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1 **TITLE:** Methods comparison for molecular diagnosis of human herpesvirus 8 infections.

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3 Manon Corgiat^a, Vincent Calvez^a, Anne-Geneviève Marcelin^a, Aude Jary^a

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5 ^aSorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique
6 (iPLESP), AP-HP, Pitié Salpêtrière Hospital, Department of virology, F-75013 Paris, France

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9 **Corresponding author:** Aude Jary, 47-83 boulevard de l'Hôpital, 75013 Paris, +33 1 42 17

10 74 01, aude.jary@aphp.fr

11 **Alternate corresponding author:** Pr Anne-Geneviève Marcelin, 47-83 boulevard de

12 l'Hopital, 75013 Paris, +33 1 42 17 74 01, anne-genevieve.marcelin@aphp.fr

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26 **Highlights**

- 27 - HHV-8 DNA viral load in blood is an accurate marker for diagnosis and management
28 of HHV-8 associated diseases.
- 29 - None of the commercial assays for HHV-8 diagnosis by real-time PCR were
30 comparable with the in-house PCR.
- 31 - The HHV-8 R-geneTM assay overestimated by in median 0.46 log₁₀ copies/10⁶ cells
32 the results obtained by the in-house PCR
- 33 - The HHV-8 Clonit® assay overestimated by in median 1.16 log₁₀ copies/10⁶ cells the
34 results obtained by the in-house PCR

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52 **ABSTRACT**

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54 Background: Human herpesvirus 8 (HHV-8) virological diagnosis and monitoring relies
55 mainly on real-time PCR.

56 Objectives: To evaluate two real-time PCR commercial kit (HHV-8 Premix R-geneTM and
57 Clonit® HHV-8) and compare with in-house real-time PCR.

58 Study design. Twelve samples (3 undetectable and 9 detectable with viral load ranging from
59 10^1 to 10^5 per reaction) were tested for HHV-8 detection and quantification with the 3
60 methods. Methods comparison was supported with regression curve and diagram presenting
61 difference or ratio between commercial and in-house PCR results and plotted against the in-
62 house PCR results. Statistical analyses, specifically Student tests and Spearman correlation,
63 were performed.

64 Results: In both cases, qualitative results obtained with commercial kit and in-house PCR
65 were identical and HHV-8 quantitation results were significantly correlated (Clonit®, $R_s = 1$,
66 $p < 0.001$ and R-geneTM $R_s = 0.98$, $p < 0.001$). However, Clonit® results were significantly
67 higher compared to the in-house results with an overestimation in median [IQR] of $1.16 \log_{10}$
68 copies/ 10^6 cells [1.12–1.18] whereas R-GeneTM results were not significantly higher, and an
69 overestimation in median of $0.46 \log_{10}$ copies/ 10^6 cells [0.37-0.52]. Otherwise, repeatability
70 and reproducibility tests of undetectable sample failed with Clonit® technique contrary to the
71 R-GeneTM.

72 Conclusions: HHV-8 R-geneTM assay seems to be the most suitable since it showed consistent
73 qualitative results with in-house HHV-8 PCR, a good quantitative correlation, an
74 overestimation not significantly different and inferior to $0.50 \log_{10}$ copies/ 10^6 cells and a good
75 repeatability.

76

77 **BACKGROUND**

78 Human herpesvirus 8 (HHV-8) is involved in all forms of Kaposi's sarcoma (KS) and
79 in two lymphoid malignancies: some forms of multicentric Castleman disease and primary
80 effusion lymphoma [1,2].

81 As others human herpesviruses, HHV-8 establishes latency after primary infection and can
82 provide symptomatic reactivations in immunocompromised patients [2]. Real-time
83 Polymerase Chain Reaction (PCR) is an effective diagnostic tool for the detection and the
84 quantification of HHV8-DNA and is based on the amplification of a conserved gene within
85 different HHV-8 subtypes [3–6]. In the peripheral compartment, HHV8-DNA viral load (VL)
86 monitoring was reported to be an accurate biomarker for assessment of the risk of further
87 disease progression [7–9], but also to evaluate response to therapy [10,11] and to distinguish
88 the three main HHV-8 pathologies at diagnosis [12]. Otherwise, whole blood seems to be the
89 best sample to evaluate HHV8-DNA VL rate since it allows quantifying latent HHV-8
90 (intracellular), replicating HHV-8 (intracellular) and free viral particles (extracellular).

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93 **OJECTIVES**

94 In our virology department, HHV8-DNA detection and quantification is performed with an in-
95 house real time PCR amplifying ORF-73 [5]. This study aimed to evaluate two real time PCR
96 commercial kits for the detection and quantification of HHV8-DNA by comparing them with
97 the in-house PCR.

98

99

100 **STUDY DESIGN**

101 *Samples*

102 Twelve whole blood samples were selected [Supplementary Table 1]: 3 with undetectable
103 HHV8-DNA VL and 9 with detectable HHV8-DNA VL ranging from 10^1 to 10^5 per reaction
104 (or from 1.56 to 5.79 \log_{10} copies/ 10^6 cells). Before extraction, DICO (Biomérieux®) internal
105 control (IC) was added in each sample.

106

107 ***HHV-8 in-house real time PCR***

108 HHV-8 in-house PCR was performed as previously reported [5]. Five quantitation
109 standards (QS): QS1, QS2, QS3, QS4 and QS5 corresponding respectively to 10, 100, 1000,
110 10 000 and 100 000 copies/reaction were used and results expressed in copies/ 10^6 cells
111 through quantification of human albumin gene [13]. Each run was validating by a negative
112 (water RNase-free) and two positive (QS2 and QS3) controls, and also by DICO IC amplified
113 in the same well as the HHV-8.

114

115 ***Clonit HHV-8 PCR (Eurobio Ingen®)***

116 This technique is based on the detection of a gene coding a minor capsid protein. The
117 kit enabled 48 reactions and was composed as follows: R1 reagent amplification mix, R2
118 primers and probes, and R6, R5, R4 and R3 quantitation standard corresponding respectively
119 to 500, 5000, 50 000 and 500 000 copies/reaction. IC corresponding to the human beta-globin
120 gene, was amplified concurrently with HHV-8. The same controls as the in-house PCR were
121 used.

122

123 ***HHV-8 Premix R-geneTM (Biomérieux®)***

124 This test is based on the amplification of a 146 base pairs long of ORF26 and allows
125 carrying out 20 reactions [14]. The quantification was performed with a calibration range
126 (Quanti HHV-8 QS R-geneTM) that included 4 QS: QS1, QS2, QS3 and QS4, corresponding

127 to 50, 500, 5000 and 50 000 copies/reaction respectively. The amplification of HHV-8 and
128 DICO was done in two separate wells for each sample. The same controls as the in-house
129 PCR were used.

130

131 Results of the two commercial kits were converted in copies/millions cells through
132 quantification of human albumin gene performed once on each extracted-DNA.

133

134 ***Methods verification***

135 We supported our method comparison using the following graphs: a regression curve
136 presenting results of commercial kit compared to the in-house technique; a diagram
137 presenting difference between commercial and in-house PCR results and plotted against the
138 in-house PCR results; a diagram presenting ratio between commercial and in-house PCR
139 results and plotted against the in-house PCR results. GraphPad software was used to perform
140 Student's t-test (risk at 5%) and Spearman rank-order correlation. Repeatability and
141 reproducibility were performed with 3 samples: each one was tested three times during the
142 same experiment (repeatability) or four times in different experiments (reproducibility).

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144

145 **RESULTS**

146 ***HHV-8 Clonit® and in-house PCR***

147 Qualitative results obtained by the two techniques were identical; samples 1, 2 and 3 were
148 undetectable whereas samples 4 to 12 were detectable [Figure 1A]. HHV-8 quantitation
149 results between the two methods were significantly correlated ($r_s=1$, $p<0.001$). However,
150 Student's t-test showed that the slope was not significantly different from 1, but the intercept
151 was from 0, suggesting that Clonit® results were significantly higher compared to the in-

152 house results [Fig 1B]. This observation was further confirmed by difference and ratio
153 analysis: respectively, Clonit® results were (i) in median [IQR] 1.16 log₁₀ copies/10⁶ cells
154 [1.12–1.18] greater compared with the in-house PCR results [Fig 1C] and (ii) in median 1.34
155 [1.24-1.44] times higher than the in-house results [Fig 1D].

156 Repeatability and reproducibility tests were performed with an undetectable sample (n⁰2), and
157 two detectable samples (n⁰5, Ct=32.87 and n⁰10, Ct=24.23). For positive samples, both tests
158 succeeded with a variation coefficient lower than the 5% accepted in our laboratory
159 [Supplementary Table 2]. However, repeatability and reproducibility tests failed with sample
160 2 which was detectable once and twice, respectively. In order to exclude a contamination, we
161 tested the same extracted-DNA and a new extracted-DNA from the same sample with the in-
162 house PCR, and HHV8-DNA remained undetectable.

163

164 ***HHV-8 R-geneTM and in-house PCR***

165 Qualitative results obtained by the two techniques were identical; samples 1, 2 and 3 were
166 undetectable whereas samples 4 to 12 were detectable [Figure 2A]. HHV-8 quantitation
167 results between the two methods were significantly correlated ($r_s=0.98$, $p<0.001$). Student's t-
168 test performed found that the slope was significantly different from 1 but the intercept was not
169 from 0, suggesting that R-geneTM results were not significantly higher compared to the in-
170 house results [Fig 2B]. Commercial test values were in median 0.46 log₁₀ copies/10⁶ cells
171 [0.37–0.52] greater compared with the in-house PCR results [Fig 2C], and thus less than 0.50
172 log₁₀ usually accepted. The ratio chart showed that R-geneTM results were in median 1.13
173 [1.12-1.14] times higher than the reference technique results [Fig 2D].

174 Repeatability test was performed with the same positive samples (no5 and 10) but with a
175 different undetectable sample (n⁰1). For all samples, repeatability tests succeeded with a

176 variation coefficient lower than 5% [Supplementary Table 2]. R-geneTM reproducibility could
177 not be achieved due to a limited number of PCR-test.

178

179

180 **DISCUSSION**

181 In this study, we determined that none of the commercial assays were comparable with the in-
182 house PCR. Indeed, although HHV-8 quantitation was correlated in both cases, there was an
183 overestimation of the values. This could be explained by a better sensitivity, with more
184 efficient amplifications and different molecular targets. However, this overestimation was less
185 than 0.5 log₁₀ with R-geneTM, as opposed to Clonit® results which overestimated by more
186 than 1 log₁₀. Clonit® results overestimation may be explained by two parameters: a non-
187 specific amplification as seen with reproducibility and repeatability tests failures of
188 undetectable sample; the QS which ranged from 500 to 500 000 copies/reaction while the in-
189 house PCR QS ranged from 10 to 10 000 copies/reaction. Moreover, Clonit® lower limit of
190 quantification was high (45 000 copies/ml after correction of dilution factor) compared to the
191 VL levels usually found in HHV-8 associated pathologies. Indeed particularly in KS, HHV8-
192 DNA values were reported to be in median 2 log₁₀ copies/10⁶ cells [12].

193 Two limitations of this study are probably the number and the type of samples tested.
194 However, although other specimens as effusion liquid and biopsies could be tested, blood
195 sample remains the main specimen for diagnosis and management of HHV-8 associated
196 diseases. Moreover, although few samples were used, wide range of VL rates including those
197 found in the three main HHV-8 pathologies were tested.

198 In conclusion, R-geneTM technique seems to be the most suitable commercial kit since it
199 showed consistent qualitative results with in-house HHV-8 PCR [14], a good quantitative

200 correlation, an overestimation not significantly different and inferior to $0.50 \log_{10}$ copies/ 10^6
201 cells and a good repeatability.

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227 providing commercial kits for this study.

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229 **Declarations of interest:** none

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326 **Figure 1: Results of methods comparison between HHV-8 Clonit[®] kit (Eurobio Ingen[®])**
327 **and HHV-8 in-house PCR. HHV-8 DNA viral load levels obtained by the two techniques are**
328 *listed in figure 1A. Correlation curve ($y=0.9586x+1.3375$) between results obtained by the*
329 *two techniques is represented in figure 1B and theirs differences and ratio respectively in*
330 *figure 1C and 1D.*

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351 **Figure 2: Results of methods comparison between HHV-8 R-gene™ kit (Biomérieux®)**
352 **and HHV-8 in-house PCR. HHV-8 DNA viral load levels obtained by the two techniques are**
353 *listed in figure 2A. Correlation curve ($y=1.0738+0.1928$) between results obtained by the two*
354 *techniques is represented in figure 2B and theirs differences and ratio respectively in figure*
355 *2C and 2D.*

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Supplementary Table 1: Characteristics of the samples selected for methods comparison

| Patient | Samples | HHV8-DNA viral load (log₁₀ copies/10⁶ cells) | HHV-8 serology | HIV status | Clinical context |
|----------------|----------------|---|---------------------------|-------------------|--|
| 1 | Whole blood | <10 | Negative | Negative | Liver transplantation No signs suggestive of HHV8-associated diseases |
| 2 | Whole blood | <10 | Negative | Positive | HIV-infected patient with inflammatory syndrome No signs suggestive of HHV8-associated diseases |
| 3 | Whole blood | <10 | Positive | Negative | Liver transplantation No signs suggestive of HHV8-associated diseases |
| 4 | Whole blood | 1.56 | NA | NA | Unknown |
| 5 | Whole blood | 1.98 | NA | Positive | Macrophage activation syndrome No signs suggestive of HHV8-associated diseases |
| 6 | Whole blood | 2.48 | Positive | Positive | Cutaneous, ganglionic and splenic KS in patient under effective ARVs. |
| 7 | Whole blood | 3.20 | NA | Positive | Multicentric Castleman disease |
| 8 | Whole blood | 3.27 | NA | NA | Unknown |
| 9 | Whole blood | 3.87 | NA | NA | Unknown |
| 10 | Whole blood | 4.68 | NA | Positive | Cutaneous KS and multicentric Castleman disease |
| 11 | Whole blood | 4.97 | Negative | Positive | Multicentric Castleman disease |
| 12 | Whole blood | 5.79 | NA | Positive | Multicentric Castleman disease |

ARV: antiretroviral; KS: Kaposi's sarcoma; HHV-8: human herpesvirus 8; HIV: Human Immunodeficiency virus; NA: not available

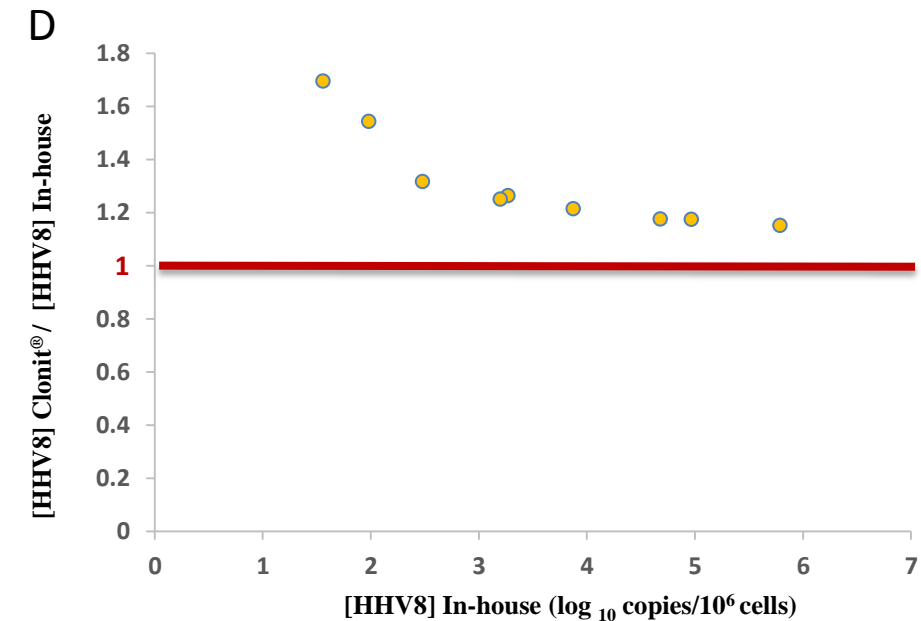
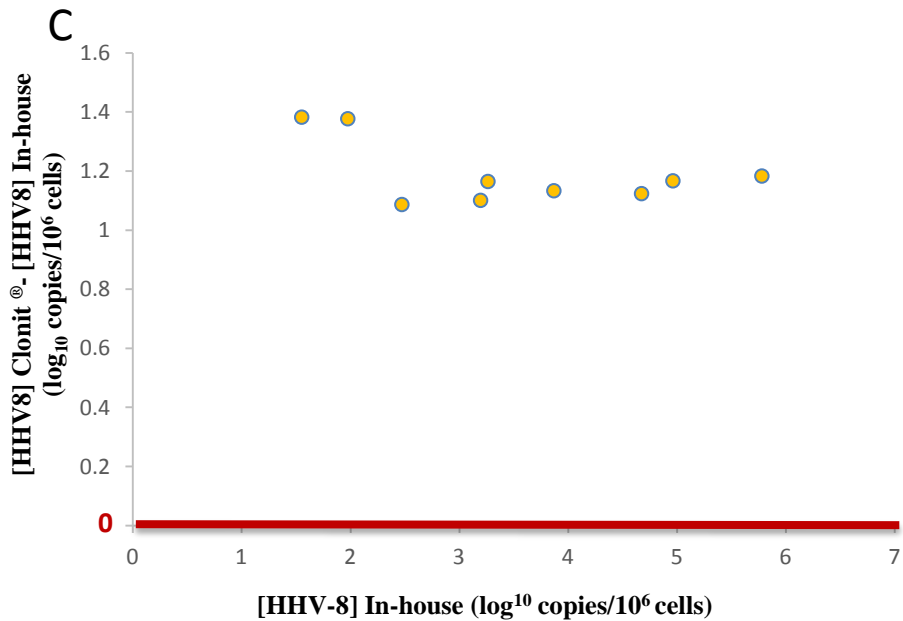
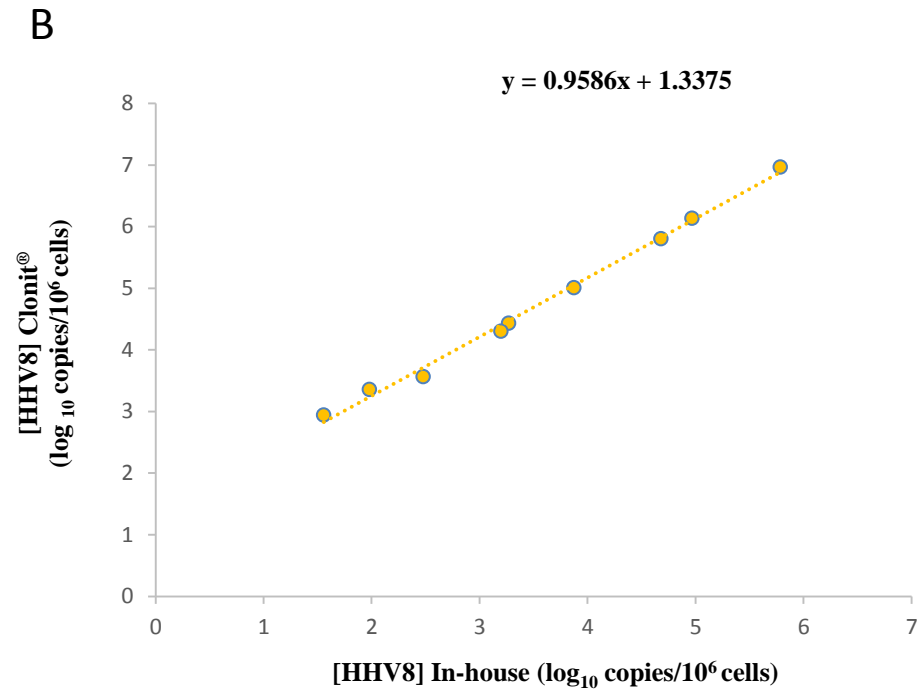
Supplementary Table 2: Repeatability and reproducibility results of HHV-8 Clonit® and HHV-8 R-Gene™ PCR assay

| HHV-8 Clonit® | | | | |
|------------------------|---------------------------|--------------|----------|-----------|
| | SAMPLES | 2 | 5 | 10 |
| Repeatability | Ct 1 | Undetectable | 32.87 | 24.23 |
| | Ct 2 | Undetectable | 33 | 24.29 |
| | Ct 3 | Undetectable | 32.78 | 24.19 |
| | Ct 4 | 36.94 | 33.17 | 24.17 |
| | Mean | NA | 32.95 | 24.22 |
| | Standard deviation | NA | 0.17 | 0.053 |
| | Variation coefficient (%) | NA | 0.51 | 0.22 |
| Reproducibility | Ct 1 | Undetectable | 33.54 | 24.51 |
| | Ct 2 | 36.94 | 33.17 | 24.36 |
| | Ct 3 | 37.03 | 33.42 | 24.63 |
| | Ct 4 | NR | 32.88 | 24.25 |
| | Mean | NA | 33.25 | 24.43 |
| | Standard deviation | NA | 0.29 | 0.17 |
| | Variation coefficient (%) | NA | 0.88 | 0.68 |
| HHV-8 R-Gene™ | | | | |
| | SAMPLES | 1 | 5 | 10 |
| Repeatability | Ct 1 | Undetectable | 33.39 | 23.92 |
| | Ct 2 | Undetectable | 33.14 | 23.78 |
| | Ct 3 | Undetectable | 33.03 | 24.03 |
| | Mean | NA | 33.18 | 23.91 |
| | Standard deviation | NA | 0.18 | 0.13 |
| | Variation coefficient (%) | NA | 0.56 | 0.52 |

NA: not applicable; NR: not realized

A

| Patients | [HHV8] In-house (log ₁₀ copies/10 ⁶ cells) | [HHV8] Clonit® (log ₁₀ copies/10 ⁶ cells) |
|----------|---|--|
| 1 | Undetectable | Undetectable |
| 2 | Undetectable | Undetectable |
| 3 | Undetectable | Undetectable |
| 4 | 1.56 | 2.94 |
| 5 | 1.98 | 3.36 |
| 6 | 2.48 | 3.56 |
| 7 | 3.20 | 4.30 |
| 8 | 3.27 | 4.43 |
| 9 | 3.87 | 5.01 |
| 10 | 4.68 | 5.80 |
| 11 | 4.97 | 6.13 |
| 12 | 5.79 | 6.97 |



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| Patients | [HHV8] In-house (log ₁₀ copies/10 ⁶ cells) | [HHV8] R-gene™ (log ₁₀ copies/10 ⁶ cells) |
|----------|---|--|
| 1 | Undetectable | Undetectable |
| 2 | Undetectable | Undetectable |
| 3 | Undetectable | Undetectable |
| 4 | 1.56 | 1.77 |
| 5 | 1.98 | 2.50 |
| 6 | 2.48 | 2.89 |
| 7 | 3.20 | 3.63 |
| 8 | 3.27 | 3.53 |
| 9 | 3.87 | 4.35 |
| 10 | 4.68 | 5.21 |
| 11 | 4.97 | 5.56 |
| 12 | 5.79 | 6.42 |

