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2 **Original articles**

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4 **Development of a human herpesvirus 6 virus species-specific immunoblotting assay**

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16 ***Running title:*** HHV-6 virus species-specific serological assay

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18 **Footnote page**

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25 ***Key words*** **:** HHV-6, virus species-specific, antibody, immunoblotting, p100, 101K

26

27 ***Abstract***

28 In order to assess the full spectrum of HHV-6A- and HHV-6B-associated diseases, we sought
29 to develop a HHV-6 virus species-specific serological assay based on immunoblot analysis.
30 The immunodominant proteins encoded by ORF U11, p100 for HHV-6A (strain U1102) and
31 101K for HHV-6B (strain Z29), were selected to generate virus species-specific antigens.
32 Recombinant p100 and 101K were produced in a prokaryotic expression system. The
33 expression of these proteins was confirmed using anti-His tag and 101K-specific monoclonal
34 antibodies. HHV-6 virus species-specific antibodies were detected by immunoblotting in
35 patient sera. Eighty seven serum samples obtained from various subjects were utilized to
36 determine the reliability of the method for clinical use. Ten of 12 exanthem subitum
37 convalescent sera reacted exclusively with 101K, while none of 12 acute sera reacted with
38 either protein. Two of three sera collected from HHV-6A-infected patients reacted with p100
39 and 101K. Although all 5 acute and convalescent sera obtained from transplant recipients
40 reacted exclusively with 101K, two of 6 convalescent sera obtained from patients with
41 drug-induced hypersensitivity syndrome reacted with both p100 and 101K. Thirty-one of 38
42 sera obtained from healthy adults were positive for 101K antibody, while 4 reacted with both
43 proteins. However, PCR analysis of PBMCs and saliva from these subjects did not detect
44 HHV-6A DNA. In conclusion, this novel serological assay based on immunoblot analysis
45 using recombinant HHV-6A p100 and HHV-6B 101K allowed to discriminate between
46 HHV-6A- and HHV-6B-specific antibodies.

47

48 Human herpesvirus 6 (HHV-6) is classified as two distinct virus species, designated
49 HHV-6A and HHV-6B (2, 3, 20). Since the overall nucleotide sequence identity between the
50 virus species is 90% (11, 17), distinguishing between the two s using serological analysis has
51 proven difficult. It has been demonstrated that primary HHV-6B infection occurs in infancy
52 and early childhood (31), and causes exanthem subitum (31, 34), a common febrile
53 exanthematous illness. Additionally, in transplant recipients HHV-6B reactivation can cause
54 several clinical manifestations such as encephalitis, bone marrow suppression, and
55 pneumonitis (32). In contrast to HHV-6B, HHV-6A seems to be less prevalent in the
56 population: it is rarely detected in transplant recipients with encephalitis (7, 10), but it has
57 been implicated in the pathogenesis of multiple sclerosis (25). *In vivo* studies have suggested
58 that HHV-6A has a stronger neurotropism than HHV-6B (1, 15). To date, however, both the
59 clinical features of primary HHV-6A infection and the full spectrum of diseases associated
60 with HHV-6A have not been elucidated.

61 Methods for the differentiation between HHV-6A and B have been developed based
62 on restriction fragment length polymorphism analysis of polymerase chain reaction (PCR)
63 products, PCR using virus species-specific primers, and Southern blotting analysis with virus
64 species-specific probes (3, 4, 12, 28). Subsequently, real-time PCR methods using virus
65 species-specific primers or probes have been introduced for easier discrimination between the
66 two virus speciess (6). Recent PCR-based molecular epidemiological analysis demonstrated
67 that HHV-6A is highly endemic in the region of sub-Saharan Africa (5, 18). However, it is
68 difficult to discriminate between active and latent infections on the basis of PCR analysis

69 because these viruses can latently infect peripheral blood mononuclear cells (PBMCs) after
70 primary infection. Furthermore, the most important problem of molecular epidemiological
71 analysis is that this analysis does not reveal precise seroepidemiology and can be affected by
72 the sensitivity of PCR method used. Thus, the lack of a virus species-specific serological
73 assay has hampered the elucidation of clinical features and epidemiology of HHV-6A
74 infection.

75 The ideal gene target for the development of a virus species-specific serological
76 assay would be a gene with low sequence homology between the two virus species encoding a
77 strong immunoreactive protein. We chose the U11 gene, which encodes a major antigenic
78 structural protein and has 81% amino acid sequence identity between HHV-6A and B (11,
79 17). Previous studies have shown that the 101 kDa HHV-6B virion protein (101K) encoded
80 by the U11 gene is highly immunoreactive in immunoblotting analysis and is a specific
81 serological marker of infection (24, 30). Therefore, we sought to develop a virus
82 species-specific serological assay based on immunoblotting analysis utilizing the U11 gene of
83 HHV-6. The reliability of this novel virus species-specific assay was examined using human
84 sera collected from patients with various types of HHV-6 infection.

85

86 **Materials and methods**

87 ***Cells and viruses.***

88 Cord blood mononuclear cells were separated by Ficoll-Hypaque gradient
89 centrifugation from heparinized cord blood samples, and stimulated for 2 days before
90 inoculation with the viruses in RPMI-1640 medium containing 20% fetal calf serum, 0.1U/mL
91 recombinant human interleukin-2, and 5μg/mL phytohemagglutinin-P. HHV-6A (U1102
92 strain) or HHV-6B (Z29 strain) were propagated in cord blood mononuclear cells. At day 7
93 post-inoculation, when the infected cells showed maximum levels of cytopathic effects, the
94 infected cells were harvested and lysed by repeated freezing-thawing. The samples were
95 stored at -20°C until the generation of expression vectors.

96 ***Patients and samples.***

97 Four panels of serum samples (total: 87 serum samples) were collected from patients
98 with different clinical symptoms. (1) Positive control sera for HHV-6A infection were
99 collected from two patients with chromosomally integrated HHV-6A (ciHHV-6A) and one
100 patient with chronic fatigue syndrome and HHV-6A infection. (2) Positive control sera for
101 HHV-6B infection were 12 paired sera obtained from patients with exanthem subitum (age, 7
102 to 17 months; median age, 11 months). Primary HHV-6B infection was confirmed by virus
103 isolation and seroconversion for IgG HHV-6 antibody. (3) As controls for HHV-6B
104 reactivation, 6 paired sera were obtained from drug-induced hypersensitivity syndrome
105 (DIHS) patients (age, 29 to 73 years; median age, 61.5 years) and 5 paired sera from
106 hematopoietic stem cell transplant (HSCT) recipients with HHV-6 reactivation (age, 10

107 months to 11 years; median age, 3 years). HHV-6B reactivation was confirmed by virus
108 isolation and serological analysis in all of the patients. (4) As control sera for considering as
109 previous infection with HHV-6B, 38 healthy seropositive adult sera (age, 20 to 88 years;
110 median age, 31 years) were used.

111 In addition to the serum samples, 24 PBMC and 38 saliva samples were collected
112 from the 38 healthy control adult subjects to assess the presence of latent virus
113 species-specific infection. All samples were stored at -20 °C until assayed.

114 As patients with primary HHV-6A infection are very difficult to identify, serum
115 obtained from macaques inoculated with HHV-6A (21) was used to determine whether this
116 assay can specifically detect HHV-6A-specific antibodies.

117 ***DNA extraction.***

118 Viral DNAs were extracted from the HHV-6A (U1102) and HHV-6B (Z29) infected
119 cord blood mononuclear cells using a QIAamp DNA Blood mini kit (Qiagen, Chatsworth,
120 CA) according to the manufacturer's instruction, and were used as the templates for the
121 generation of the expression vectors. In order to determine the genotype of HHV-6, viral
122 DNA was extracted from PBMCs and saliva obtained from 38 seropositive healthy adults
123 using the same DNA extraction kit. Then, extracted DNA were analyzed by nested PCR to
124 discriminate between HHV-6A and B.

125 ***PCR assay.***

126 Details of the nested PCR for determining the genotype of HHV-6 was described in a
127 previous report [28]. In brief, the PCR amplifies a 751 base pair (bp) DNA fragment

128 containing the gene that encodes a putative large tegument protein (U31 gene) using Taq
129 DNA polymerase (Ampli Taq Gold; Perkin-Elmer, Norwalk,CT). The identity of the virus
130 species of HHV-6 is determined by the presence or absence of a *Hind*III site in a second PCR
131 product.

132 ***Construction of HHV-6 U11 gene expression plasmid.***

133 U11 gene of HHV-6A and HHV-6B encoding for p100 and 101K protein
134 respectively were amplified with (2.5U/μl pfu) DNA polymerase (Fermentas LIFE
135 SCIENCES, MD, USA) using following primer sets. The upstream and downstream primers
136 for amplification of the U11 gene of HHV-6A (U1102) were HHV-6A U11 NcoI
137 (5'-GCGATGGCCATGGAT ATCATGGATCTGCAAAGACAT-3') and HHV-6 AB U11
138 SacI (5'-GTCGACGGAGCTCGACGACGCGATCGCTGA-3'). In order to amplify the U11
139 gene of HHV-6B (Z29), HHV-6B U11 NcoI (5'-GCGATGGCCATGGAT
140 ATCATGGATTTGAAAGCGCAG) was used for upstream primer, and HHV-6 AB U11 SacI
141 was used for downstream primer.

142 The upstream primer has a *Nco*I restriction enzyme site and the downstream
143 primer has a *Sac*I restriction enzyme site (underlining indicates restriction enzyme sites). The
144 PCR products were digested with the restriction endonucleases *Nco*I and *Sac*I (NEW
145 ENGLAND BioLabs, MA, USA), respectively. The purified fragments were ligated into the
146 histidine tagged expression vector pET22b(+) (Novagen, CA, USA) using Ligation High
147 (TOYOBO, Japan). As shown in Figure 1, the plasmids, pET22b(+)-6AU11 and
148 pET22b(+)-6BU11, consisting of a pelB leader on the N-terminal end and a 6xHistidine-tag

149 on the C-terminal end, were used to transform the competent *E. coli* strain Nova Blue
150 (Invitrogen, CA, USA).

151 ***Expression of p100 and 101K.***

152 *E. coli* strain BL21 cells (Invitrogen) were transformed using the recombinant
153 pET22b(+)-6AU11 and pET22b(+)-6BU11 plasmids. Recombinant p100 and 101K were
154 expressed by optical induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3
155 hours at 30°C. The bacteria were harvested and the cell pellets were frozen at -80°C overnight.
156 The frozen pellets were thawed, resuspended in 200μl of lysis buffer (PBS with 0.05% NaN_3)
157 supplemented with protease inhibitor (Roche Applied Science, IN, USA). The suspensions
158 were sonicated and the extracts were used for immunoblot analysis.

159 ***Immunoblot assay.***

160 Recombinant p100 and 101K were resolved by 10% sodium dodecyl
161 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred
162 to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). The membrane was
163 incubated in blocking buffer (5% skim milk in PBS) overnight at 4°C. Next, it was incubated
164 for 1 hour at room temperature with the primary antibodies, mouse anti-His tag antibody
165 (MBL, 6C4, M089-3), and mouse anti-101K (Chemicon, MAB8535, C3108-103), diluted to
166 1:1500 using antibody dilution buffer (5% skim milk in PBS). Meanwhile, human sera
167 samples were diluted to 1:500 for use as primary antibodies. Preliminary experiment for
168 determining an appropriate dilution of serum samples was carried out, and finally 500 time
169 dilution was the optimal dilution of this IB assay. After the membrane was washed with PBS,

170 it was incubated for 1 hour at room temperature with horseradish peroxidase

171 (HRP)-conjugated goat anti-mouse IgG (MBL, Nagoya, Japan) or HRP-conjugated mouse

172 anti-human IgG (MBL) antibodies. The enzyme-labeled antibodies were detected by ECL

173 plus Western Blotting Detection System (GE Healthcare Bio-Sciences, UK) according to the

174 manufacturer's instructions.

175 ***Immunofluorescence assay (IFA).***

176 Antibody titers to HHV-6 were measured by indirect IFA as described previously.

177 The detection limit of IFA titer was <8 (34). The representative strain of HHV-6A (U1102

178 strain) and B (Z29 strain) were used as the standard antigens. The antibody titer was defined

179 as the reciprocal of the serum dilution showing specific fluorescence.

180

181 **Results**

182 ***Expression of HHV-6A and B U11 gene-encoded proteins.***

183 To determine whether the HHV-6A and B U11 gene expression plasmids

184 (pET22b(+)-6AU11 and pET22b(+)-6BU11) expressed the expected proteins,

185 immunoblotting analysis was carried out using an anti-His tag antibody and an anti-101K

186 antibody (Fig. 1B). As expected, the pET22b(+)-6AU11 product was recognized as a 100-kDa

187 band, and the pET22b(+)-6BU11 product was recognized as a 101-kDa band using the

188 anti-His tag antibody. Moreover, only the pET22b(+)-6BU11 product was detected by the

189 anti-101K HHV-6B-specific monoclonal antibody, demonstrating HHV-6B-specific protein

190 expression. As an HHV-6A U11 gene product-specific monoclonal antibody was not available,

191 it was impossible to determine whether the pET22b(+)-6AU11 product was virus species

192 specific. As shown in Fig. 1 B, these plasmids synthesized the specific proteins without IPTG

193 induction, and not only up-regulation of the proteins synthesis but also production of

194 additional sized proteins was demonstrated after IPTG induction.

195 ***Evaluation of the assay for clinical applications.***

196 Assessment of HHV-6A-infected patients (no information with regard to HHV-6B

197 infection) revealed that two of the three sera (Pt. 2 and 3) reacted with both p100 and 101K

198 (Table 1). Representative results of immunoblotting analysis in patients 2 and 3 are shown in

199 Figure 2A and B, respectively. Serum collected from patient 1, a ciHHV-6A patient with

200 central nervous system manifestations, did not react with either protein despite having low

201 titers of HHV-6A and B antibodies based on IFA analysis.

202 Among acute sera from 12 patients with primary HHV-6B infection (exanthem
203 subitum), none reacted with either p100 or 101K (Table 1 and Fig. 2C). However, 10 of the 12
204 (83.3%) convalescent sera reacted exclusively with 101K (Table 1 and Fig. 2C). In contrast to
205 the immunoblotting, seroconversion against both HHV-6A and B was demonstrated in all 12
206 patients by IFA analysis, reflecting the presence of cross-reactive epitopes. As shown in Table
207 1, the two negative sera were collected on days 10 (Pt. 8) and 11 (Pt. 7) after onset of the
208 illness, and showed relatively low IFA antibody titers. Contrary to our expectation, monkey
209 sera obtained from macaques inoculated with HHV-6A were found to be reactive with both
210 p100 and 101K (data not shown). It is not clear whether this finding reflects the presence of
211 pre-existent antibodies against a monkey variant of HHV-6B or cross-reaction between A and
212 B antibodies.

213 Sera from DIHS patients with HHV-6 reactivation revealed reactivity with 101K in
214 only three of the 6 acute sera (50.0%). However, all convalescent sera reacted with 101K
215 (Table 1), and two of them (Pt. 16 and 17) reacted not only with 101K but also with p100
216 (Table 1). In contrast, all acute and convalescent sera collected from HSCT recipients reacted
217 exclusively with 101K (Table 1).

218 In healthy adults considered to have previous infection with HHV-6B, 31 of the 38
219 (81.6%) sera were positive for 101K-specific antibodies (Table 2 and Fig. 2D). Three (no. 21,
220 26, and 33) of the 7 negative sera contained low titers of HHV-6B IgG antibodies (x8) as
221 determined by IFA, while in the other 4 sera IFA titers of HHV-6B IgG antibodies were 1:16.
222 Four (no. 5, 25, 28 and 29) of the 38 sera (10.5%) reacted with both 101K and p100 (Table 2

223 and Fig. 2E). In order to determine whether these subjects were latently infected with the two
224 HHV-6 virus species, detection of viral DNA from PBMCs or saliva was carried out using
225 nested PCR and genotyping (Table 2). HHV-6B DNA was detected in 9 (37.5%) of the 24
226 PBMCs, and 27 (71.1%) of the 38 saliva samples. None of the samples contained HHV-6A
227 DNA. Four (no. 8, 9, 26, and 33) of the seven cases without p100 and 101K antibodies
228 contained HHV-6B DNA in either PBMCs or saliva. Additionally, only HHV-6B DNA was
229 detected in either PBMCs or saliva collected from three (Pt. 5, 28, and 29) of the 4 cases
230 having both p100 and 101K antibodies, while the fourth was negative for both HHV-6A and B
231 DNA.
232

233 **Discussion**

234 The development of a virus species-specific serologic assay for HHV-6A and B
235 antibodies is urgently needed to evaluate the full pathogenic potential of the two virus species.
236 Using the U11 gene products, p100 (HHV-6A) and 101K (HHV-6B), we have developed a
237 novel assay that enables differentiation of antibodies directed against these two viruses.
238 Quality controls demonstrated that recombinant p100 (HHV-6A) and 101K (HHV-6B) were
239 correctly expressed by immunoblot analysis, and that 101K was HHV-6B specific. Although it
240 is important to note that it was impossible to prove that the p100 antigen was HHV-6A
241 specific because no appropriate monoclonal antibody is currently available, specificity was
242 demonstrated using human sera from patients with primary HHV-6B infection (exanthem
243 subitum) which failed to cross-recognize the p100 antigen. In contrast, we were unable to
244 discriminate between primary HHV-6A and B infection by IFA assay, even though the IFA
245 assay was more sensitive than the immunoblotting assay. This is likely due to the fact that IFA
246 utilizes whole virus as an antigen, which contains multiple cross-reactive epitopes between
247 the two virus species. These findings suggest that our immunoblotting assay could be useful
248 for distinguish between primary HHV-6A and B infections; however, the assay sensitivity
249 could be improved in future developments.

250 One of the two sera collected from patients with ciHHV-6 A and one serum collected
251 from a chronic fatigue patient with persistent HHV-6A infection were found to react with both
252 p100 and 101K antigens. However, neither p100 antibody nor 101K antibodies were detected
253 in the serum obtained from one of the ciHHV-6A patients. As IFA antibody titers against

254 HHV-6A and B were relatively low in this patient, in comparison to the other two patients, it
255 is possible that the patient (Pt. 1) had low levels of HHV-6A or B antibodies, below the
256 detection limit of immunoblotting analysis. Although it has been suggested that ciHHV-6
257 patients develop HHV-6 antibodies (14, 19, 23), to our knowledge this is the first report
258 examining virus species-specific antibodies in ciHHV-6 patients. Not only p100- but also
259 101K-specific antibodies were detected in patient 2, in whom the HHV-6A genome was
260 integrated. One possible explanation for the detection of 101K-specific antibodies is that the
261 patient was previously infected with HHV-6B. To confirm the hypothesis, ciHHV-6 patients
262 should be prospectively monitored to determine whether they could be infected with another
263 HHV-6 virus species.

264 At present, if patient has both p100 and 101K antibodies on the basis of our
265 immunoblotting assay, there are two possibilities; one is cross-reaction between HHV-6A and
266 B antibodies (patient infected with only HHV-6B) and another is double infection of HHV-6A
267 and B (patient infected with two viruses). In order to perform seroepidemiological analysis, it
268 is critical to determine whether p100 antigen is HHV-6A specific or not. Thus, serum obtained
269 from patients with primary pure HHV-6A infection would be necessary to evaluate the
270 specificity of this assay for detection of HHV-6A antibodies; indeed, such patients are difficult
271 to identify, except possibly among children in HHV-6A endemic region (5). A recently
272 reported microwell adapted immunoblot assay for HHV-6 virus species specific serological
273 analysis contains same problem. They used pooled serum samples collected from adult and
274 child donors to test assay reliability. As infectious status of HHV-6 virus species in the

275 subjects were not clear, it is impossible to prove whether the assay was virus species specific
276 or not (29).

277 Instead of serum collected from patients with primary HHV-6A infection, monkey
278 serum collected from macaques inoculated with HHV-6A were used to determine whether
279 p100 antigen contains HHV-6A specific epitope or not. In contrast to our expectation, the
280 monkey serum reacted with both p100 and 101K. In addition to possibility of cross-reactivity
281 between HHV-6A and B, as the presence of antibodies to HHV-6 has previously been
282 demonstrated in macaques (16), it is possible that an HHV-6B-like virus persistently infects
283 macaques inducing cross-reactive antibody that were detected by the present assay. Although
284 serum collected from macaques before virus inoculation would be useful for this experiment,
285 no such samples was available because pooled serum samples obtained in the previous study
286 were used in this experiment.

287 All ten paired sera obtained from HSCT recipients with HHV-6B reactivation reacted
288 exclusively with 101K antigen. According to the seroepidemiological studies conducted in
289 Japan (33, 35), most of transplant recipients are HHV-6 seropositive. A previous molecular
290 epidemiological study demonstrated that all HHV-6 isolates recovered from transplant
291 recipients in Japan were HHV-6B (13). Thus, the present findings are consistent with previous
292 seroepidemiological and molecular epidemiological studies. In contrast to transplant
293 recipients, three of the 6 acute sera samples obtained from DIHS patients did not contain
294 101K-specific antibodies. As suggested in previous studies (22, 26), sera from acute-phase
295 DIHS patients appeared to have relatively low levels of HHV-6 IgG antibodies as measured

296 by IFA assay. Thus, the three DIHS patients might have had low levels of 101K-specific
297 antibodies, which were below the detection limit of the present immunoblotting assay.
298 Interestingly, although the isolates from peripheral blood of the two DIHS patients were
299 HHV-6B, seroconversion against p100 antigen was observed in these patients. This finding
300 both suggests that HHV-6A reactivation might occur in anatomical sites (e.g. salivary glands
301 or central nervous system) other than peripheral blood, which have been suggested as the sites
302 of HHV-6 latency (1, 6, 14, 15), and that HHV-6B might be cross reactivation.

303 Thirty-one of the 38 (81.6%) serum samples collected from healthy adults reacted
304 with 101K. The 7 negative sera samples had low levels of IgG antibodies (x8 to x16) as
305 measured by IFA. Thus, it is likely that the 7 negative sera contained low levels of
306 HHV-6B-specific antibodies that were below the detection limit of our assay. As HHV-6 B
307 DNA was detected in four (no. 8, 9, 26, and 33) of the seven cases without both antibodies,
308 nested PCR, which detect latently infected HHV-6B, is likely to be more sensitive than our
309 immunoblotting assay. Four of the 38 (10.5%) of the sera samples also reacted with the p100
310 antigen. Molecular epidemiological studies have suggested that the prevalence of ci HHV-6A
311 population is low both in Western countries (14) and in Japan (27). However, recent reports
312 have demonstrated a high prevalence of HHV-6A in sub-Saharan Africa (5, 18). Although
313 molecular epidemiological examination was carried out in PBMCs and saliva obtained from
314 our healthy subjects, no HHV-6A was identified in any of these samples. Most of the previous
315 molecular epidemiological studies examining PBMCs or saliva detected a high frequency of
316 latent HHV-6B infection (1, 8). However, it has been suggested that mixed infection of the

317 two virus species frequently occurs in lung tissues (9); thus, we speculate that persistent

318 infection with HHV-6A might occur in lung tissue of the patients with p100 antibody.

319 In conclusion, although further serological analysis using samples collected from

320 patients with primary HHV-6A infection is necessary, we have developed a new immunoblot

321 assay that permits to reliably identify antibodies to HHV-6A and B. Further

322 seroepidemiological studies using the immunoblot analysis in combination with molecular

323 epidemiological study will be necessary to clarify the full spectrum of HHV-6A infection.

324 Additionally, it will be important to develop an enzyme-linked immunosorbent assay using

325 these recombinant proteins for the high-throughput analysis of samples.

326

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333

334 **Figure legends**

335 **Figure 1.**

336 (A) Construction of HHV-6 U11 gene expression plasmids, pET22b(+)-6AU11 and

337 pET22b(+)-6BU11. These recombinant proteins expressed 6×Histidine-tag on C-terminal

338 end.

339 (B) Expression of recombinant proteins, p100 (6A) and 101K (6B), and immunoblot analysis.

340 Induction was carried out using 1mM IPTG for 3 hours at 30℃ (+) or without 1mM IPTG (-).

341 I.C.-Induction control was expressed by 1mM IPTG for 3 hours at 30℃. Molecular mass

342 standards are indicated in kDa on the left.

343 Detection of recombinant p100 and 101K using an anti-His tag antibody. The anti-His tag

344 antibody reacted with both recombinant p100 (6A) and 101K (6B) (lanes 1 and 3 of the left

345 panel, respectively).

346 Detection of recombinant 101K using an anti-101K antibody. The anti-101K antibody reacted

347 with recombinant 101K (lane 3 of the right panel).

348 *IC, Induction Control

349

350 **Figure 2.**

351 Immunoblot analysis with human sera collected from patients with various types of HHV-6

352 infections against recombinant p100 and 101K antigens. All human sera were diluted to 1:500

353 for the assay. The patient sera collected from ci-HHV-6A patient (Pt. 2: A) and CFS patient

354 (Pt. 3: B) reacted with p100 (lane 1) and 101K (lane 2). (C) The paired sera obtained from