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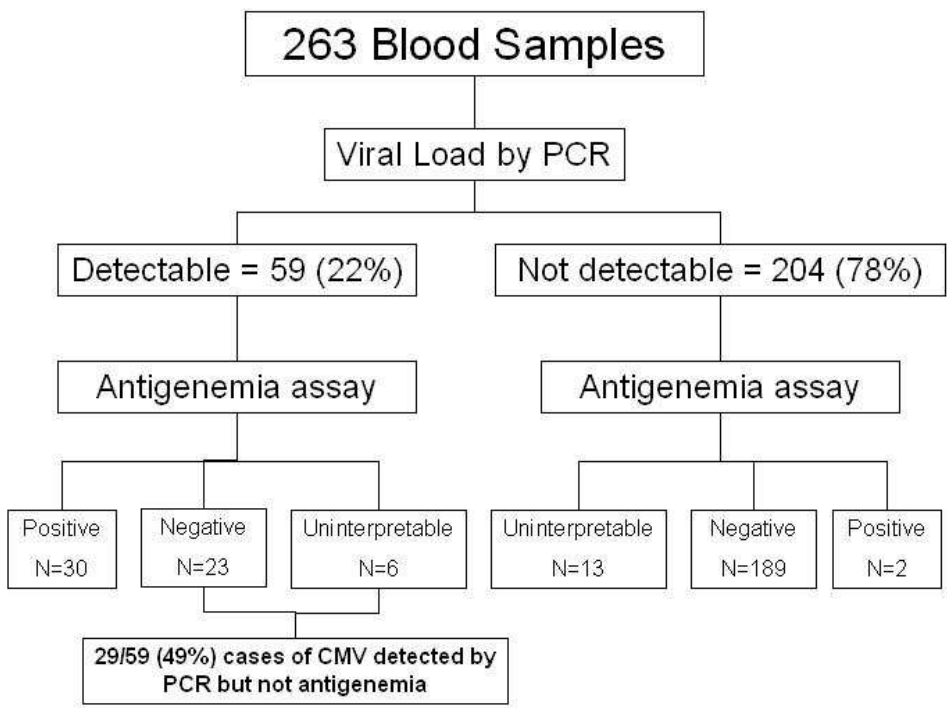


**Algorithm based on CMV kinetics DNA viral load for
preemptive therapy initiation after hematopoietic cell
transplantation**

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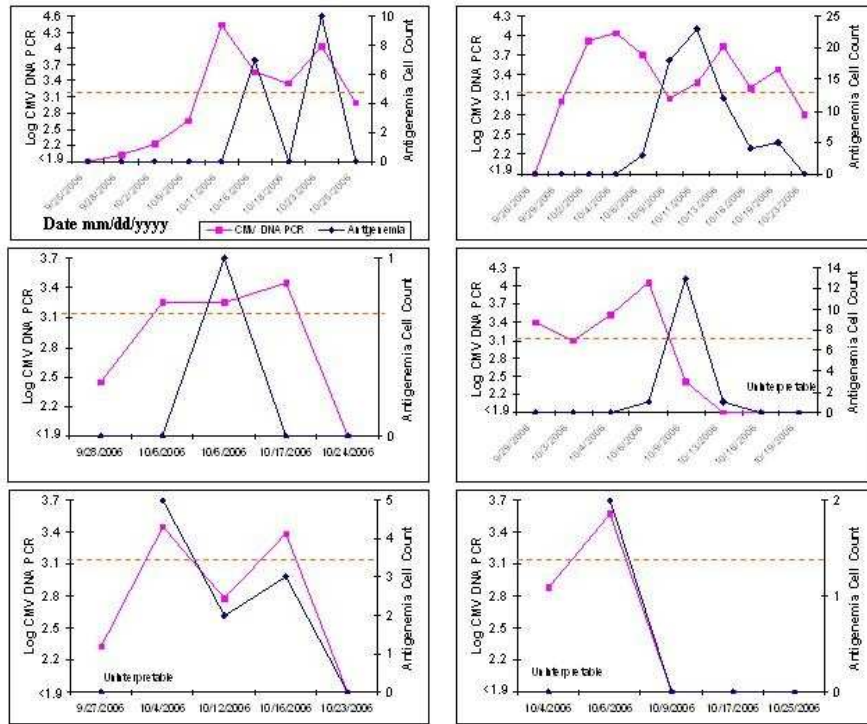
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Review

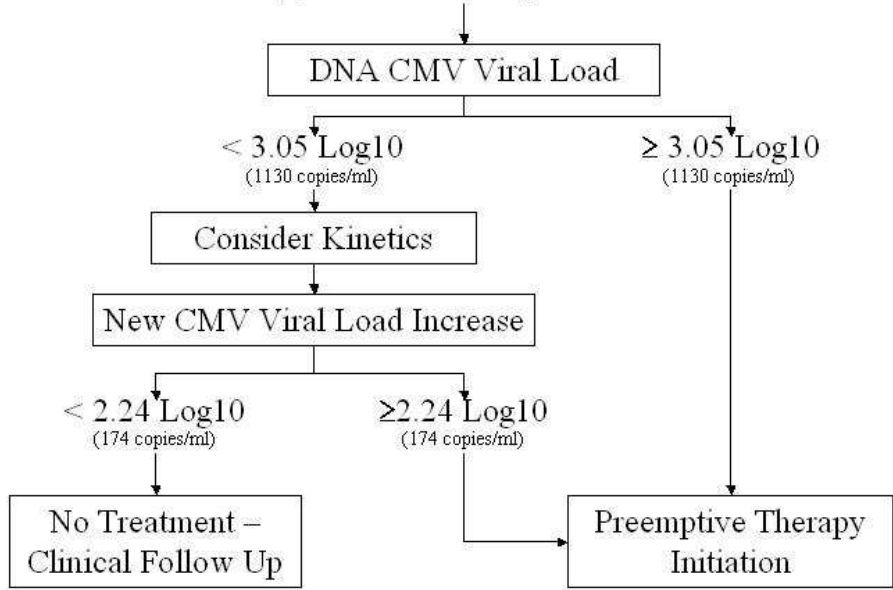
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Decision Ongoing CMV Preemptive Therapy in Allograft Patients



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Review

Algorithm based on CMV kinetics DNA viral load for preemptive therapy initiation after hematopoietic cell transplantation

Running Head: CMV viral load for preemptive therapy

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Abstract:

Preemptive therapy in hematopoietic cell transplantation is initiated by a diagnostic technique at first detection of cytomegalovirus (CMV).

The aim of this study was to use the viral dynamics of CMV DNA viral to start preemptive therapy, and to prospectively compare the CMV viral load kinetics to pp65 antigenemia.

263 blood samples were collected prospectively from 93 patients. All clinical decisions regarding use of preemptive therapy were based on CMV antigenemia.

Based on the positivity of the antigen assay and clinical CMV outcome in allotransplant patients, an optimal threshold of 3.05 log₁₀ (1130 copies/ml) was found to discriminate patients who required preemptive therapy and those who did not (sensitivity, 71%; specificity, 65%). A DNAemia level increase of 2.24 log₁₀ (174 copies/ml) per day was the optimal threshold to discriminate between patients who required preemptive therapy and those who did not (sensitivity, 93%; specificity, 43%). Sensitivity of PCR assay was 92.4% compared with 39% for the antigen assay ($p < .001$).

A standardized real-time PCR assay is more appropriate than the antigen assay for detecting CMV. It allowed earlier diagnosis of active CMV infection and monitoring of the response to anti-CMV treatment.

Keywords: Cytomegalovirus, plasma real-time PCR, Preemptive therapy

24 Background

25 CMV remains one of the most important complications after allogeneic hematopoietic
26 stem cell transplantation (HCT) [Boeckh et al., 2003; Krause et al., 1997; Razonable
27 and Emery, 2004]. Both DNA and CMV pp65 antigen detection methods have largely
28 replaced viral tube cultures and shell vial culture in CMV surveillance because of
29 their improved sensitivity, predictive value, and rapidity [Boeckh et al., 1996; Boeckh
30 et al., 2004; Boivin et al., 2000; Caliendo et al., 2007; Caliendo et al., 2000; Einsele
31 et al., 1995]. The CMV load detected in blood and in tissues is an important factor in
32 predicting subsequent or ongoing CMV disease in transplant patients and individuals
33 infected with human immunodeficiency virus (HIV)[Ghaffari et al., 2008; Humar et al.,
34 2009]. Antigenemia assay and culture assays are imprecise, have poor sensitivity, or
35 require long time processing. These drawbacks may be improved by real time PCR
36 with automated DNA extraction [Abbate et al., 2008; Gimeno et al., 2008; Gouarin et
37 al., 2007; Ksouri et al., 2007; Tang et al., 2008]. Earlier studies suggested that
38 plasma-based PCR assays result in delayed detection of CMV DNA because the
39 sensitivity of these assays is lower than that of cell-based and whole blood assays
40 [Razonable et al., 2002]. Plasma does not require lengthy cell separation and offers a
41 much better opportunity to detect CMV viremia during periods of severe cytopenia
42 when cell-based assays perform poorly. Plasma-based assays were validated
43 clinically by direct comparison with CMV detection [Kalpoe et al., 2004; Lengerke et
44 al., 2006]. Quantitation of virus load may also be useful in preemptive treatment
45 strategies; a certain threshold or increasing levels of viral load could be used to start
46 antiviral therapy [Boeckh and Ljungman, 2009; Lilleri et al., 2004; Machida et al.,
47 2000; Mori et al., 2002]. Recent guidelines confirmed that initial viral load is probably
48 the best indicator of viral dynamics because it indicates the slope of CMV replication
49 without intervention [Boeckh and Ljungman, 2009; Verkruyse et al., 2006]. These
50 guidelines reported that an initial viral load of 1000 copies/ml should be considered
51 as the parameter to start preemptive therapy. It was demonstrated that the doubling

52 time of CMV is only 1 to 2 days on average and that the degree of
53 immunosuppression determines *in vivo* replication dynamics [Emery et al., 1999].
54
55 The primary aim of the present study was to use the viral dynamics of CMV viral load
56 as a parameter to start preemptive therapy. The secondary objective was to compare
57 prospectively the CMV viral load kinetics to pp65 antigenemia.

58

59 **Study design**

60

61 All patients were recruited at the hematology unit of the Institute Paoli Calmettes in
62 Marseille, France between September 2006 and October 2006. A total of 263 blood
63 samples were collected prospectively from 93 patients (59 men, 34 women) and
64 were subjected the same day to a pp65 antigenemia assay and a quantitative real-
65 time PCR assay of plasma DNA (**Table 1**).

66

67 **Monitoring and management of active CMV infection.** The surveillance for active
68 CMV infection was performed routinely with the pp65 antigenemia assay and the
69 real-time PCR assay. An episode of active CMV infection was defined either by a
70 single positive PCR, by antigenemia, or by both. Two consecutive negative results on
71 both tests defined the end of a given episode. Preemptive therapy with oral
72 valganciclovir (900 mg/12 h) and intravenous ganciclovir (5 mg/kg of body weight/12
73 h) was initiated upon a positive antigenemia result (≥ 1 pp65-positive cells/200,000
74 cells) as indicated previously.

75

76 ***pp65 antigenemia assay***

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78 The CMV pp65 antigenemia assay is an indirect immunofluorescence test used for
79 the detection of internal matrix phosphoprotein (pp65) in peripheral blood leukocytes.

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80 It was carried out using the CINAKit® (Argene, France) according to the
81 manufacturer's recommendations. The presence of one or more pp65-positive cells
82 was considered a positive result.
83

84 **Real-Time PCR assay.** Blood samples were obtained in EDTA-treated tubes and
85 were processed within 2 hours. Polymorphonuclear leukocytes (PBMCS) and plasma
86 were separated by the standard dextran sedimentation method. Real-time PCR with
87 the Artus CMV LC PCR kit (Qiagen GmbH, Hilden, Germany) was performed using
88 the ABI PRISM 7000 system (Applied Biosystems Inc., Foster City, CA), or the
89 m2000RT (Abbott Molecular) according to the manufacturer's instructions. The limit of
90 detection of the assay was 64.2 copies/ml (linear range: [200;10⁶ copies/ml]. DNA
91 extractions were performed using the Abbott sample preparation system DNA kit on
92 the m2000 instrument (Abbott Molecular) according to the manufacturer instructions
93 [Gimeno et al., 2008].

94 **Data analysis.** Non parametric Cuzick test for trend was used to compare multiple
95 distributions [Cuzick, 1985]. Chi-square test was used to compare proportions.
96 Statistical significance was considered if $p < 0.05$. Receiver operating curve (ROC)
97 was used and Youden index was used to determine determine the most appropriate
98 DNAemia threshold. All calculations were assessed using SAS 9.1.3 (SAS Institute
99 Inc. Cary, NC).

100

101 **Results**

102

103 ***Performance of PCR and pp65 antigenemia assays for detection CMV.***

104 Among the 263 samples from the 93 patients, 59 (22%) had a detectable viral load
105 by PCR and 204 (78%) had non detectable viral load (**Figure 1**). With the pp65

106 antigenemia assay, 32 samples (12%) were positive, 30 among the 59 detectable by
107 PCR, and 2 among the 204 non detectable by PCR. Nineteen samples could not be
108 analyzed by the antigenemia assay because of low neutrophil counts, among which
109 six were detectable by PCR. CMV was detected by both assays in 30 samples (11%)
110 **(Table 2)**. Among the 59 samples with detectable viral load by PCR, 29 (49%) were
111 not detected by the antigenemia assay. Two samples were positive by the
112 antigenemia assay but non detectable by the PCR assay. These samples were
113 reanalyzed by PCR using different aliquots and found to be undetectable (the internal
114 control was amplified, ruling out the possibility of PCR inhibition or inefficient DNA
115 extraction). The concordance between the two assays was 90% (kappa=.65) and
116 PCR assay was more sensitive than antigenemia assay (92.4% versus 39.0%
117 $p<.001$) for detection of CMV in blood.

118 In samples detectable by PCR, CMV DNA increased significantly with antigenemia
119 cell number (Cuzick test for trend $p=0.02$) (data not shown).

120 Based on the follow-up of the positive detection by PCR and/or the CMV clinical
121 outcome, 14 episodes of active CMV infection were diagnosed **(Table 3)**.

122

123 ***Discordant cases***

124 - *Antigenemia positive and PCR negative*

125 Two cases were non detectable by PCR assay and positive by antigenemia assay. At
126 time point "October 4, 2006", log CMV DNA PCR was <1.90 , and antigenemia cell
127 count was 1. This case might be considered as antigenemia false positive because
128 CMV virus detection quickly turns negative after that time point. The same can be
129 expected for another case: at time point "October 2, 2006", log CMV DNA PCR was
130 <1.90 , and antigenemia cell count was 1. The follow-up of both PCR and
131 antigenemia assays of these two cases reveals negative results. These cases were
132 not linked with any CMV clinical outcome and were considered as antigenemia false
133 positive.

134 - *Antigenemia negative and PCR positive*

135 **Figure 2** shows the kinetics of six cases that were CMV DNA PCR detectable and
136 antigenemia negative. Antigenemia turned positive respectively 19 days, 8 days (for
137 three cases), 7 days, and 2 days after PCR assay.

138

139 ***Guiding preemptive therapy on the basis of plasma CMV DNA quantitation.***

140 - *Initial viral load threshold*

141 Based on the positivity of the antigenemia assay in allotransplant patients, an optimal
142 threshold of 3.05 log₁₀ (1130 copies/ml) was found to discriminate patients who
143 required preemptive therapy and those who did not (sensitivity, 71%; specificity,
144 65%). The same analysis was performed on the entire cohort: 3.17 log₁₀ (1494
145 copies/ml) was the optimal threshold for ongoing preemptive therapy (sensitivity,
146 72%; specificity, 61%).

147 - *Kinetics viral load threshold*

148 In allotransplant patients, as long as CMV DNAemia is below a cut-off value of 3.05
149 log₁₀ (1130 copies/ml), any slope of viral load change ≥ 2.24 log₁₀ per day should
150 set off an alarm for preemptive therapy. This threshold slope corresponds with a viral
151 load increase of at least 174 copies/ml/day. The analysis performed on the entire
152 cohort showed that the same threshold (2.24 log₁₀) was optimal.

153

154 **Discussion**

155

156 Using CMV viral load in PCR tests for CMV DNA detection in place of pp65 antigen
157 test in order to identify CMV infections earlier constitutes an important medical
158 challenge for many reasons. Firstly, the positive predictive value of plasma PCR for
159 the development of CMV disease has been evaluated in different settings with
160 somewhat contradictory results [Boeckh et al., 2004; Boeckh and Ljungman, 2009;

161 Caliendo et al., 2007; Caliendo et al., 2000; Gerna et al., 2008; Gerna et al., 1998].

162 The consistently positive CMV DNA results during CMV infection, whereas pp65 tests
163 shows false negatives, lead to a more accurate description of CMV
164 dynamics/kinetics. Secondly, the quantitative nature of the assay may increase its
165 specificity by using a certain viral load threshold or increases over time, thereby
166 avoiding unnecessary treatment of patients who are at low risk of progression to
167 disease. CMV DNA has a much higher stability in blood/plasma than pp65 antigen in
168 polynuclear cell lymphocyte and is therefore less sensitive to transport and does not
169 require immediate processing of samples [Boeckh and Ljungman, 2009; Boivin et al.,
170 2000; Ksouri et al., 2007; Weinberg et al., 2002; Yakushiji et al., 2002].

171 In the present study, based on the positivity of the antigenemia assay and clinical
172 CMV outcome, it has been demonstrated that an optimal initial viral load threshold of
173 3.17 log₁₀ (1494 copies/ml) discriminated patients who required preemptive therapy
174 (sensitivity, 72%; specificity, 61%). A threshold of 3.00 log₁₀ was applied as to fit
175 with a recent study by Boeckh and Ljungman [Boeckh and Ljungman, 2009]: Results
176 were similar with the ones obtained with 3.17 log₁₀ threshold: sensitivity of 75%,
177 specificity of 57%, negative predictive value of 62%, and positive predictive value of
178 71%.

179 In the present study, the PCR assay was more sensitive than the antigenemia assay
180 for detecting CMV in blood (sensitivity, 92.4% versus 39.0% respectively, $p < .001$). In
181 fact, 14 episodes were defined solely on the basis of positive results with the PCR
182 assay, whereas only two non detectable PCR/antigenemia-positive episodes were
183 observed. If the decision to initiate preemptive therapy in our cohort had been based
184 upon either the first detection of CMV DNA in plasma or the occurrence of two
185 consecutive detectable PCR results, the first detection at 3.05 log₁₀ (1130 copies/ml)
186 would have allowed preemptive therapy to be started 8 days before the first positivity
187 of antigenemia. On the other hand, as long as CMV DNAemia is below a cut-off
188 value of 3.05 log₁₀ (1130 copies/ml), any slope of viral load change ≥ 2.24 log₁₀ per

189 day should set off an alarm for preemptive therapy. This threshold slope corresponds
190 with a viral load increase of at least 174 copies/ml/day. Using this cut-off would have
191 allowed preemptive therapy to be started 3 days before the first positivity of
192 antigenemia.

193 Recently, consensus criteria have been reported for the initiation of anti-CMV
194 preemptive therapy on the basis of a quantitative PCR monitoring strategy in this
195 clinical setting. The coefficient of variation of most PCR assays for viral loads close to
196 the limit of detection may be as high as 30% [Boeckh and Boivin, 1998; Boeckh et
197 al., 2004; Von Muller et al., 2002]. Thus, increases of less than 0.5 log₁₀ (or 3 times
198 the baseline level) may not represent true increases. Based on these principles, a
199 preset viral threshold of 1,000 copies/ml has been in use for some years [Boeckh and
200 Ljungman, 2009; Verkruyse et al., 2006]. Data presented in this study are in
201 accordance with those reported in earlier studies evaluating the performance of
202 several laboratory-developed plasma real-time PCR assays in comparison with the
203 antigenemia assay [Abbate et al., 2008; Boeckh et al., 2004; Caliendo et al., 2007;
204 Ghaffari et al., 2008; Gimeno et al., 2008; Kalpoe et al., 2004; Ksouri et al., 2007;
205 Mori et al., 2002; Pumannova et al., 2006]. The concordance between the
206 antigenemia assay and the Artus CMV LC PCR assay results was 90% ($k=0.65$), also
207 reported by others [Gimeno et al., 2008; Pajand et al., 2008; Solano et al., 2001].
208 Analysis of the kinetics of CMV DNAemia—but not that of the antigenemia—either at
209 the time of initiation of preemptive therapy or early thereafter may allow the prediction
210 of the response to CMV treatment.

211 An algorithm based on CMV kinetics for initiation of preemptive therapy after
212 allogeneic hematopoietic stem cell transplantation was suggested (**Figure 3**). This
213 algorithm is based on a single initial determination of CMV viral load; in case of value
214 above 1130 copies/ml, one should consider to start preemptive therapy; in case of
215 value below this cut-off, one should consider the kinetics of CMV viral load. If the

1 216 value, even on the following day, were at least 174 copies/ml higher, one should
2 217 consider to initiate preemptive therapy.
3
4 218 One limitation of this study might be the lack of standardization of CMV DNA
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6 219 monitoring. Such a standardization could be used to calibrate all the different PCR
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8 220 methods, but it represents a major obstacle in obtaining universally acceptable and
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10 221 validated thresholds for preemptive therapy [Deback et al., 2007]. This limitation can
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12 222 be improved by the development of an international standard using International
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14 223 Units. Another point to be discussed is the use of whole blood (which is known to get
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16 224 a higher viral load than plasma) for assessing CMV DNA with a possible earlier
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18 225 detection of CMV DNA in whole blood, due to the higher sensitivity of cell-based and
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20 226 whole blood assays.
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28 228 In conclusion, data presented in this study indicate that the Qiagen Artus CMV LC
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30 229 PCR kit, a standardized assay, is more suitable than the antigenemia assay for
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32 230 detecting CMV in blood. It allowed earlier diagnosis of active CMV infection and
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34 231 monitoring of the response to anti-CMV treatment. This study confirmed the threshold
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36 232 plasma viral load of 1000 copies/ml as a cut-off for initiating preemptive therapy
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38 233 [Boeckh and Ljungman, 2009]. In addition, in case of initial viral load detection of
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40 234 CMV DNA below this range, the kinetics of CMV DNA should be monitored every
41
42 235 three days and preemptive therapy initiated when DNAemia level increases by 2.24
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44 236 log₁₀ (174 copies/ml) per day.
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52 239 **Conflict of interest disclosure:** All authors declare that they have no conflict of
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54 240 interest.
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379 **Figure Legends**

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3 **Figure 1: Description of the 263 blood samples included**

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5 383

6 **Figure 2: Discordant cases analysis (detected by PCR assay and negative by Antigenemia).**

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8 **Horizontal dotted lines indicates the optimal initial viral load threshold of 3.17 log₁₀**

9 **discriminating patients requiring preemptive therapy.**

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12 **Figure 3 Algorithm for initiation of preemptive therapy after allogeneic hematopoietic stem cell**

13 **transplantation**

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390 **Table 1: Clinical characteristics of the 263 study samples**

	Clinical Context
<i>Allotransplant (N=168)</i>	Asymptomatic (N=159); Fever (N=2); Not Defined (N=7)
<i>Other (95):</i>	
Autotransplant (N=4)	Cytolysis* (N=2) ; Asymptomatic (N=1) ; Other (N=1)
AML (N=12)	Asymptomatic (N=4) ; Cytolysis (N=2) ; Fever (N=1) ; Arthralgia (N=1); CMV Pneumonia (N=1); Not Defined (N=3)
CLL (N=9)	Asymptomatic (N=6); Fever (N=2); Not Defined (N=1)
Lymphoma (N=9)	Fever (N=4); Cytolysis* (N=1); CMV Pneumonia (N=1); Not Defined (N=3)
Myeloma (N=9)	Asymptomatic (N=3) ; Fever (N=2) ; Cytopenia (N=2) ; Not Defined (N=2)
TRT (N=21)	Asymptomatic (N=11) ; CMV Pneumonia (N=3) ; Cytolysis* (N=2) ; Fever (N=2); Cutaneous Rash (N=1); Other (N=1); Not Defined (N=1)
Not Defined (N=30)	Asymptomatic (N=6) ; Fever (N=3) ; CMV Pneumonia (N=2) ; Not Defined (N=22)

391 *Cytolysis is considered when >2 x upper normal limit

392 **Table 2: Comparison of PCR assay and the antigenemia assay for detection of CMV in the 263**
 393 **blood samples**

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Result of antigenemia assay	Viral Load by PCR assay	
	Positive (59)	Negative (204)
Positive (32)	30	2
Negative (212)	23	189
Uninterpretable (19)	6	13

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396 **Table 3: characteristics of the 14 patients, corresponding to the 14 episodes**

397

Patient N°	Gender	Clinical Context	Episode beginning			Date of 1 st positive Ag	End of follow-up
			Date	viral load (cp/ml)	Ag* (cell nb)		
1	Male	Asymptomatic	09/28	283	0	10/06	10/24
2	Female	Asymptomatic	10/09	10133	5	10/09	10/19
3	Male	Asymptomatic Fever	10/04	766	0	10/06	10/25
4	Male	Asymptomatic	09/28	105	0	10/10	10/25
5	Male	Pneumonia Fever Cytolysis Cutaneous rash	09/29	2529	0	10/06	10/19
6	Male	Asymptomatic	09/26	321	4	09/26	10/24
7	Male	Asymptomatic	09/21	241	0	-	09/29
8	Male	Asymptomatic	09/27	216	0	10/04	10/23
9	Male	Asymptomatic	09/29	997	0	10/06	10/23
10	Female	Asymptomatic	09/25	1823	33	09/25	10/25
11	Male	Asymptomatic Fever	09/10	1402	1	09/10	10/24
12	Male	-	09/29	148	0	-	10/12
13	Female	Asymptomatic Fever	10/02	0	1	10/02	10/16
14	Female	Asymptomatic	10/09	49	4	10/09	10/23

398 *Ag: antigenemi