

Clinico-Molecular Detection of Human Parvovirus B19 in Post-Exchange Transfusion, Encephalitis, and Ewing's Sarcoma: From Development to Utilization of a Molecular Detection Assay in a Tertiary Hospital Setup

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Article Summary Line

A validated, cost-effective in-house PCR assay for human parvovirus B19 reveals diverse
genotype circulation and post-transfusion detection in Central India, highlighting critical gaps
in blood safety surveillance and the need for accessible molecular diagnostics in resource-
limited settings.

Running Title

Parvovirus B19 PCR Assay Validation, India

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Abstract

Background

Human parvovirus B19 (HB19V) infection poses significant clinical challenges in resource-limited settings, particularly regarding blood safety surveillance and diagnosis of associated

syndromes. Limited access to validated diagnostic assays restricts clinical management and transfusion safety protocols.

Objective

To develop and validate an in-house duplex real-time PCR assay for HB19V detection and evaluate its clinical utility in a resource-limited setting.

Methods

A duplex real-time PCR assay targeting the conserved NS1 gene of HB19V with RNase P as endogenous control was developed. Analytical validation assessed amplification efficiency, limit of detection (LOD), precision (intra- and inter-assay coefficients of variation), and cross-reactivity. Clinical validation compared qualitative agreement with a CE-certified commercial kit using Cohen's kappa statistic. Positive samples underwent Sanger sequencing for genotype characterization. Clinical specimens from suspected HB19V infections were tested and results correlated with patient presentations.

Results

The assay demonstrated 98.82% amplification efficiency with LOD of 6 copies per reaction. Precision metrics showed excellent reproducibility (CoV <1% intra-assay, <5% inter-assay) with no cross-reactivity. Qualitative agreement with the commercial reference was 100% (Cohen's kappa >0.95). Clinical application identified three significant cases: post-transfusion HB19V in neonatal bilirubin encephalopathy, HB19V consideration in severe anaemia with ventriculitis, and active infection in paediatric Ewing sarcoma with chemotherapy-induced cytopenias requiring IVIG therapy. Genotyping revealed genotypes 1a and 3, confirming regional viral diversity.

Conclusion

This validated in-house duplex real-time PCR assay provides a cost-effective, locally sustainable diagnostic tool for HB19V detection. It addresses critical gaps in blood safety

surveillance and expands diagnostic capacity for HB19V-associated syndromes in resource-limited settings, while highlighting the necessity of contextual clinical interpretation for optimal patient management.

1. Introduction

Human Parvovirus B19 (HB19V), first identified in 1975, is a small, non-enveloped, single-stranded DNA virus of the genus *Erythroparvovirus* (Parvoviridae). (1) Its ~5.6 kb genome encodes the non-structural protein NS1 for replication and structural proteins VP1/VP2 forming the capsid (2). HB19V spreads primarily via respiratory droplets, exhibits strong tropism for erythroid progenitors in bone marrow and fetal liver via P-antigen and $\alpha 5 \beta 1$ integrin, causing transient erythropoiesis arrest. (3), (4) Genetically, it comprises genotype 1 (worldwide), 2 (sporadic), and 3 (geographically restricted) (3)

HB19V infection ranges from asymptomatic to well-defined syndromes. (5). In children, it typically causes erythema infectiosum; adults, particularly women, may develop arthralgia or arthritis. Systemic associations include autoimmune, renal, and haematological disorders such as transient aplastic crisis or pure red cell aplasia (6). Vertical transmission can cause adverse fetal outcomes, including hydrops, thrombocytopenia, and neurological complications. (7). Because of its role in unexplained anaemia and perinatal morbidity, HB19V has gained renewed clinical importance (8). HB19V is also linked, though rarely, to encephalitis and other neurological conditions, underscoring its diverse pathogenic potential (9).

HB19V transmission via blood products is a key concern. While U.S. and European guidelines set risk-based DNA limits ($\sim 10^4$ IU/mL) rather than absolute exclusion, India lacks mandatory screening or quantitative thresholds. Given high donor seroprevalence and

recommendations for targeted testing in vulnerable recipients, this regulatory gap may leave residual clinical risk unaddressed (10) (11).

HB19V clinical manifestations are not fully understood, and treatment is mainly supportive. IVIG is used selectively but with variable efficacy, and although recombinant vaccines exist, widespread commercial use is limited (2).

Serology for anti-B19V IgM/IgG is useful but limited in early infection, reactivation, or immunocompromised patients who may not mount an antibody response (12). NAATs, particularly real-time PCR, detect low-level viremia and are the diagnostic gold standard (13). Commercial kits (e.g., Genes2Me HB19V PCR, IVD certified, India) exist but are costly and rely on external supply chains, limiting routine regional use.

To improve diagnostic autonomy and affordability, validated in-house assays can be developed and optimized for local workflows. Following MIQE and WOAHA 2023 validation frameworks, this study aimed to design, develop, and analytically validate a real-time PCR assay targeting the conserved NS1 gene of HB19V, benchmarking its performance against the Genes2Me IVD kit.

The validated assay was applied to clinical samples from tertiary-care hospitals in Central India, including both haematology, and obstetric patients; identifying three clinically significant infections: HB19V-associated bilirubin encephalopathy, hydrocephalus in transfused neonates, and Ewing sarcoma with febrile cytopenia during chemotherapy.

Sequencing and phylogenetic analysis confirmed genotypes 1a and 3, supporting circulation of globally predominant HB19V lineages.

The present paper, therefore, aims to:

1. Develop, and standardize an in-house real-time PCR assay for HB19V using the NS1 gene target;
2. Validate its analytical performance in accordance with MIQE, and WOAHA standards;

3. Compare its results with a commercial reference assay (HB19V Real-Time PCR IVD Kit Genes2Me); and
4. Apply a validated assay to clinical samples from patients presenting with anaemia, adverse pregnancy outcomes, and other overlapping clinical features suggestive of HB19V infection, based on clinician-guided differential diagnosis in Central India, to demonstrate diagnostic utility, and epidemiological relevance.

2. Materials and Methods

2.1. Study design, setting, and ethical approval

This study was conducted in the Infectious Disease Laboratory, Department of Translational Medicine, All India Institute of Medical Sciences (AIIMS), Bhopal, Central India, from May 2023-June 2025. The work comprised three phases: (i) assay optimization, and standardization; (ii) analytical validation; and (iii) clinical application.

Ethical approval was obtained from the Institutional Human Ethics Committee (IHEC), AIIMS Bhopal (IHEC-LOP/2023/EL065 dated 14.02.2023; IHECSR/AIIMSBPL/Jan25/95 dated 06.02.2025). Written informed consent/assent was obtained from participants, and/or legally authorized representatives (LAR) for prospective sample collection, and use of specimens for assay development and clinical investigation. (Paediatric assent was taken in patients < 12 years of age). Clinical samples requested by treating clinicians were screened using the HB19V PCR assay, with positives confirmed by sequencing.

2.2. Assay optimization and standardization

2.2.1. Primer and probe design

Primers, and probes targeting the conserved NS1 region of HPB19V and Human RNase P (RPP30) were selected from published sources (14); (15) with minor modifications for optimal assay performance (Table 1). Oligonucleotide sequences were evaluated for specificity using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to minimize cross-

173 reactivity with human and non-target microbial genomes. Design, and selection criteria were
174 aligned with accepted qPCR standards (16). Oligonucleotides were synthesized by Sigma-
175 Aldrich, and reconstituted to 100 μ M stock solutions.

176 **2.2.2. Real-time PCR assay setup and optimization**

177 Assay optimization was performed using gradient PCR (55–65°C) to identify the optimal
178 annealing/extension temperature, followed by titration of primer, and probe concentrations.
179 The finalized reaction volume was 25 μ L and contained: 1 $\times$ TaqMan Fast Virus 1-Step
180 Master Mix (Applied Biosystems), 320 nM of each primer, 120 nM probe, and 1 μ L synthetic
181 positive control template. Amplification was performed on a QuantStudio 5 Real-Time PCR
182 System (Applied Biosystems, USA) using the following cycling conditions: initial
183 denaturation at 95°C for 4 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s.
184 Each run included no-template (NTC), positive, and extraction controls. Contamination was
185 minimized by physically separating pre-PCR, sample handling, and post-PCR workflows.
186 Runs were accepted only if NTC and extraction controls were negative.

187 **2.2.3. Analytical validation**

188 Analytical performance was evaluated according to MIQE guidelines and WOAHA (2023)
189 validation principles (parameters in Table 2). Comparative validation used 43 clinical
190 samples from patients with adverse pregnancy outcomes, tested with a CE-certified IVD kit
191 (Genes2Me). A panel of 3 positive and 40 negative samples was established, excluding 7
192 borderline samples near the C_q cut-off. Samples (EDTA whole blood or serum) were
193 transported under cold chain and processed within 4 hours. Viral DNA was extracted using
194 the QIAamp DNA Mini Kit (Qiagen, Germany), quantified by Nanodrop, and stored at
195 –80°C. RNase P served as an endogenous control, and 2 μ L of extracted DNA was used per
196 25 μ L PCR reaction.

197 **2.3. Sanger sequencing and phylogenetic analysis**

Positive samples were subjected to Sanger sequencing using the NS1 primers (Table 3).

Amplicons (~588 bp) were purified using a QIAquick PCR Purification Kit (Qiagen), and sequenced bidirectionally on an ABI-3730 Genetic Analyzer.

2.4. Data management and statistical analysis

Cq values were reported as mean $\pm$ standard deviation (SD). Standard curve linearity was assessed using regression analysis, and amplification efficiency was derived from the slope.

Repeatability, and reproducibility were expressed as coefficients of variation (%CV).

Statistical analyses, and figure preparation were performed using GraphPad Prism v10, and Microsoft Excel-365. Evolutionary analysis was conducted in Mega-12.

2.5. Clinical application

2.5.1. Screening of Clinical Samples

20 specimens screened (whole-blood/ serum) from clinical cases from AIIMS OPD, requesting HB19V screening. Three cases with significant findings are being included as case reports.

3. Results

3.1. Assay Optimization and Standardization

The duplex real-time PCR assay targeting the NS1 region of HB19V and the endogenous control RNase P was successfully optimized. Gradient PCR experiments identified 60 °C as the optimal annealing/extension temperature, providing consistent amplification efficiency and optimal fluorescence signal for both targets. Under the finalized conditions, all NTC, and extraction controls remained negative, confirming assay specificity and absence of contamination. The assay reliably amplified HB19V, and endogenous RNase P in all positive samples, demonstrating robust performance for clinical application.

3.2. Analytical Performance Evaluation

3.2.1. Linearity and Dynamic Range

The assay exhibited excellent linearity over 10-fold serial dilutions ranging from 6×10^{10} to 60 copies/ μ L of synthetic NS1 control. Linear regression analysis of Cq values against $\log_{10}$ copy number demonstrated a strong linear relationship ($R^2 = 0.999$) across the entire dynamic range tested (Figure 1).

3.2.2. Amplification Efficiency

The amplification efficiency of the assay was 98.817%, with a slope of -3.351 , within the acceptable range recommended by MIQE guidelines (Figure 1)

3.2.3. Limit of Detection and Quantification

The limit of detection (LoD) was determined to be 6 copies per reaction, with $\geq 95\%$ detection across replicates and appropriate amplification curve characteristics. The limit of quantification (LoQ) was established at 60 copies per reaction, demonstrating acceptable precision with coefficients of variation $\leq 25\%$.

3.3. Precision and Robustness

3.3.1. Repeatability (Intra-assay Precision)

Intra-assay precision assessed using five replicate reactions at high, medium, and low target concentrations showed minimal variability, with SD ranging from 0.02 to 0.03 Cq, and CV consistently below 1%.

3.3.2. Reproducibility (Inter-assay Precision)

Inter-assay reproducibility evaluated across three operators and two instruments demonstrated comparable performance, with % CV values well below 5%, indicating high robustness of the assay.

3.4. Analytical Specificity

3.4.1. *In-silico* Analysis

In-silico BLAST analysis demonstrated no non-target sequences with sufficient homology to permit primer or probe binding under the assay conditions, supporting high analytical specificity.

3.4.2. Wet-lab Exclusivity Testing

No cross-reactivity was observed when the assay was tested against a panel of non-target bacterial and viral pathogens, including *Brucella* spp., *Coxiella burnetii*, *Chlamydia* spp., *Listeria monocytogenes*, Group B *Streptococcus*, Human bocavirus, HBV, HSV-1/2, CMV, EBV, Enterovirus, and HEV. All non-target samples were negative in the NS1 detection channel.

3.5. Contamination Control

All NTC, and extraction controls included in each run remained negative, confirming the effectiveness of contamination prevention measures.

3.6. Comparative Evaluation with Commercial Assay

Parallel testing of 43 clinical samples demonstrated 100% qualitative agreement between the in-house assay and the CE-certified Genes2Me commercial kit. No discordant results were observed. Cohen's kappa coefficient and PABAK values exceeded 0.95, indicating excellent agreement, and absence of systematic bias.

3.7. Clinical Matrix Interference

Spike-in experiments performed in serum samples at a concentration of 10^6 copies/ μ L showed minimal matrix interference, with ΔC_t values ≤ 1 cycle (+0.88 cycles), and CV $\leq 2\%$, supporting assay reliability in clinical matrices.

3.8. Sequencing Confirmation

All qPCR-positive clinical samples were confirmed by Sanger sequencing of the NS1 region. Bidirectional sequencing generated high-quality consensus sequences, validating the

specificity of the assay results. The genotype 1a and 3 were detected with phylogenetic analysis described in Figure 2.

3.9. Clinical Application

A total of 20 clinical specimens (whole blood and/or serum) received for HB19V testing were screened using the developed assay. Three cases exhibiting clinically significant and diagnostically informative findings were identified, and are being reported separately as individual case reports.

Case 1: Bilirubin encephalopathy with incidental Human Parvovirus B19 detection post-exchange transfusion

A late-preterm neonate (~35–38 weeks; birth weight 2.3 kg) born by spontaneous vaginal delivery to a 30-year-old mother (G3P3L3) developed jaundice on day 2 of life, and was admitted with severe hyperbilirubinemia, and clinical features consistent with bilirubin encephalopathy with Rh incompatibility. On admission, the neonate was tachycardic (heart rate 213/min), and tachypnoeic (respiratory rate 60/min), with oxygen saturation 98%, and BP 64/31 mmHg. Laboratory evaluation demonstrated marked indirect hyperbilirubinemia: total serum bilirubin 35.6 mg/dL with indirect fraction 33.9 mg/dL. A reactive sepsis screen, including CRP was noted. Intensive phototherapy was initiated, followed by exchange transfusion due to very high bilirubin levels. A total of 380 mL of whole blood was transfused over 38 exchange cycles. Supportive care included cefotaxime, phenobarbital, isoprenaline, and vitamin K. Following intervention, total bilirubin decreased to 24.8 mg/dL; direct bilirubin rose from 1.7 to 4.23 mg/dL during the same period. Subsequent virological testing performed after exchange transfusion detected HB19V. This positivity was documented as an incidental post-transfusion observation, and was not considered etiologically related to bilirubin encephalopathy, which was attributed to severe hyperbilirubinemia secondary to Rh incompatibility.

Case 2: Hydrocephalus/ventriculitis phenotype with severe anaemia; Human

Parvovirus B19 considered in differential diagnosis

A preterm neonate (~33–34 weeks) delivered by emergency LSCS for foetal distress, and suspected hydrops was admitted on day 2 of life with lethargy, severe pallor, respiratory distress requiring support, generalized edema, and mild hepatosplenomegaly. Maternal antenatal history was unremarkable with negative HIV/HBsAg/HCV screening, and no TORCH exposure or transfusion history. Initial haematology showed profound anaemia (haemoglobin 6.4 g/dL; haematocrit 20%) with low reticulocyte count, and no evidence of haemolysis; peripheral smear showed normocytic normochromic anaemia without nucleated RBCs or blasts. Inflammatory markers were not supportive of bacterial sepsis (CRP negative), and blood cultures remained sterile. Mild unconjugated hyperbilirubinemia was present (total bilirubin 2.1 mg/dL; indirect 1.6 mg/dL) with mildly elevated transaminases. During hospitalization, repeated CSF analyses showed a ventriculitis-like profile with persistently very low glucose (as low as 2.42 mg/dL), markedly elevated protein (420–1516 mg/dL), and neutrophilic pleocytosis, yet serial aerobic CSF cultures were sterile. Neuroimaging documented progressive ventriculomegaly evolving from communicating to obstructive hydrocephalus, necessitating neurosurgical interventions (reservoir placement, serial CSF taps, and later VP shunt). CSF viral PCR detected CMV, prompting valganciclovir. Given severe anaemia with reticulocytopenia, absence of haemolysis, sterile cultures, and encephalitis-like presentations, HB19V (with other neurotropic viruses) was considered in the differential diagnosis. The patient was discharged against medical advice; follow-up was unavailable.

Case 3: Ewing sarcoma case with Human Parvovirus B19 positivity presenting as febrile cytopenias during chemotherapy

A 17-year-old male with newly diagnosed Ewing sarcoma of the proximal right femur was initiated on multimodal chemotherapy (COG B-VDC/IE regimen) following pathological confirmation. During the second cycle of treatment, the patient developed profound chemotherapy-induced cytopenias (haemoglobin 7.2 g/dL, total leukocyte counts $2.1 \times 10^3/\mu\text{L}$) complicated by febrile neutropenia (temperature 38.8°C) that did not respond to conventional broad-spectrum antimicrobial therapy. Despite appropriate infectious workup and antimicrobial coverage, the cytopenia persisted beyond the anticipated recovery window of 10-14 days post-chemotherapy, raising suspicion for an underlying viral etiology. Comprehensive virological screening was subsequently undertaken, which revealed positive HB19V IgM serology. QPCR-based molecular confirmation demonstrated a high viral load ($>10^7$ copies/mL), consistent with active parvoviral infection. Given the immunocompromised state of the patient, and the documented role of HB19V in persistent cytopenias in this population, IVIG therapy was administered. Clinical response was rapid; within 10 days of IVIG administration, repeat QPCR demonstrated a significant reduction in viral load (>3 -log reduction), with concurrent haematological recovery (haemoglobin 9.8 g/dL, total leukocyte counts $5.2 \times 10^3/\mu\text{L}$, platelets $180 \times 10^3/\mu\text{L}$). The patient subsequently tolerated resumption of chemotherapy without recurrence of severe cytopenias. At two-year follow-up post-completion of cancer-directed therapy, the patient remained in remission with sustained haematological recovery and normal blood counts.

4. Discussion

In this study, we developed, analytically validated, and clinically applied in-house duplex real-time PCR assay for HB19V targeting the conserved NS1 gene, with RNase P (RPP30) as an endogenous internal control. The assay demonstrated strong analytical performance consistent with MIQE and WOA (2023) (16, 17) validation principles, and

showed excellent agreement with a CE-certified commercial reference assay. Importantly, clinical application across tertiary-care settings in Central India highlighted the assay's diagnostic utility in real-world, clinician-guided differential diagnosis, and enabled genotypic characterization (genotype 1a and 3), supporting ongoing circulation of both globally predominant, and regionally restricted lineages.

The assay showed robust quantitative performance, with $R^2 = 0.999$ and 98.8% amplification efficiency. Analytical sensitivity and precision were confirmed, achieving an LOD of 6 copies/reaction and LOQ of 60 copies/reaction in accordance with MIQE standards. This sensitivity is clinically important because HB19V can present with low-level viremia in early infection, resolving infection, or reactivation states, and because immunocompromised patients may not mount detectable serologic responses. The LOD (6 copies/reaction) ensures sufficient sensitivity for clinically relevant viremia, as viral loads during acute infection typically range from 10^3 to 10^7 copies/ml. The use of RNase P as an endogenous control strengthened the assay by verifying extraction adequacy, and nucleic acid integrity, reducing false negatives due to poor sampling or inhibitors.

Repeatability, and reproducibility testing demonstrated minimal intra- and inter-assay variation, with CV below the stated acceptance thresholds ($\leq 5\%$ and $\leq 10\%$, respectively). These results align with earlier validation studies by Servant et al. (2002) and Qiu et al. (2017), which reported comparable performance for NS1-targeted assays. Robust performance across operators, and instruments is particularly relevant for regional laboratories, where staff rotation, and platform variability are common. Analytical specificity was supported both *in-silico* (BLAST), and experimentally; no cross-reactivity was observed with a comprehensive panel of bacterial and viral pathogens, including herpesviruses, and other common causes of fetal/neonatal morbidity, and hematologic syndromes. Consistently

negative NTC and extraction controls further support effective contamination control, which is essential for sensitive DNA assays.

The in-house assay showed 100% qualitative agreement with the Genes2Me IVD kit across the validation panel (3 positives, and 40 negatives), with Cohen's kappa and PABAK >0.95. Although the number of positives was limited reflecting real-world prevalence in the sampled cohort this concordance indicates that the in-house method can perform comparably to a commercial assay for routine qualitative diagnosis. Excluding borderline Cq samples near cut-off from validation reduced misclassification risk; however, it also highlights an important practical reality; defining and managing "grey zone" results is critical in HB19V testing. Future work could formalize an equivocal range with repeat testing and/or confirmatory sequencing, particularly for low-viremia specimens.

The clinical case series illustrates how HB19V PCR can influence interpretation across diverse syndromes, while also underscoring that detection does not necessarily imply causality.

Case 1 emphasizes this distinction clearly. The neonate's presentation severe indirect hyperbilirubinemia, Rh incompatibility, and requirement for exchange transfusion strongly supports haemolytic disease as the etiology. HB19V detection after exchange transfusion was documented as incidental and not causal. The case highlights a blood-safety concern, as HB19V can resist some pathogen-reduction methods and may be transmitted via blood products. In the absence of mandatory screening or quantitative thresholds, incidental post-transfusion positivity can complicate interpretation, particularly in evaluating unexplained anaemia or hydrops, underscoring the value of context-aware reporting and targeted surveillance in high-risk recipients. These recommendations, based on similar scenarios, have also been made by previous authors (12, 18).

392 **Case 2** represents a complex syndrome where HB19V is plausibly relevant but not
393 definitively proven as a single unifying cause. The hematologic pattern characterized by
394 profound anaemia, low reticulocytes, and no haemolysis is typical of suppressed
395 erythropoiesis and aligns with the known HB19V tropism for erythroid progenitors.
396 Concurrent neuroinflammatory findings, and progressive hydrocephalus broaden the
397 differential, including congenital infections, and viral ventriculoencephalitis. Detection of
398 CMV in CSF offers a compelling alternative etiology for the neurophenotype, yet it does not
399 exclude HB19V coinfection or contribution, particularly to the anemia. This case underscores
400 the value of local sensitive HB19V PCR for early detection in suggestive anemia, even with
401 other pathogens or atypical neuroimaging, and highlights that molecular results should be
402 interpreted alongside clinical timing, transfusion history, reticulocyte response, and, when
403 available, serial viral loads to support causality. Similar neurological symptoms with
404 detection of HB19V have been reported previously (19, 20).

405 **Case 3** demonstrates another common real-world scenario; HB19V may be detected during
406 workup of symptoms that overlap with viral arthropathy or bone pain, but the primary
407 diagnosis may be non-infectious. Here, imaging, and pathology supported Ewing sarcoma.
408 This case underscores the importance of targeted virological investigation in paediatric cancer
409 patients presenting with prolonged chemotherapy-induced cytopenia and febrile neutropenia
410 despite appropriate antimicrobial management. HB19V should be considered in the
411 differential diagnosis of refractory cytopenias in immunocompromised hosts, and IVIG
412 therapy may offer rapid clinical benefit when parvoviral infection is documented.
413 Nonetheless, its detection in an oncology patient may have clinical relevance if
414 immunosuppression develops later, given the risk of persistent infection, and chronic anaemia
415 (21-23).

416 Sequencing of qPCR positives strengthens assay specificity and provided epidemiological
417 insight. Detection of both genotype 1a (globally predominant) and genotype 3 (more
418 geographically restricted) in Central India suggests diverse HB19V circulation and highlights
419 the value of NS1-targeted assays against conserved regions. Ongoing sequencing surveillance
420 can track genotype shifts and monitor for primer/probe mismatches that may affect assay
421 sensitivity.

422 A key contextual contribution of this work is the framing of HB19V as a transfusion-relevant
423 pathogen in India, with international guidelines focusing on limiting viral DNA in plasma
424 pools rather than excluding all positives. In India, the combination of donor seroprevalence,
425 absence of mandated thresholds, and vulnerable recipient populations (pregnancy, neonates,
426 hemoglobinopathies, immunocompromised patients) creates a gap where undetected high-
427 titre donations could carry disproportionate risk. While this study was not designed as a
428 donor-screening assessment, the incidental post-transfusion detection in Case 1 adds practical
429 weight to the argument that targeted testing strategies (or defined thresholds for high-risk
430 components/recipients) may be more feasible, and clinically meaningful than attempting
431 “zero-tolerance”.

432 Key strengths include the duplex design with endogenous control, comprehensive analytical
433 validation, benchmarking against a commercial IVD assay, contamination safeguards, and
434 sequencing-confirmed genotype reporting. The study also reflects pragmatic tertiary-hospital
435 workflows and highlights real-world interpretive challenges.

436 Limitations include the modest number of positives in the comparative panel, exclusion of
437 borderline Cq samples, and limited follow-up in Case 2, restricting causal inference. The
438 study did not report viral loads in IU/mL or calibrate against WHO standards, which would
439 enhance comparability and support transfusion-policy thresholds. Future work should expand
440 validation across larger, and diverse cohorts, establish an equivocal zone algorithm (repeat

testing, replicate confirmation, sequencing), align quantification to international standards, and evaluate performance across HB19V genotypes more broadly, including *in-silico* and wet-lab inclusivity.

5. Conclusion

The validated in-house NS1 duplex real-time PCR assay provides a robust, cost-effective tool for HB19V detection in regional diagnostic laboratories. Its strong concordance with a CE-certified commercial kit underscores its reliability for routine clinical use. Clinical application demonstrates the assay's value in supporting clinician-guided differential diagnosis, particularly in transfused neonates and patients with complex co-infections. Detection of genotypes 1a and 3 confirms genetically diverse HB19V circulation in Central India, emphasizing the need for locally validated molecular diagnostics to inform patient management and public health surveillance.

6. Declaration of Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Dr Megha Katare Pandey reports financial support was provided by Directorate of Medical Education Govt of MadhyaPradesh India. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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533 **Table 1. Real Time q-PCR Oligonucleotides**

Target	Gene / Assay	Oligo type	Name	Sequence (5'→3')	Label / Quencher
Human Parvovirus B19	NS1	Forward primer	NS1-F	CCACTATGAAAACCTGGGCAATA	
Human Parvovirus B19	NS1	Reverse primer	NS1-R	GCTGCTTTCACTGAGTTCTTCA	
Human Parvovirus B19	NS1	Probe	NS1-P	AATGCAGATGCCCTCCACCCAG	Texas Red / BHQ2
Endogenous control	RNase P	Forward primer	RPP30-F	AGATTGACCTGCGAGCG	

Endogenous control	RNase P	Reverse primer	RPP30-R	GAGCGGCTGTCTCCACAAGT	
Endogenous control	RNase P	Probe	RPP30-P	TTCTGACCTGAAGGCTCTGCGCG	Cy5 / BHQ2

534

535 **Table 2: Analytical Validation Matrix**

Validation parameter	Method	Accepted Criteria
Linearity/ dynamic range	10-fold serial dilutions of synthetic NS1 control across 6×10^{10} to 60 copies/ μ L; each dilution in triplicate; regression of Cq vs log ₁₀ copies	$R^2 \geq 0.98$; linear across stated range
Amplification efficiency	Calculated from slope: $E = (10^{(-1/\text{slope})} - 1) \times 100$	90–110% (slope ~ -3.1 to -3.6)
Limit of detection (LoD)	The lowest concentration of target nucleic acid (copies/reaction) that can be reliably distinguished from background noise.	$\geq 95\%$ hit rate; correct curve shape; valid controls
Limit of quantification (LoQ)	The lowest concentration at which the target can be quantified with acceptable precision and accuracy.	% CV $\leq 25\%$ and consistent quantification
Robustness (Repeatability/intra-assay precision)	Same run, same operator: high/med/low concentrations tested in five replicates (n=5)	% CV of Cq $\leq 5\%$
Robustness (Reproducibility/inter-assay precision)	Across 3 operators, same concentrations as repeatability testing performed in 2 separate instruments	%CV of Cq $\leq 10\%$
Analytical specificity (<i>in-silico</i>)	NCBI BLAST for primers/probes against human genome and related viruses	No significant off-target homology at the intended annealing temperature
Analytical specificity (wet-lab exclusivity)	Tested panel of non-target pathogens: Brucella, Coxiella, Chlamydia, Listeria, GBS, Human BocaVirus, Hepatitis B, HSV 1/2, CMV, EBV, Enterovirus, Hepatitis E virus (Both with Positive Synthetic constructs and clinical samples)	No amplification in the NS1 channel
Contamination controls	NTC in each run; extraction control in every extraction batch; separate pre/post-PCR areas	NTC/Extraction control must be negative
Comparative agreement vs Genes2Me	Parallel testing of all 43 samples; qualitative agreement;	Qualitative agreement $\geq 95\%$; Cohen kappa and PABAK (> 0.95)

	sensitivity; specificity, Cohen k, PABAK	
Clinical matrix / interference	Spike-in Protocol: positive control added serum with a concentration of 10 ⁶ copies/μL	Acceptable %CV: ≤2% Acceptable ΔCt due to matrix: ≤1 cycle

Table 3. Human Parvo B19 (NS1) sequencing Oligonucleotides

Gene / Region	Oligo type	Sequence (5'→3')
NS1	Forward primer	ACTATGACCCCCTATTGGTGGG
NS1	Reverse primer	GGTGGAGGGCATCTGCATTAAT

Table 4. Clinical summary of cases with Human Parvovirus B19 detection

Case	Age group	Primary syndrome	Key supporting data	Parvovirus B19 role (as documented)
1	Neonate	Severe indirect hyperbilirubinemia → bilirubin encephalopathy	TSB 35.6 mg/dL, Rh incompatibility; exchange transfusion	Incidental post-transfusion detection, not causal
2	Preterm neonate	Severe anaemia + ventriculitis-like CSF + hydrocephalus	Hb 6.4, low retics, no haemolysis; sterile cultures; progressive hydrocephalus; CMV in CSF	Differential diagnosis (aplastic anaemia pattern + neuro features)
3	Adolescent	Bone pain/swelling → Ewing sarcoma	MRI lesion; cytology/HPE Ewing; CBC counts cytopenic	Positivity, considered in differential for febrile neutropenia for IVIG administration

Figures

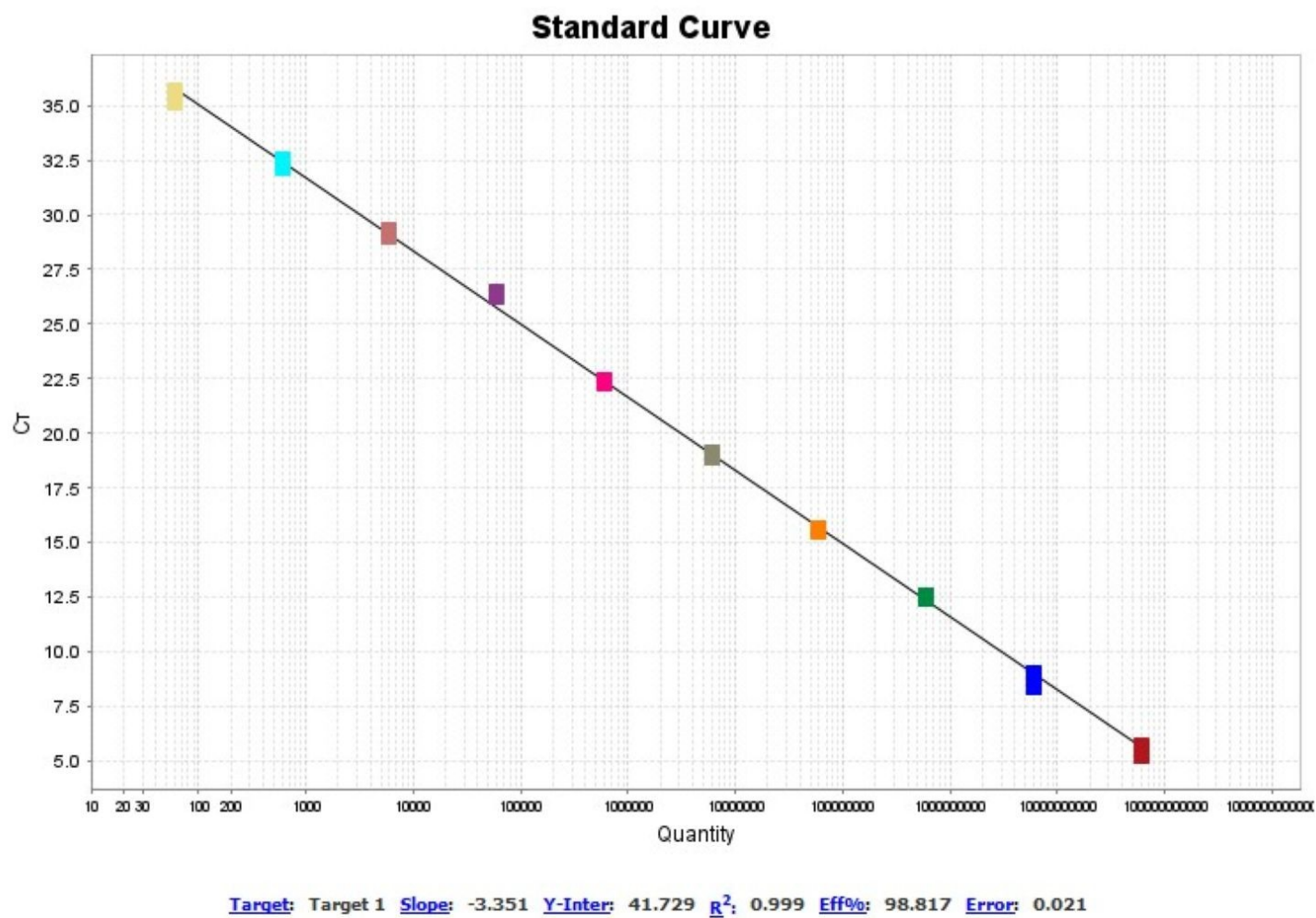


Figure 1

Legend not included with this version

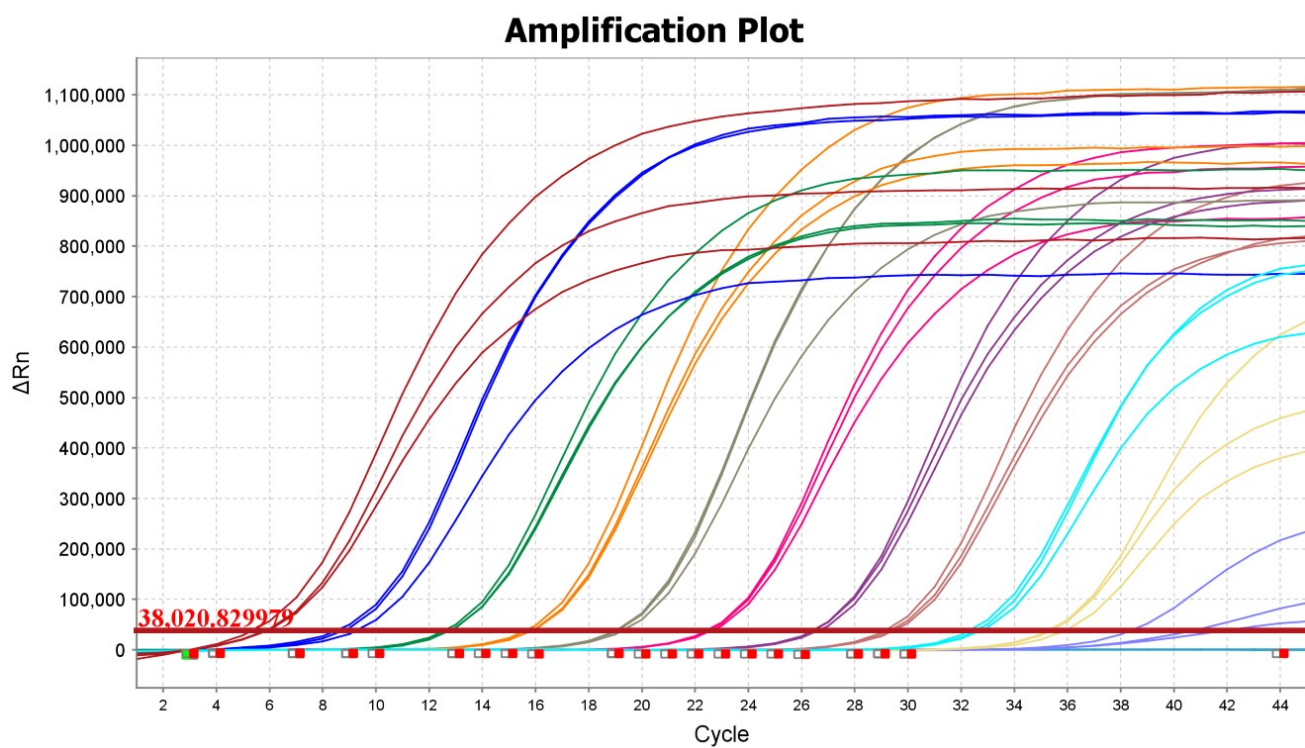


Figure 2

Legend not included with this version

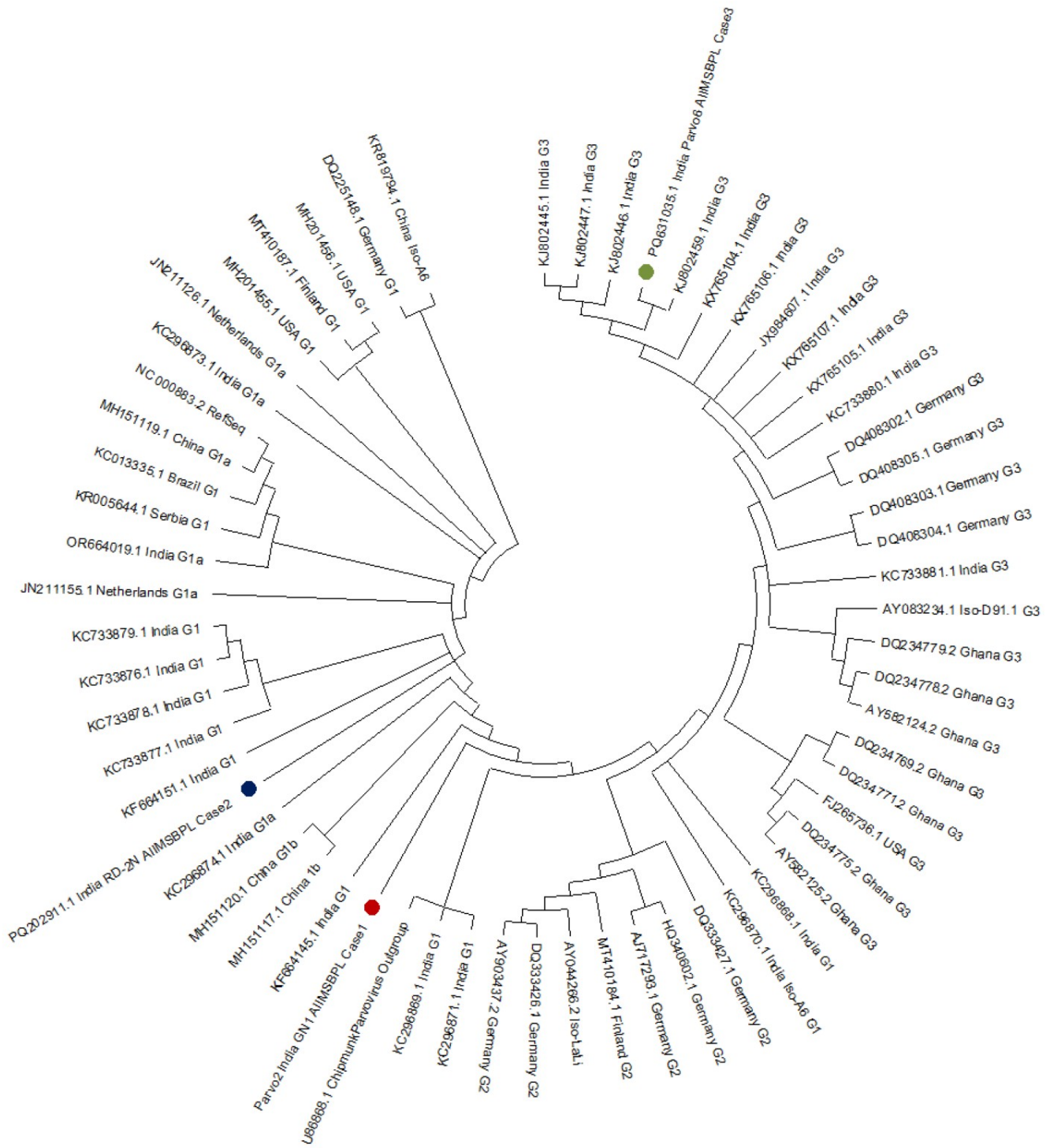


Figure 3

Legend not included with this version