

Title: Monitoring of human herpesviruses-6 and -7 DNA in saliva samples during the acute and convalescent phases of exanthem subitum.

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Supplemental material: one figure.

## **Abstract**

The amounts of the DNAs of human herpesviruses-6 (HHV-6) and -7 (HHV-7) in saliva samples were monitored during the acute and convalescent phases of exanthem subitum (ES) to elucidate the kinetics of virus shedding after ES. A total of 247 saliva samples were collected from 17 children (5 males and 12 females: 8 to 31 months old at onset). The monitoring period ranged from 152 to 721 days after onset, and in 15 children it was longer than one year. Among the 17 cases, 16 were attributed to HHV-6B, while a single case was attributed to HHV-7. Detection rates and average amounts of HHV-6 DNA in saliva samples after ES attributed to HHV-6B were low in the acute phase, increased to the maximum in the convalescent phase at 3-7 months, and then decreased. In addition, to investigate the source of infection, saliva samples from the older siblings ( age 3-9 years) and parents of ES patients and children with a history of ES were also examined. The detection rate of HHV-6 DNA in saliva samples from 3-9 year-old children was significantly higher than the rate in adult saliva samples. Taken together, these findings suggest that the saliva of children in the convalescent phase of ES might be a more likely source of HHV-6 infection than that of adults.

(218 words)

## **Introduction**

Exanthem subitum (ES; also referred to as roseola infantum) is a benign febrile pediatric disease with maculopapular eruption [Caserta MT, 2011; Yamanishi et al., 2013; Agut et al., 2015]. It is caused by primary infection of human herpesvirus 6 (HHV-6) [Yamanishi et al., 1988] or human herpesvirus 7 (HHV-7) [Tanaka et al., 1994]. HHV-6 is now classified into two distinct species, HHV-6A and -6B [Ablashi et al., 2014], and the latter is widely prevalent and implicated as a major causative agent of ES [Yamanishi et al., 2013]. These viruses exhibit life-long latent infection in most of the affected children, and older children and adults sometimes continue to shed virus DNA in saliva [Caserta MT, 2011]. Therefore, saliva is believed to be a source of infection, although the details of the kinetics of virus shedding after ES and the mode of transmission are still unclear. In this study, in order to elucidate the kinetics of virus shedding after ES, the amounts of DNA of HHV-6 and HHV-7 in saliva samples were monitored during the acute and convalescent phases of ES. We also sought to investigate the source of infection and mode of transmission of HHV-6 and HHV-7. For this purpose, saliva samples of 3-9 year-old children and adults, including siblings and parents of monitored cases, were also examined.

## **Materials and Methods**

### **Subjects and sample collection**

Children diagnosed as having ES based on clinical signs were enrolled in this study after providing informed consent. The research protocols were approved by the ethic committee of Okayama University (No. 919). The clinical criteria used for the diagnosis of ES were as follows: a fever higher than 37.5 °C for one day or more followed by a maculopapular skin rash that appeared around the time of defervescence and disappeared within 1-4 days [Caserta MT, 2011].

Blood and saliva were collected at the time of diagnosis of 17 children, and saliva was sequentially collected approximately once a week in the first month and once a month thereafter. Saliva samples were collected with PurFlock Ultra Swabs® (Puritan Medical Products, Guilford, ME), suspended in a universal transport medium (UTM) (UniTranz-RT®; Puritan Medical Products) and transported to the laboratory. The diluted saliva sample is referred to as the UTM-saliva mixture hereinafter.

Virus shedding in saliva was also monitored for older children and adults. They were 23 older siblings (age 3-9 years) and parents of ES patients or children with a history of ES, although most of the diagnoses as having ES were based on clinical signs. Four older siblings (two brothers of Case #14, a brother of Case #1 and a sister of Case

#6) and two parents (the mother of Case #1 and the mother of Case #6 ) of the 17 ES cases were included. Saliva samples were collected with the same method as described above after providing informed consent when they visited the clinics with ES patients or children with a history of ES. The above-mentioned research protocols included saliva sampling from family members.

#### **DNA extraction and real-time PCR**

DNA was extracted from 200 µl of whole blood, or from 200 µl of the UTM-saliva mixture using a QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). The elution volumes of the saliva and blood samples were 50 and 100 µl, respectively. Copy numbers of HHV-6 and HHV-7 were measured by the real-time PCR method based on TaqMan® technology as previously reported elsewhere [Tanaka et al., 2000; Hara et al., 2002; Yamamoto et al., 2014]. Briefly, primer sets and probes were used in the assay to amplify and detect the U31 gene for HHV-6 and the U57 gene for HHV-7. Five microliters of the eluted sample was used for each reaction.

#### **Virus isolation**

Virus isolation from peripheral blood mononuclear cells (PBMCs) and saliva was also performed as described previously [Takahashi et al., 1989; Asada et al., 1989;

Yoshida et al., 1998]. PBMCs were separated from 800 µl of whole blood, while 800 µl of the UTM-saliva mixture was used after filtration with a 0.45 µm membrane filter (Millex-HA<sup>®</sup>; Merck Millipore, Tullagreen, Ireland). As host cells to support viral replication, cord blood mononuclear cells (CBMCs) or MT-4 cells were used. Protocols for the use of CBMCs were approved by the ethic committee of Okayama University (No.1587). Isolated viruses were identified using specific monoclonal antibodies against HHV-6 and HHV-7 as described previously [Tsukazaki et al., 1998].

### **Typing of HHV-6**

Typing of the two species of HHV-6 based on the size difference of amplified products of the U90 gene using a common primer set was performed as described elsewhere [Okuno et al., 1995]. DNA extracted either from the blood or from CBMCs infected with HHV-6 isolates from the blood was used as a template.

### **Antibody assay**

An indirect immunofluorescent antibody assay to determine the plasma antibody titer against HHV-6B and HHV-7 was performed as described previously [Yoshida et al., 2002].

### **Statistical analysis**

Differences in detection rates between children and adults were compared using Fisher's exact tests. *P* values less than 0.05 were considered to indicate statistical significance.



## **Results**

### **Virological confirmation of 17 ES cases as primary infection with either HHV-6B or HHV-7**

A total of 247 saliva samples were collected from 17 children (5 males and 12 females). The monitoring periods ranged from 152 to 721 days after the first episode, and in 15 children the period was more than one year (Table 1). The onset ages were 8 to 31 months (average: 13.7 months). Among the 17 diagnostic blood samples, HHV-6 was detected and/or isolated from 16 children, while HHV-7 was detected from one child.

Typing of HHV-6 revealed that all HHV-6 detected or isolated from 16 diagnostic blood samples were HHV-6B. In addition, the antibody titers ( $<10$ ) of the diagnostic blood samples were consistent with a primary infection of HHV-6 or HHV-7. Thus, the causative agent was inferred to be HHV-6B in 16 of the 17 cases, and HHV-7 in a single case (Case #12), although direct proof of primary infection was not established in some cases.

### **Kinetics of the saliva shedding of HHV-6 and HHV-7 DNA after ES attributed to HHV-6B**

The kinetics of HHV-6 and HHV-7 DNA in the 15 representative cases during the first 7 months after the onset of ES attributed to HHV-6B is shown in Fig. 1. To

characterize the kinetics precisely, the levels of virus detection were coded and the pattern of saliva secretion was defined using a 3 letter code as shown in Table 2. The results are shown in Fig. 1 and summarized in Table 2B. Low detection rates and low levels of HHV-6 DNA in the first samples and the samples in acute phase (within 30 days) were observed in 81.3% of cases (among the 16 cases there were 7 ooA, 3 aaA, 1 aoA, 1 oaA, and 1 ooa case), while the DNA level became high after two months of ES onset in most cases (15/16). These data showed that the amounts of viral DNA in saliva were below the detection level or low both in first samples at the febrile period and those in the acute phase within 30 days of onset, and that they increased during the convalescent phase (in the second month or later after onset of ES).

As shown in Table 2A, 68.7% (11/16) of the first saliva samples (collected at 2-6 days of ES onset) were negative for HHV-6DNA. In addition, saliva samples of 93.7% (15/16) of patients were negative for HHV-6 DNA at least once either in the first samples in the febrile period or the samples in the acute phase (within 30 days) (Fig. 1). The median and average numbers of days before the amount of HHV-6 DNA increased to more than 1000 copies/ml were 64 and 76.8 days, respectively, indicating the presence of a considerable “low-shedding period” in the acute and early convalescent phases.

Figure 2 shows the amounts and detection rates of HHV-6 and HHV-7 DNA in

saliva during the two-year follow-up after the onsets of ES attributed to HHV-6B.

Detection rates of HHV-6 DNA in saliva samples were low in the acute phase (within 4 weeks), increased to the maximum at the third to seventh month after the onset of ES, and then decreased. The average amounts of HHV-6 DNA in saliva samples were also low in the acute phase, and elevated to the maximum level at the third to sixth month. In six ES cases attributed to HHV-6B, HHV-7 DNA was detected later during the convalescent phase, without any concomitant clinical manifestations (Table 1 and Fig. 2).

As shown in supplemental Fig. S1, extremely large amounts (more than 10,000 copies/ml) of HHV-6 DNA were detected during the period of the second to seventh month after the onset of ES, and the maximum DNA amount in each case (average: 11,755.9 copies/ml) was observed during the period from 44 to 367 days (average: 130.2 days; median: 109.0 days) after the onset of ES.

#### **Detection rates of saliva samples from older children and adults**

The detection rates of HHV-6 and HHV-7 DNA in saliva samples from older siblings (age 3-9 years) and parents of ES patients or children with a history of ES are compared in Table 3. The detection rate of HHV-6 in saliva samples from children aged

3 to 9 years was significantly higher than the rate in the saliva samples of the adults ( $P < 0.01$ ).

### **Virus isolation**

As shown in Table 1, the isolation of HHV-6 from PBMCs in the acute phase of ES was mostly successful. On the other hand, the isolation of HHV-6 from saliva using CBMCs or MT-4 cells was unsuccessful in all 16 cases, even when HHV-6 DNA was abundant. In contrast, HHV-7 was sometimes isolated from the saliva samples when HHV-7 DNA was detectable and CBMCs were used for virus isolation (Cases #1, #3 and #7). Figure 3 shows the amounts of HHV-6 and HHV-7 DNA and the results of virus isolation in a representative case (Case # 1) in which detections of HHV-6 DNA were followed by those of HHV-7 DNA. In this case, HHV-7 was sometimes isolated from saliva after HHV-7 DNA detection began. However, trials to isolate HHV-6 from saliva were always unsuccessful, even though the amounts of HHV-6 DNA and HHV-7 DNA were comparable. Two another cases (Cases #3 and #7) also exhibited similar patterns of virus isolation.

## Discussion

In the present study, the detection rates and average amounts of HHV-6 DNA in saliva samples after ES attributed to HHV-6B were low in the acute phase, became maximal in the convalescent phase at 3-7 months, and then decreased. These findings were consistent with the previous reports [Suga et al., 1998; Zerr et al., 2005], although the present study design was quite different from the latter report. Zerr et al. prospectively studied a large cohort of 277 children from birth through the first two years of life without any virological confirmation of ES using blood samples, and showed that the salivary viral load tended to be low at week 1, and high by week 8 after primary HHV-6 infection, and that approximately one-third of patients had at least one saliva sample in which HHV-6 DNA was undetectable in the first 4 weeks after the initial detection [Zerr et al., 2005].

Although HHV-7 was sometimes isolated from saliva samples during the convalescent phase, our attempts to isolate HHV-6 from saliva samples were unsuccessful. Several limitations bear mention in terms of the sensitivity of our virus detection and isolation methods. First, to obtain saliva samples from infants in a safer way, saliva samples were collected with PurFlock Ultra Swabs® and suspended in UTM medium. This process diluted the samples approximately 1:10, whereas in the previous studies the saliva was used directly [Yamamoto et al., 2014; Fujiwara et al., 2000].

Secondly, samples were transported overnight from the clinics to the viral laboratory to perform DNA quantitation and virus isolation of both HHV-6 and HHV-7. For this purpose, we initially used CBMCs. However, to minimize the possible loss of infectivity during transport, the protocol for virus isolation was changed so that the MT-4 cells were inoculated with saliva samples at the clinic. Dilution experiments using the laboratory strain Z29 of HHV-6B and some fresh isolates of HHV-6B from PBMCs of patients with acute phase ES revealed that the efficiency of these two cell lines was comparable. Moreover, it was confirmed that both CBMCs and MT-4 cells expressed a sufficient amount of CD134, the cellular receptor specific for HHV-6B entry [Tang et al., 2013]. Even using the alternative protocol, however, HHV-6 isolation was not successful.

Similar results were obtained previously with saliva samples of healthy adults [Fujiwara et al., 2000]. However, there have also been some reports of the successful isolation of HHV-6 from saliva samples [Levy et al., 1990; Harnett et al., 1990]. Nonetheless, it seems likely that confusion between HHV-6 and HHV-7 was the real reason for these earlier successes, since the discovery of HHV-7 was reported in the same year [Frenkel et al., 1990].

In the present study, we showed that the detection rates and average amounts of HHV-6 DNA in saliva samples were elevated to the maximum in the convalescent phase of ES attributed to HHV-6B and that the detection rate of HHV-6 DNA in saliva samples from children aged 3 to 9 years was significantly higher than the rate in those from adults. Taken together, these findings suggest that the saliva of children in the convalescent phase of ES might be a more likely source of HHV-6 infection than that of adults. In some previous reports, older siblings were also considered to serve as a source of HHV-6 transmission [Zerr et al., 2005; Rhoads et al., 2007].

In Japan, a decrease in the morbidity and an increase in the onset ages of ES have been reported, and both phenomena seem to be related to the decreased chances of infection due to the decline in the number of births and a trend toward nuclear families [Torigoe et al., 2013]. In this setting, not only older siblings, but also children in the same or older age group in nursery schools, kindergartens and orphanages [Okuno et al., 1991] might be considered potential sources of infection.

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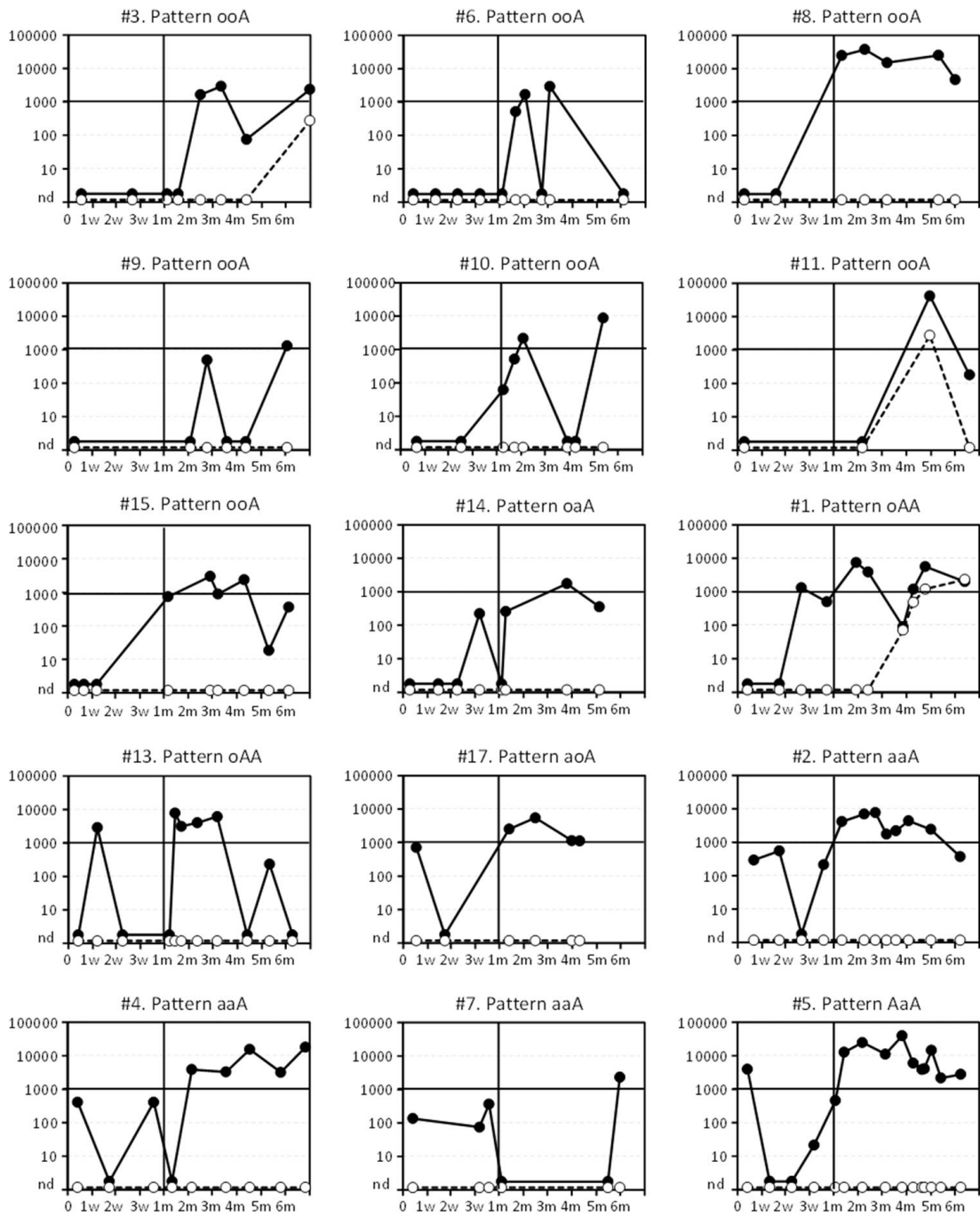


fig. 1

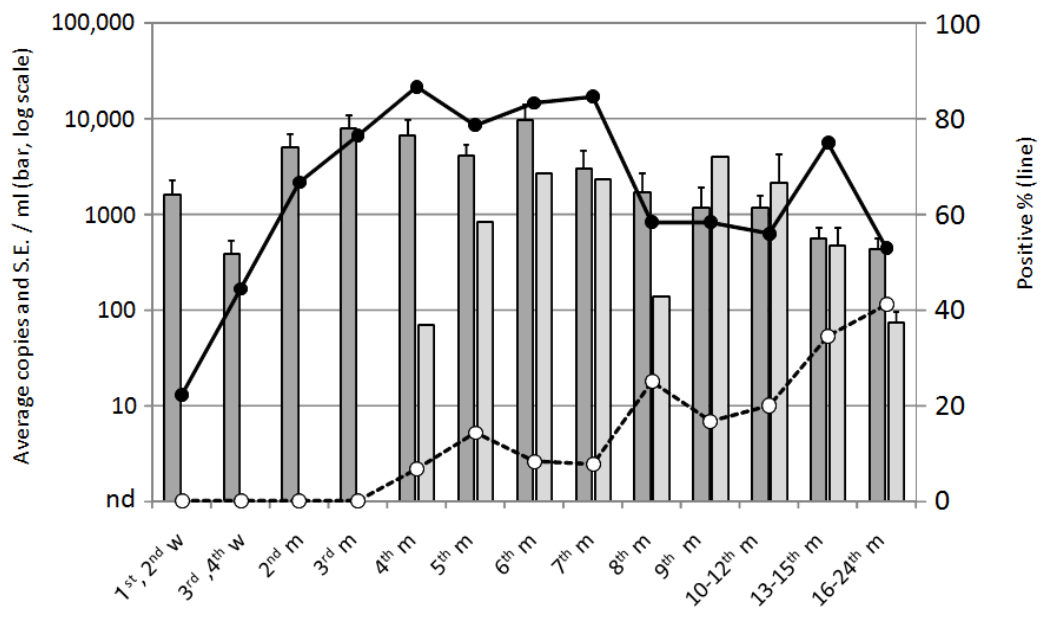


fig. 2

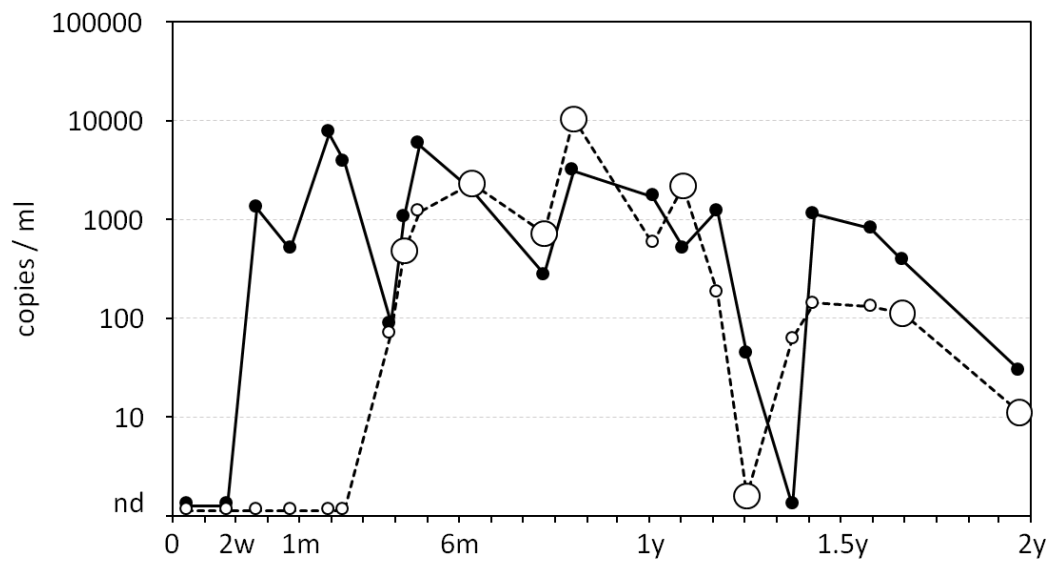
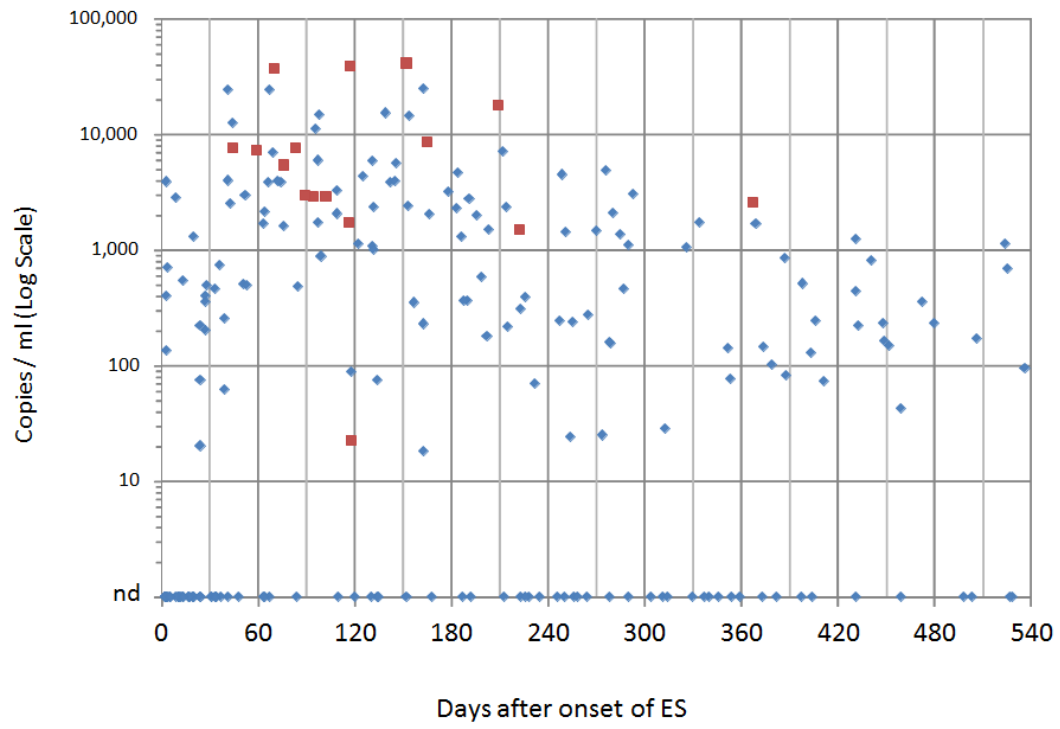


fig. 3



Supplemental figure



### Legend of figures

**Fig. 1.** Kinetics of HHV-6 (●—●) and HHV-7 (○---○) DNA amounts in saliva during the first 7 months after onset of ES attributed to HHV-6B. Fifteen representative cases are shown. The X axis indicates time after onset of ES by week (w) in the first month and by months (m) after the second month. The Y axis indicates copy number/ml of the UTM-saliva mixture by log scale. nd: not detected. The case number and the pattern of saliva secretion of viral DNA defined as a 3 letter code in Table 2 is shown at the top of each case. Nine cases in which HHV-6 DNA was not detected at the first saliva sampling in the acute phase are presented first.

**Fig. 2.** Amounts and detection rates of HHV-6 and HHV-7 DNA in saliva during two-year follow-up after the onset of ES attributed to HHV-6B. The amounts of HHV-6 (black bar) and HHV-7 (grey bar) DNA are shown as the average copy number/ml with the standard error (S.E.) of positive samples, and the detection rates of HHV-6 (●—●) and HHV-7 (○---○) DNA are also shown.

**Fig. 3.** Kinetics of HHV-6 (●—●) and HHV-7(○---○) DNA amounts in saliva and the results of virus isolation from saliva in Case # 1. A large mark indicates that virus

isolation was positive, while a small one indicates that virus isolation was unsuccessful.

Detections of HHV-6 DNA were followed by those of HHV-7 DNA. The X axis indicates the time after onset of ES by week (w) in the first month, by months (m) after the second month and by years (y) after the second year.

#### Supplemental figure legend

Fig. S1. Amounts of HHV-6 DNA in saliva during the one and a half year follow-up after the onset of ES attributed to HHV-6B. The results of all 16 cases are plotted using blue diamonds (◆) and the maximum values for each case are highlighted with red squares (■).

Table 1 List of 17 ES cases whose saliva samples were monitored for secretion of HHV-6 and HHV-7

Case No.	Sex	Months of age at the onset of ES	Days from ES onset to blood sampling	Detection from blood samples in acute phase (copies / ml)		Isolation from blood samples in acute phase		Typing of HHV-6 detected or isolated from blood samples	Plasma antibody titer in acute phase against		Periods of saliva collection, days	Times of saliva collection	Days of first detection in the saliva after onset	
				HHV-6	HHV-7	HHV-6	HHV-7		HHV-6	HHV-7			HHV-6	HHV-7
#1	f	11m	3	+(1,362)	-	+	-	HHV-6B	<10	<10	3 - 720	21	20	118
#2	f	10m	5	+(399)	-	+	-	HHV-6B	<10	<10	5 - 327	17	5	-
#3	f	31m	4	+(374)	-	+	-	HHV-6B	<10	<10	4 - 721	12	76	214
#4	f	10m	3	+(5,477)	-	-	-	HHV-6B	<10	<10	3 - 689	15	3	-
#5	f	9m	3	+(298)	-	+	-	HHV-6B	<10	<10	3 - 667	27	3	-
#6	f	11m	3	+(5,318)	-	+	-	HHV-6B	<10	<10	3 - 555	20	51	-
#7	f	11m	3	+(11,325)	-	-	-	HHV-6B	<10	<10	3 - 568	14	3	213
#8	f	10m	2	+(26,787)	-	nt	nt	HHV-6B	<10	<10	2 - 433	12	41	-
#9	m	11m	2	+(53)	-	+	-	HHV-6B	<10	<10	2 - 498	12	85	-
#10	f	13m	5	-	-	+	-	HHV-6B	<10	<10	5 - 525	12	39	-
#11	m	13m	2	+(16,551)	-	-	-	HHV-6B	<10	<10	2 - 427	7	152	152
#12	m	22m	1	-	+(338)	-	-	nt	<10	<10	1 - 522	18	455	75
#13	f	13m	3	+(481)	-	-	-	HHV-6B	<10	<10	3 - 411	14	9	118
#14	m	12m	2	+(14,754)	-	+	-	HHV-6B	<10	<10	2 - 373	13	24	-
#15	m	19m	2	+(155,883)	-	+	-	HHV-6B	<10	<10	2 - 404	15	36	214
#16	f	8m	2	-	-	+	-	HHV-6B	<10	<10	2 - 152	8	118	-
#17	f	19m	4	+(450)	-	+	-	HHV-6B	<10	<10	4 - 374	10	4	-

nt: not tested.

Table 2 Detection level of viral DNA in acute and convalescent phases of ES attributed to HHV-6B (panel A) and pattern of saliva secretion of viral DNA after the onset of ES (panel B)

A

Code for detection level (maximum of the period)	Number of cases (%)		
	Acute phase	Convalescent phase	
	First sample	Samples within 30 days	second month or later
o: not detected	11 (68.7%)	9 (56.2%)	0 (0%)
a: less than 1,000 copies	4 (25.0%)	5 (31.3%)	1 (6.3%)
A: 1000 copies or more	1 (6.3%)	2 (12.5%)	15 (93.7%)
<b>Total (%)</b>	<b>16 (100%)</b>	<b>16 (100%)</b>	<b>16 (100%)</b>

B

Pattern of saliva secretion*	Code for detection level (Maximum of the period)			Number of cases (%)
	Acute phase		Convalescent phase	
	First sample	Samples within 30 days	Second month or later	
ooA	o	o	A	7 (43.5%)
aaA	a	a	A	3 (18.8%)
aoA	a	o	A	1 (6.3%)
oaA	o	a	A	1 (6.3%)
oAA	o	A	A	2 (12.5%)
AaA	A	a	A	1 (6.3%)
ooa	o	o	a	1 (6.3%)
<b>Total (%)</b>	<b>16 (100%)</b>			

\*The pattern of saliva secretion was defined as a 3 letter code, with each letter having one of the 3 values used in panel A to describe the detection levels: the first letter represented the amount at the first saliva sampling, the second letter represented the maximum amount in the second and later samples in the acute phase (within 30 days), and the third letter represented the maximum amounts in the convalescent phase (second month or later).

Table 3: Detection rates of HHV-6 and HHV-7 DNA in children and adults

	HHV-6	HHV-7
Children (3-9 years of age) n=23	12 / 23 52.2%	6 / 23 26.1%
Adults n=23	1/23 4.3%*	13/23 56.5%

\*Significant difference between children (3-9 years old) and adults at  $p < 0.01$  by Fisher's exact test.