of the manufacturer (Invitrogen Co., Carlsbad, CA). MRC-5 cells were transfected simultaneously with plasmid DNA and viral DNA using LipofectAMINE PLUS™ Reagent (Life Technologies, Inc., Gaitherburg, MD) according to the instructions of the manufacturerf. After the appearance of cytopathic effects (CPE), the virus was released from the infected cells by sonication.

Then, the virus was cultured twice in the presence of the 10 μM GCV, and finally plaque-purified

twice. The location of the mutations was confirmed by sequencing.

2.10. Growth curve

The HEL monolayers in culture tubes were inoculated with the laboratory strain AD169 or GCV-resistant HCMV strain at a multiplicity of infection (M.O.I.) of 0.2. After adsorption at room temperature for 60 min, the inocula were aspirated off and 1 ml of maintenance medium was added to each culture tube. The culture fluid in duplicate was collected everyday and stored at −80°C until titrated for extra-cellular virus. The infected cells were washed once with maintenance medium and resuspended in the same volume of medium. Then, the infected cells were disrupted by ultrasonic treatment with a Branson 1200 (Branson Ultrasonic Division, Emerson-Japan) at an output of 60 W for 20–30 s and centrifuged at 2000 rpm for 10 min. The supernatant was stored at −80°C until titration for intracellular virus. Extra- and intracellular viruses were plaque titrated separately and then added up.

3. Results

3.1. Isolation of GCV-resistant mutant

A GCV-resistant virus which can replicate in the presence of 10 μM was isolated from the HCMV laboratory strain AD169. This virus was plaque purified twice and used as the GCV-resistant strain throughout our experiments.

3.2. Susceptibility of the selected mutant against various agents

The susceptibility of wild-type HCMV strain AD169 and the mutant strain against various antiviral agents was determined by 50% plaque-reduction test. The IC_{50} of GCV for the mutant strain was 10 μM, that is five times higher than that of GCV for the wild-type strain (Table 1). However, both strains showed the same degree of susceptibility to PAA and CDV, suggesting that the mutations occurred in the UL97 gene region.

3.3. Molecular analysis of viral UL97 coding region

The UL97 coding regions of both the wild-type and mutant HCMV strains were compared by PCR–SSCP analysis. Of two fragments amplified by PCR using biotin-labelled primer pairs, one fragment pair, nucleotide range from 1634 to 1990, showed the different mobility in SSCP analysis. Then, the fragment was sequenced to identify the mutations. When the sequences for the wild-type and mutant were compared, the mutant had a single base substitution (change from A to C) at nucleotide position 1796, resulting in an amino acid change at codon 599 of lysine by threonine. To exclude the possibility of any mutation in the DNA polymerase gene, seven overlapping fragments were also examined by PCR-SSCP analysis. However, there was no difference in the mobility between wild-type and mutant strains (data not shown).

3.4. Identification of mutation site by PCR and RFLP

Identification of the mutation at codon 599 was accomplished by means of RFLP of the selected PCR product, nucleotide range from 1634 to 1990. In wild-type strain this region contained one GTAC recognition site for *Rsa*I. In the mutant strain, however, the sequence GTAA had changed to GTAC, which produced an additional recognition site for *Rsa*I. Consequently, the codon 599

Table 1
Susceptibility of wild-type and mutant HCMV (AD169) strains to various agents

Antiviral agents	IC_{50} (μM)		Mutant
	Wild-type strain	Mutant strain	Wild-type
Ganciclovir	1.8	10	5.6
Phosphonoacetic acid	20	22	1.1
Cidofovir	0.12	0.17	1.4

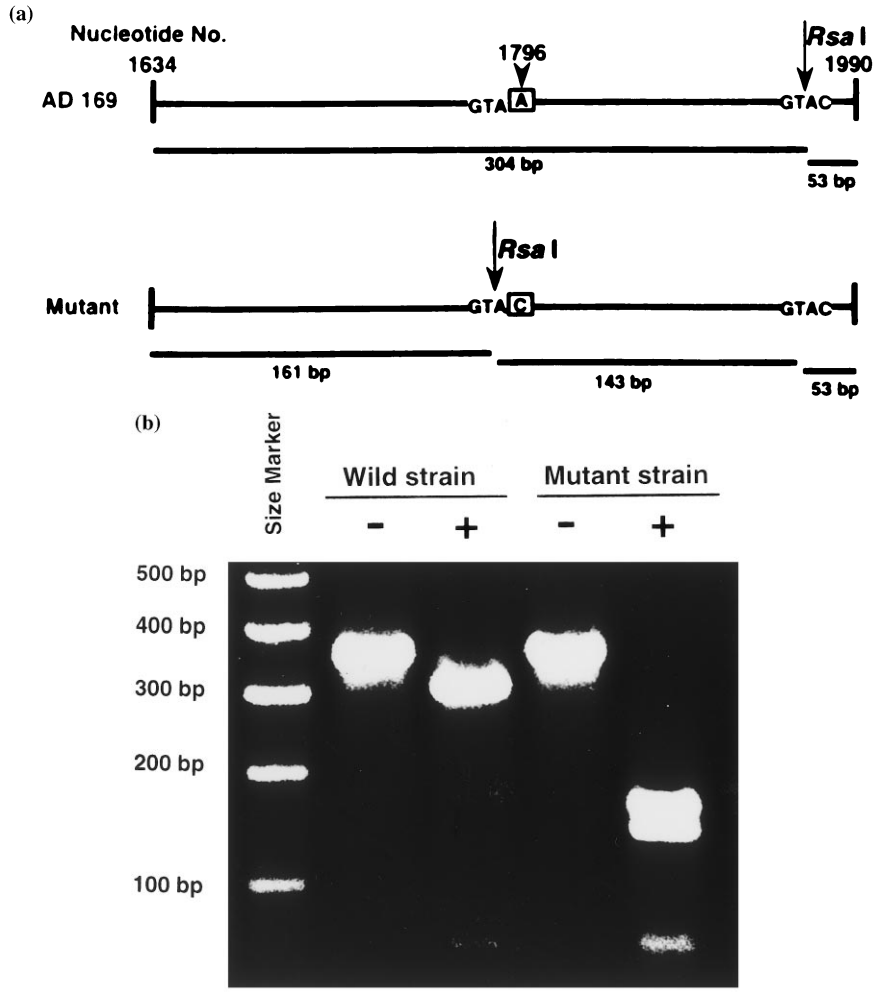

Fig. 2. Rapid screening of the mutation at codon 599. (a) Schematic representation of *Rsa*I cleavage sites of wild-type and mutant strains. (b) The selected PCR products from wild-type and mutant strains were digested with (+) or without (−) *Rsa*I and subjected to 2% agarose gel electrophoresis.

AAG (lysine) had changed to ACG (threonine). The PCR products amplified from wild-type and mutant strains were digested with *Rsa*I and subjected to 2% agarose gel electrophoresis. The PCR product from the wild-type strain produced two bands, 304 and 53 bps. The PCR product from the mutant strain produced three bands, 161, 143 and 53 bps as shown in Fig. 2(a) and (b). Therefore, the mutation was easily detected by digestion with *Rsa*I.

3.5. Marker transfer of the mutant to wild-type AD169 background

The plasmid DNA containing the mutated UL 97 region, together with the DNA of wild-type strain AD169, was transfected to MRC-5 cells. Recombinant virus was selected twice in the presence of 10 μM GCV. The IC_{50} of the recombinant virus was 9.6 μM, almost equivalent to that of the mutant strain. In addition, the recombinant virus

contained the same mutation in UL97 gene as the mutant did (data not shown).

3.6. Replication of the wild-type strain and mutant AD169 strains in HEL cells

To investigate whether there was any biological difference between the two strains, the replication of these strains in HEL cells was compared by plaque titration (data not shown). The progeny viruses of both strains appeared on day 3 post infection. Replication of the GCV-resistant strain was only slightly slower compared to that of the wild-type strain. The yield of progeny GCV-resistant virus strain at 5 days post infection was 2.6×10^6 PFU as compared to 3.2×10^6 PFU for the wild-type strain. Plaques of the GCV-resistant strain were also slightly smaller than those of the wild-type strain AD169. Overall, however, there was no marked difference in replication pattern between the two strains.

4. Discussion

We isolated and characterized a GCV-resistant mutant of HCMV from the laboratory strain AD169. Drug-susceptibility tests showed that the GCV-resistant mutant had the same sensitivity to CDV and PAA as the wild-type strain. The pattern of drug sensitivity thus found points to the UL97 gene region as the site of the mutation. In fact, molecular analysis of the mutant revealed a novel mutation in the UL97 gene, namely, a single base substitution (from A to C) at nucleotide no. 1796 in the UL97 gene region with an amino acid substitution (from lysine to threonine) at codon 599. It was confirmed by marker transfer experiments that this mutation conferred resistance of HCMV to GCV.

The GCV-resistant mutations in the UL97 gene reported so far resulted from point mutations (or deletions) at codon 460, 520, 590, 591, 592, 594, 595, 596, 600, 603, 607, 665 (Stanat et al., 1991; Sullivan et al., 1992; Lurain et al., 1994; Alain et al., 1995; Baldanti et al., 1995a,b; Hanson et al., 1995; Wolf et al., 1995; Baldanti et al., 1996; Chou et al., 1995a,b; Baldanti et al., 1996; Chou et al., 1997; Erice et al., 1997; Smith et al., 1997). Mutations in the UL97 gene which confer GCV resistance cluster in two regions that are highly conserved in protein kinases (Hanks et al., 1988). One of these is designated subdomain VI, a region which possesses an ATP-binding site and may play a role in substrate recognition. The mutation at codon 460 is the most prevalent one in this region. The second region of importance is designated subdomain IX. Although its functional role remains unclear it may have substrate-inhibitor binding activity (Hanks et al., 1988). In this subdomain the mutations at codons 594 and 595 are prevalent. In our GCV-mutant, the mutation had occurred at codon 599 within the catalytic domain of UL97 gene, where GCV-resistant mutations are usually found. No mutation was found in the DNA polymerase gene region. Thus, it is tempting to conclude that the GCV resistance of our mutant is due to the codon 599 mutation in the UL97 region.

It has been suggested that the UL97 gene function may be essential for the replication of HCMV. Actually, UL97-deficient HCMV, obtained by recombination was shown to be growth-defective (Michel et al., 1996). In our experiments, GCV-resistant HCMV had a mutated UL97 gene but still showed replicative ability comparable to that of the GCV-sensitive wild-type. Our mutant was selected by serial passages in the presence of GCV, and the mutation was obviously nonlethal. Thus, it is conceivable that a point mutation in the UL97 gene confers GCV resistance and the mutated UL97 gene produces a protein that remains functional in HCMV replication. However, the role of the UL97 gene product in HCMV replication or pathogenesis still remains to be elucidated, and analysis of the various HCMV mutants may foster our insight into the function of the UL97 gene region.

Drug susceptibility tests include: plaque-reduction assay, yield reduction assay (Rasmussen et al., 1984), DNA hybridization assay (Danker et al., 1990), and in situ enzyme immunosorbent assay (Tatarowicz et al., 1992). A late antigen reduction assay (Pepin et al., 1992) allows rapid and direct determination of HCMV susceptibility to GCV and gives a clear distinction between drug-sensitive and -resistant strains of HCMV. The major limita-

tion of these methods is the necessity for sufficient amounts of cell-free HCMV. The amplified fragment which contains mutations can be detected by SSCP analysis. SSCP followed by restriction enzyme cleavage provides a rapid sensitive detection of certain mutations in PCR-amplified DNA fragments, although negative results of this method do not necessarily mean that the virus does not carry any UL97 drug resistant mutation. In our case, the mutation at codon 599 (AAG to ACG mutation) was detected by *Rsa*I digestion (recognition sequences GTAC). However, this identification is specific only for AAG to ACG mutation. If the mutation were not AAG to ACG, it would not have been detected by this method. However, the A to C mutation nucleotide No. 1796 could be specifically detected by the ligase chain reaction (Bourgeois et al., 1997).

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