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Optimized nested PCR enhances biological diagnosis and phylogenetic analysis of human parvovirus B19 infections

Maxime Pichon^{1,2} · Clément Labois¹ · Véronique Tardy-Guidollet³ · Delphine Mallet³ · Jean-Sébastien Casalegno^{1,2} · Geneviève Billaud¹ · Bruno Lina^{1,2} · Pascal Gaucherand⁴ · Yahia Mekki¹

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Abstract

Diagnosis and epidemiological analysis of human parvovirus B19 (hB19V) infections are essential for disease management in severely ill patients. This study aimed to evaluate the performance of an optimized NS1-VP1u nested PCR for detection and sequencing of viruses in clinical samples using 224 clinical and five reference samples. PCR sensitivity, specificity, and positive and negative predictive values were perfect (100%). While phylogenetic analysis of a 615 bp-long fragment demonstrated that the viruses in all of the samples belonged to genotype 1, this study confirmed that this optimized PCR could detect all known hB19V with high performance.

Human parvovirus B19 (hB19V) is a small (20-nm-diameter) non-enveloped virus that belongs to the genus *Erythro-parvovirus* [1]. Like bocaviruses, which belong to the same family, hB19V is known to be pathogenic in humans [2, 3]. hB19V possess a single positive or negative-stranded DNA genome of about 5,600 nucleotides. This genome encodes three major proteins: one nonstructural protein (NS1) and two structural proteins (VP1 and VP2) [4]. Composed of 671 amino acids, NS1 is involved in the transactivation of proteins required for viral DNA replication and is known to have pro-inflammatory properties [5]. The VP1 unique

region (“VP1u”) is a 277-amino-acid-long region of VP1 that differs from VP2 [6]. This region is exposed on the surface of infected cells and is the target of neutralizing antibodies in humans [7]. It has also been shown to be useful for phylogenetic analysis of hB19V strains [8].

Different routes of transmission have been described, with respiratory infection being the most common, but par-enteral (through blood transfusion) and vertical (maternofetal) transmission have also been observed [9–11]. One of the most important consequences of hB19V infections is that they can progress to severe disease in pregnant women, leading to lethal fetal hematologic failure [12]. hB19V targets erythroid progenitor cells and can be measured in burst-forming units and colony forming units [13]. hB19V infection is responsible for a large spectrum of clinical manifestations among children, ranging from the classical “fifth disease” to complete red cell aplasia in immunosuppressed patients or chronic hemolytic anemia [14]. Reports have suggested that hB19V infections can be associated with several diseases, such as arthritis, hepatitis, myocarditis and encephalitis, especially in young children [15–17]. Fortunately, most of the patients develop protective and lifelong immunity during childhood [18].

Although serology remains the diagnostic procedure used in numerous laboratories, molecular testing has become the most useful strategy for this purpose. Some molecular biology testing procedures demonstrate good but not perfect performance and do not allow analysis of the amplicons that are generated [19]. A nested PCR, which enhances analytical

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performance and generates amplified cDNA, is well suited to clinical and epidemiological applications.

Optimal management of severe disease associated with hB19V infection requires rapid and efficient diagnostic tools. The aim of this study was to evaluate an optimized version of the NS1-VP1u nested PCR assay for the detection of hB19V infections in clinical samples and reference samples that can be used to obtain sequences suited for phylogenetic analysis.

Samples (blood [n = 189], amniotic fluid [n = 17], necropsy [n = 10], placenta [n = 6] and bone marrow [n = 2]) from 195 patients, representative of the samples from immunosuppressed patients and pregnant women received by the virology laboratory of the University Hospital of Lyon, France, were collected between January 2005 and June 2012. All routine laboratory results were obtained using the Parvo B19 PCR Kit (Abbott Laboratories, IL, USA), and since no samples were excluded because of a viral load, these samples were representative of the epidemiological range.

WHO reference samples (n = 3) and several hB19V variants of the French NRC-V9 strain (n = 2) were also tested, in order to confirm the range of PCR detection and sequencing of the samples [20].

To confirm absence of cross-contamination or false amplification during the analytical step, three negative controls were added to the cohort and subjected to the same analytical process.

After routine management of the clinical samples (analysis within 5 days after reception at room temperature), all clinical specimens were stored at -20 °C until inclusion in this study. DNA was extracted from samples using the EasyMag platform (bioMérieux, Marcy l'Étoile, France) according to the manufacturer's instructions. Extracted DNA was stored at -20 °C before amplification.

All of the samples were tested by three different methods (targeting the NS1 gene of hB19V): a commercially available real-time PCR carried out as suggested in the package insert ("cPCR", Parvo B19 PCR kit, Abbott Laboratories, IL, USA), a nested PCR carried out exactly as published previously by Servant *et al.* ("nPCR"), and the optimized version of the NS1-VP1u PCR ("nnPCR") [20]. The final volume was 50 µl for nPCR and nnPCR and 25 µl for cPCR, including viral extracts, divalent ions, dNTP, primers and polymerase.

NS1-VP1u gene sequences from hB19V genotypes 1 (GenBank accession numbers AF162273 and AY504945), 2 (GenBank accession number AY064476) and 3 (GenBank Accession numbers AY582124 and AY582125)) were used to design primers with AmplifX software v1.5.4. These primers were tested against recently published sequences of hB19V to assess their performance.

For the nested PCRs, the forward and reverse primers for the first step were 5'-CACAAAGCTGGGACCACT

ATGAA-3' and 5'-GCGACTTCCGGTAAACTTCCTT-3', respectively. The amplicons produced in this step were 1253 bp long (nt 2020 to 3273 in hB19V genotype 1 sequence). For the second step, the forward and reverse primers were 5'-TTGTACGCTAACTTGCCCAGGCTT-3' and 5'-CAT TTGTCGGAAGCCCAGTTTCCT-3', respectively. The amplicons produced during this step were 699 bp long (nt 2272 to 2971, corresponding to a portion of the non-structural protein NS1 gene in the hB19V genotype 1 sequence).

The thermocycling parameters for the first step were 94 °C for 6 min, 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and finally, 72 °C for 7 min. For the second step, they were 6 min at 94 °C, 50 cycles of 30 s at 94 °C, 30 s at 50 °C and 50 s at 72 °C, and finally, 72 °C for 7 min. All amplicons were then kept at 4 °C in the thermocycler before gel-detection of the final amplicons.

Finally, all positive amplicons were sequenced. After purification on a column (Healthcare Bio-Sciences, Uppsala, Sweden), hB19V amplicons were sequenced using an Applied Biosystems 3130 Genetic Analyzer (Life Technologies, CA, USA) in accordance with the manufacturer's instructions.

The biological gold standard was defined as a "concordant" positive or negative result obtained using at least two molecular tests. All non-concordant samples were analyzed by Sanger sequencing, when amplicons were available, on an Applied Biosystems 3130 Genetic Analyzer (Life Technologies) to confirm the presence or absence of the virus genome and to verify the phylogeny of the examined cohort. Note that cPCR did not produce usable amplicons, and therefore isolated samples that were positive by cPCR could not be verified.

The sequences that were obtained were analyzed using Molecular Evolutionary Genetic Analysis (MEGA) v6.06 software. Consensus nucleotide sequences were aligned using the ClustalW package, and all sequences with insufficient length (< 50% of the longest sequences) were excluded from the phylogenetic analysis. Phylogenetic trees were constructed using the Bio-NJ method with 1,000 bootstrap replicates. Sequences were used for clustering (GenBank accession numbers: i/ genotype 1A: AF162273; AY504945; DQ225151; DQ408301; NC_000883; and Z68146; ii/ genotype 1B: DQ357064 and DQ357065; iii/ genotype 2: AY064476, DQ333426 and DQ333428; iv/ genotype 3A: AY582125 and DQ234769; and v/ genotype 3B: AY582124, DQ234778, DQ408304). The five reference samples (three WHO and two French national references) were sequenced at the same time to enhance the quality of the phylogenetic analysis. Trees were rooted using a bocavirus sequence [GenBank accession number KP710213].

Performance parameters such as sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and accuracy (Ex) were evaluated

using GraphPrism software v6.0. In addition, concordance between molecular techniques (cPCR, nPCR, and nnPCR) and sequencing was evaluated using the Cohen kappa test. Confidence intervals for sensitivity and specificity were determined by the Wilson score method. Confidence intervals for positive and negative predictive values were calculated by the method described by Simel *et al.* [21].

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was presented to the institutional medical ethical committee (Hospices Civils de Lyon, France). For this type of study, formal consent is not required. All clinical specimens, collected as part of standard clinical procedures, were obtained independently from patients and de-identified prior to this study.

From January 2005 to June 2012, 2793 samples from patients with suspected HB19V infection were received by the laboratory at Hospices Civils de Lyon, France, for molecular diagnosis. From among these clinical samples, 224 were selected randomly after being tested in routine practice by cPCR. Examination of the patients' clinical characteristics showed that the analyzed population was representative of the one undergoing hB19v diagnosis (mainly immunosuppressed patients and pregnant women; 66.5%, 149/224; Table 1). Samples were tested using nPCR and nnPCR, and the results were validated by Sanger sequencing. Reference samples (n = 5) were added to the cohort to assess the detection of all the currently circulating genotypes. All the reference samples were detected by both nnPCR and nPCR. Negative samples (n = 3) were added to the cohort and remained negative at the end of the study (absence of false amplification or cross-contamination).

Concordance between the cPCR or nPCR assay and the nnPCR was evaluated, as was the performance of the three tests (Table 2). No determination of Sp and PPV was possible with the ParvoB19 PCR kit because 28 of the positive samples could not be sequenced due to the absence of usable amplicons. Of the remaining clinical samples, 170 were detected by all of the techniques, whereas 13 were not detected by nPCR, four by cPCR, and two by neither cPCR nor nPCR. The cPCR assay had an Se and NPV of 95 and 91%, respectively, while the nPCR assay had an Se, Sp, PPV and NPV of 91, 100, 100 and 84 %, respectively, and the nnPCR assay had Se and NPV values of 100%. Accuracy, *i.e.*, the proportion of correct results given by the molecular test, was estimated to be 0.94 and 1.00 for nPCR and nnPCR, respectively.

Phylogenetic analysis of the 615-bp amplicons was done on 104 available samples (53.3%) after exclusion of the samples that did not fulfill the quality requirements. As described previously, clustering was consolidated by using

Table 1 Clinical characteristics of the study population. Blood and amniotic fluid were obtained by puncture during normal clinical management. Necropsies were heart (n = 8) or nervous system necropsies (n = 2). Immunosuppressive diseases included cancers, hemopathy, AIDS-stage HIV infection, and nephritic syndrome. Immunosuppressive therapies were cancer chemotherapies and steroids. Anemia was defined as a hemoglobin count below normal values for the patient's gender and age. Neurological symptoms were represented by paralysis, paresthesia, fainting and convulsions. Cardiologic symptoms included heart failure (including abnormal ECG) or rhythm-related disorders. Respiratory symptoms were defined as respiratory distress or radiological signs

		Number of samples (n = 224)
Median age (min-max)		33 (0-83)
Gender (male/female)		64/131
Sample type	Blood (%)	189 (84.4 %)
	Amniotic fluid (%)	17 (7.6 %)
	Necropsy (%)	10 (4.5 %)
	Placenta (%)	6 (2.7 %)
	Bone marrow (%)	2 (0.9%)
Immunomodulation	Pregnancy (% among women)	68 (51.9 %)
	Graft (%)	36 (16.0 %)
	Immunosuppression (%)	45 (20.1%)
Symptoms	Anemia (%)	37 (16.5 %)
	Respiratory symptoms (%)	24 (10.7 %)
	Dermatological symptoms (%)	21 (9.4 %)
	Cardiologic symptoms (%)	18 (8.0 %)
	Neurological symptoms (%)	14 (6.3 %)
	Arthralgia (%)	12 (5.4 %)
	Death (%)	4 (1.8 %)

reference sequences (n = 17) from the GenBank database and by sequencing WHO and NRC samples. Analysis of the sequences showed that only genotype 1 was present in the infected population from Lyon (Fig. 1). Sequences were deposited in the GenBank database (accession numbers MN088124 - MN088309)

This is one of the largest studies aiming to develop a robust diagnostic PCR able to produce usable amplicons for sequencing in cases of suspected hB19V infection.

Although hB19V has been known for years, molecular diagnosis needs to be optimized in order to improve clinical detection and management of hB19V infection [22]. As long as the safety of blood transfusions and blood-derived products cannot be fully ensured, detection of these viruses could have an important impact in immunosuppressed patients, pregnant women and recipients of blood or blood components [22–24]. Indeed, detection of hB19V remains a major public-health need. It has been reported previously that there are inconsistencies in the detection of different

Table 2 Performance of the three tested PCR tests. **a)** Analytical performance. ND: No determination of Sp and PPV was possible due to the exclusion of test-positive sample (n = 28) that were positive for cPCR but could not be sequenced). †, significant difference compared to nnPCR; ‡, significant difference compared to nPCR; •, significant difference compared to cPCR. All samples used to measure the per-

formance of the nnPCR satisfied the criteria of the study reference. **b)** Comparison of each technique with sequencing and comparison between techniques. nnPCR demonstrated higher concordance with Sanger sequencing than both cPCR and nPCR, showing very robust results. Concordances between all three methods, compared two by two, were not statistically different

a)	cPCR (n = 187)	nnPCR (n = 195)	nPCR (n = 195)
Sensitivity (95% CI)	95% (90-98)‡	100 (97-1.00)‡	91 (84-95)†
Specificity (95% CI)	ND	100 (95-1.00)	100 (94-100)
Positive predictive value (95% CI)	ND	100 (95-1.00)	100 (97-100)
Negative predictive value (95% CI)	91 (83-96)†	100 (97-1.00)‡•	84 (74-90)†
b)	Kappa value		Confidence interval
Sequencing versus cPCR	0.908		[0.845; 0.9]
Sequencing versus nPCR	0.85		[0.772; 0.928]
Sequencing versus nnPCR	1		[0.999; 1]
cPCR versus nPCR	0.811		[0.726; 0.896]
cPCR versus nnPCR	0.93		[0.875; 0.985]
nPCR versus nnPCR	0.858		[0.784; 0.932]

hB19V genotypes by commercially available assays [25, 26]. These data underscore the wide variation in their ability to detect different hB19V genotypes. Although genotype 1 is the main circulating genotype, reliable detection must be ensured in cases of travelers or new variants. Moreover, sequence determination could be of interest to ensure that the clinical impact not be associated with a particular genotype or mutation.

In our study, variations in performance can be explained by cycling temperature, DNA input, and the number of cycles for each protocol. As the nnPCR was performed using an nPCR thermocycler protocol, only the primers differed between the two methods. The nature of the PCR (nested *versus* conventional) as well as the lower melting temperature (50-55 °C *versus*. 60 °C) used in the nPCR and the nnPCR could have an important impact on specificity when compared to cPCR. Because of the reference criteria used in this study (concordance of the two molecular tests or sequencing), the specificity of cPCR could not be analyzed. To our knowledge, the performances of cPCR have not been evaluated previously, and this study is the first to describe these differences. Nevertheless, in the manufacturer's notes, it is stated that the positive and negative concordance between cPCR and nPCR was found to be 90.4% [82.1; 95.0] and 99.2% [97.0; 99.9), respectively. Moreover, the use of fewer cycles in this cPCR can explain its lower performance when compared to nnPCR (but not the differences with nPCR). Finally, a lower volume of viral extract is needed for nPCR and nnPCR than for cPCR (10 µL *versus* 20 µL), permitting optimization of the sample, especially in fetal sample management, in which only a limited volume of sample is available. Moreover, although nested

PCR could be associated (by design) with contamination, we have shown that, with respect to standard-of-care practices, this problem could be overcome, and good performance was achieved. One limitation is the absence of confirmation of the false or true positive nature of the discordant samples that were found positive by cPCR alone because this test did not permit the use of the generated amplicons for sequencing.

This study demonstrates the quality of the optimized NS1-VP1u nested PCR, an nnPCR, (Se, Sp, PPV and NPV showed all perfect performance). This study showed variable performances for nPCR and the cPCR kit. After confirmation by sequencing, thirteen samples tested positive by cPCR and nnPCR only. Four samples were found positive by nPCR and nnPCR only. As described above, two additional samples tested positive by nnPCR, and 169 samples were found positive by the three tested PCRs. In our university hospital laboratory, 1,723 samples (1,723/3,530; 48.8%) were analyzed for parvovirus and remained negative when tested by the routine procedure using cPCR. Because nnPCR identified six additional positive samples from this experimental cohort (verified by sequencing), we can assume that the application of nnPCR in routine practice would have permitted a correct diagnosis for 3.1% cPCR false-negative samples during normal sample management. Theoretically, more than 53 additional samples in which virus was not detected by cPCR would have been correctly identified, leading to appropriate modification of the clinical management. This has important clinical implications for patient management. Indeed, patients who are affected by this diagnosis are often immunosuppressed, have had an organ graft, or are pregnant. Viral detection would allow better

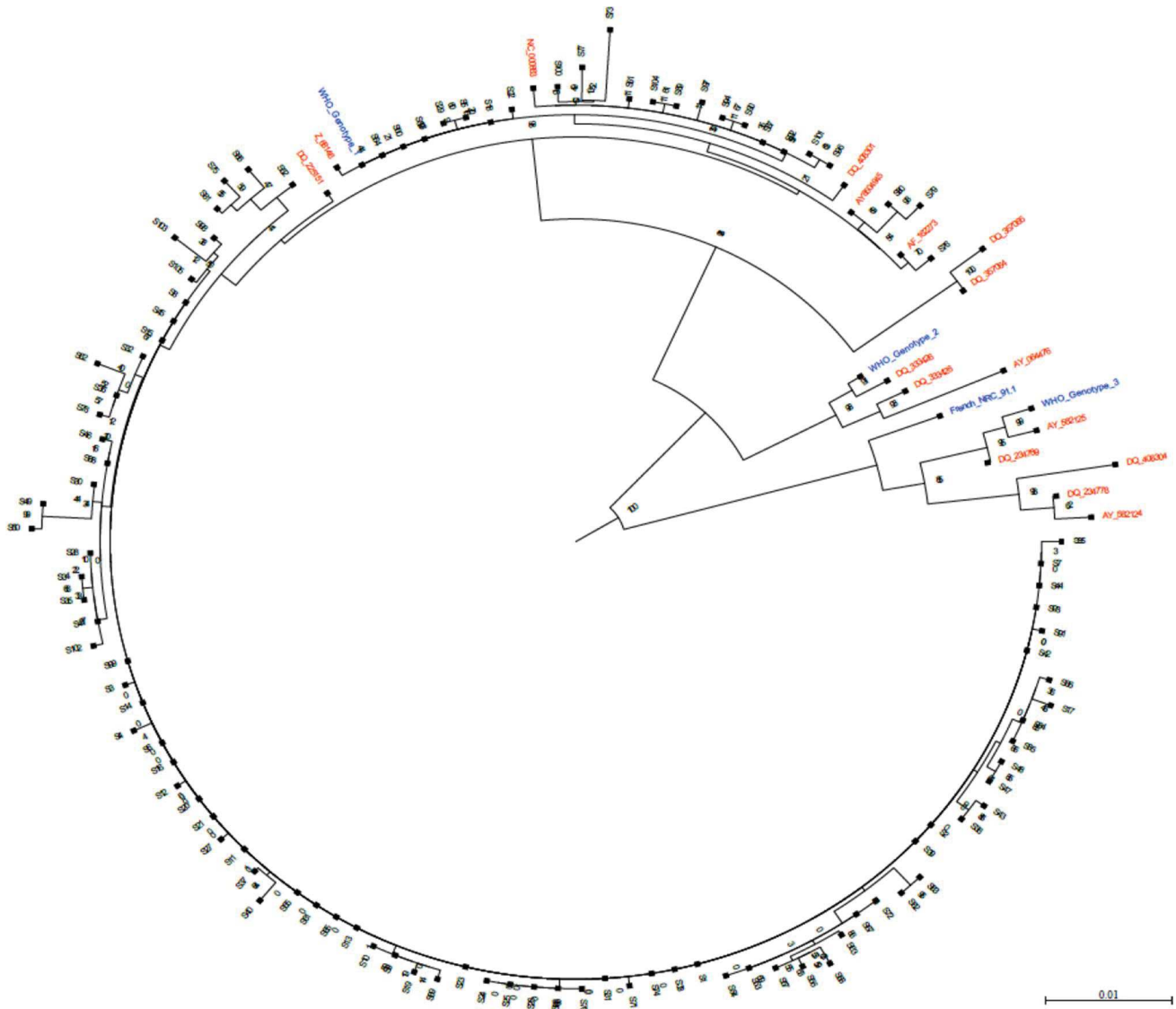


Fig. 1 Phylogenetic representation of sequenced samples. Phylogenetic analysis was performed using the BioNJ method. Bootstrap values (1000 replications) are indicated at the nodes. Sequences identified with “WHO” or “NRC” correspond to reference samples

sequenced in this study. Clinical sequences are shown in black. WHO reference samples and reference sequences are shown in red and blue, respectively

management of these patients, including therapeutic modifications (transfusion, limitation of immunosuppression, or polyclonal immunoglobulin administration) by highlighting the cause of some clinical symptoms (e.g., dermatologic rash and neurological signs) [27]. Although the “Society of Gynecologists and Obstetricians of Canada” recommends testing for hB19V only in women suspected of infection or with an a history of contact with a patient currently infected with B19V, this society has also suggested that optimization is required in order to apply biological diagnostic tests systematically during follow-up of pregnant women [28]. Efficient diagnostics are the key not only to limiting unnecessary testing (e.g., testing for genetic pathologies) but also

to limiting the psychological impact (during limitation of therapy) of the therapeutic intervention [29, 30]. In immunosuppressed patients (transplant recipients, AIDS patients, and patients receiving cancer therapy), improved monitoring of the patient’s hB19V status would facilitate the adaptation of immunosuppressive therapy and proper application of symptomatic treatment such as transfusion.

Finally, this optimized nested PCR assay could help to optimize hB19V epidemiological follow-up. The primers used in this assay can be used to generate hB19V sequences for phylogenetic analysis. Although, Sanger sequencing showed that all of the positive samples from this French cohort clustered with hB19v genotypes 1A and 1B, nested

endpoint PCR remains a useful method for diagnosing viral infections for possible epidemiological analysis.

Despite some limitations, this remains one of the most important studies with the aim of improving viral diagnosis of hB19V. Clinicians need an efficient diagnostic procedure to optimize management of patients. Biological diagnosis is an important part of these decisions, especially during hB19V infections. Although the primers have been verified using WHO and French NRC reference samples for the three main genotypes to be a perfect match, it would be interesting to assess their performance on “real-life samples” from a larger prospective cohort. This work, describing a very robust method to sequence hB19V, represents an advance in parvovirus research, with a potentially dramatic impact on clinical management of the disease.

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Compliance with ethical standards

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