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Determination of Frequency of HHV-6 IgG by an In-house ELISA and Detection of Antibody Avidity by Application of Diethylamine

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In the present study an indirect in-house ELISA was optimized to detect IgG antibody against HHV-6 in sera from different groups of children. Regarding designation of in-house ELISA, all corresponding steps were standardized by checker boading after virus culture and purification of antigen. The results were calculated according to Specific Binding Ratio (SBR) and cut-off methods. Inter and intra assay helped to investigate different technical aspects of in-house ELISA. When accuracy of ELISA was confirmed the protocol was applied in screening of sera from different hosts including immunocompetent and immunocompromized that facilitated examination of their clinical aspects. Furthermore, seropositivity and seronegativity had to be proved real or false so Diethylamine (DEA) in 20 and 70 mM concentrations were used in order to assay IgG antibody avidity. Primary HHV-6 infection and seroconversion are reported most commonly between 6 and 18 months of age and among children older than 3 years most subjects are seropositive. Nonetheless, a high prevalence of HHV-6 IgG antibodies was documented in all children if all SBR and/or cut-off positive HHV-6 IgG ELISA readings were considered to represent seropositivity. No evidence of a decline in HHV-6 antibody positivity was seen with increasing age amongst children. The HHV-6 IgG antibody avidity indices were stable throughout childhood in immunocompromized patients. The in-house HHV-6 ELISA proved efficient, reproducible and quite concordant with results obtained by previous workers in terms of IgG distribution of the virus.

Key words: Human herpes viruses, HHV-6, in-house ELISA, IgG avidity, DEA

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INTRODUCTION

Human herpesvirus type 6 (HHV-6), a member of *herpesviridae* family and β-herpesvirinae subfamily, was isolated by Salahuddin *et al.* (1986). These investigators derived six isolates of a new virus from peripheral blood mononuclear cells (PBMCs) of adults with HIV infection or lymphoproliferative diseases (Leach *et al.*, 1992). HHV-6 infects virtually all children during the early years of life and like other herpes viruses causes latency after primary infection (Zerr, 2006). The virus can be reactivated in the immunosuppressed patients especially after bone marrow and orthotopic, liver and renal transplants (Clark, 2000; Nash *et al.*, 2004). Different variants of HHV-6 have been isolated from AIDS patients from different countries. All isolates of HHV-6 have been grouped into two groups of A and B on the basis of biological, immunological and molecular characteristics (Bernerman and Ablashi, 1992; Ablashi *et al.*, 1991; Clark, 2000).

The routes of transmission of HHV-6 have not been clearly recognized although virus has been found in the saliva of the vast majority of healthy adults (Leach *et al.*, 1992; Fox *et al.*, 1995; Ward, 2005a). The oral secretions serve an important source of HHV-6 among children; the latter glands may be a major site of viral latency or chronic semi-permissive infection or both. Nevertheless, HHV-6 has been associated with several diseases categorized as: Roseola infantum, other childhood febrile and exanthematous illnesses, mononucleosis-like illnesses, post-viral chronic fatigue syndrome, malignancy and lymphoproliferative disorders like: Canale-Smith syndrome, Hodgkin's disease, African Burkitt's lymphoma, acute lymphocytic leukaemia and many other disorders (Kruger *et al.*, 1992; Clark, 2000; Nash *et al.*, 2004; Ward, 2005b).

Amongst all above mentioned disorders presumably the most common is roseola infantum, which was first reported by Yamanishi *et al.* (1988) its association with HHV-6. Roseola infantum is a common disease of infants characterized by high fever for a few days and the appearance of a rash coinciding with subsidence of the fever (Clark, 2000). Hence, the virus is highly seroprevalent and has world wide distribution and infection usually occurs within the first two years of life (Clark, 2000). The peak age range for roseola is believed to be 6 months to 3 years and that precisely fits the peak range for primary HHV-6 infection (Leach *et al.*, 1992; Dewhurst *et al.*, 1993).

Most seroepidemiological studies of HHV-6 have been carried out by IIF or Anti-complementary Immunofluorescence (ACIF) and only a few by ELISA (Lopez, 1993, Fox *et al.*, 1995; Neilsen and Vestergaard, 1996).

HHV-6 ELISA is considered to be one of the most trustworthy techniques for seroepidemiological study by many investigators (Neilsen and Vestergaard, 1996; Sloots *et al.*, 1996; Neilsen and Vestergaard, 2002; Gutierrez *et al.*, 2002) and so forth. Therefore an avidin-biotin amplified ELISA for detection of HHV-6 IgG antibodies was designed and optimized in present study. This assay was highly sensitive because of the very strong bonds (dissociation constant 10^{-15}) formed between avidin and biotin (Green, 1964).

As a result of worldwide distribution of HHV-6 and its involvement in different infections, we were encouraged to carry out this project preferably through an in-house ELISA since efficiency of commercial kits and cost-effectiveness is obscure subsequently differentiation of true positivity from negativity by avidity assay, as a reliable tool, introduced by different workers (Thomas and Morgan-Capner, 1991; Thomas *et al.*, 1993; Stiller *et al.*, 2006) was our objective as well.

MATERIALS AND METHODS

As stated above ELISA was the key protocol in most of seroepidemiological studies but these investigators employed commercial kits instead of in-house ELISA. The latter is the main difference between current study and theirs. This project was carried out in Manchester Medical School, England and samples were collected within few years from different centers as Booth Hall children hospital and Preston general hospital. Cultivation of the virus, preparation of the materials, optimization of the protocol and the rest plus analysis of the data were all long-lasting procedures to complete as the authors describe each and every step below.

Antigen production: In order to design an in-house ELISA, the first requirement is to have appropriate antigen as HHV-6 replicates in the T-lymphocyte cell line known as HSB-2. The uninfected and HHV-6 infected HSB-2 cells were kindly donated by Dr H.K. Osman, Department of Virology, University of Newcastle, Royal Victoria Infirmary, Newcastle. They were supplied as actively growing cells as experience in this laboratory and elsewhere indicates that recovery of HSB-2 cells following storage even in liquid nitrogen is unreliable. The procedure for antigen production was as follows:

Two flasks of 25 cm^3 were received; one contained uninfected and next infected cells both in growth medium. The contents of both flasks were spun at 1000 rpm in a bench top centrifuge for 5 min and sediments of both flasks were resuspended in 5 mL of growth medium. Next, in a sterile tissue culture flask 1 mL cell suspension plus 0.2 mL virus suspension was added along with

approximately 20-25 mL of growth medium. The flasks were then incubated in a CO_2 incubator at 37°C; it took about 7 days for the cell suspension to regrow to approximately the original density and for the viral CPE to appear. When the cells reached complete confluence and the viruses showed CPE they were spun for 10 min at 1000 rpm, the sediment of both HHV-6 and HSB-2 cultures kept at -20°C for an hour and they were then thawed at room temperature. The preceding freeze-thaw cycle was repeated once more and 2 mL Sterile Distilled Water (SDW) was added to each flask and by using a rubber policeman, the cells were scraped into the water, the cycle of freezing and thawing was then repeated twice. Fluids from all flasks were collected in a sterile 30 mL glass universal and centrifuged in a bench top centrifuge (Heraeus Omnifuge 2.ORS) at 1000 rpm for 10 min. Supernatant was then collected and aliquoted in 0.2 mL amounts in sterile plastic vials and stored at -40°C.

In order to assay produced antigen from infected and uninfected cells Coomassie protein assay kit (Pierce: Blue G-250 reagent, Product no: 23200) was employed.

Standard ELISA protocol: ELISA had proved previously by different workers the most reliable technique in seroepidemiological studies (Shen *et al*., 1992, Yamanaka *et al*., 1992) hence, the key protocol in this project was ELISA, it had to be optimized by checkerboarding and if necessary modified with respect to antigen and antibody concentrations, blocking buffer, the optimal dilution of monoclonal antibody (anti-human IgG), conjugate and finally washing of the plates after coating with antigen and after anti-antibody binding.

The wells of a microtitre plate (Falcon 3912 Micro Test III) were coated with 140 μL optimally diluted antigen (1:50 extracted by checkerboarding) in carbonate bicarbonate buffer (pH: 9.6) and then incubated overnight at 4°C in a moist chamber. The next day the plate was washed with wash buffer 5 times with the same volume i.e. 140 μL (PBS-A with 0.05% tween 20 (Sigma No 5362) and each well was incubated with 140 μL of blocking buffer (1 gram skimmed milk powder dissolved in 100 mL of PBS-A) in moist chamber for an hour and later washed 5 times with wash buffer.

In the next step 140 μL of optimally diluted serum (1:50) in serum diluent (blocking buffer plus 0.5% W/V tween 20) was added to each well. Of course two wells for each serum dilution and on every plate were used as blank (no serum only diluent). The plate was then incubated over night in moist chamber at 37°C. The next day plate was washed according to optimized number of washes i.e. ×15. Afterward 140 μL of optimally diluted anti-human IgG mouse monoclonal antibody i.e., 1:1000 (biotin labeled; Sigma product no: B-3773) in diluent, which was exactly as blocking buffer, was added to appropriate wells. Incubation period was then half an hour at 37°C in moist chamber. The plate was again washed 5 times and incubated for 5 min at 37°C before addition of 140 μL of optimally diluted avidine peroxidase (Sigma product No: A-3151) in suitable diluent (100 mL PBS-A plus 4.0 gram bovine serum albumin and 4.0 g goat serum supplied by Sara Lab Ltd., Sussex, U.K). After incubation and washing of plate 3 times 140 μL of o-phenylenediamine (OPD) dihydrochloride (Sigma product No: P-8287) 10 mg mL^{-1} dissolved in citrate buffer (pH: 5.0) was added to the wells. The plate was then incubated at 37°C away from light in moist chamber for half an hour. In final step 50 μL stop solution (1N, H_2SO_4) was added to the wells and the absorbance was read at 492 nm wave length against mean of column blank.

IgG avidity assay: IgG avidity assays plays an important role in distinguishing primary from other categories of viral infections, as it was aimed in this project to measure of IgG avidity (also used as a measure of denaturant-induced dissociation of binding) the approach was as following.

Diethylamine (DEA) (Sigma product no: D-31) in different concentrations (20 and 70 mM) were chosen as IgG denaturants. Standard ELISA was carried out for this purpose except that after antigen-antibody binding, the plate was washed 15 times with wash buffer containing one of the denaturant instead of wash buffer alone. In order to measure IgG avidity it is essential to determine OD of the sample both in standard ELISA and in the avidity assay procedure. Therefore for every batch of samples different plates had to be prepared as:

- plate coated with viral antigen
- plate coated with control antigen (uninfected cells)
- plates coated with viral antigen but subjected to avidity assay by adding required denaturants

Calculation of ELISA results: The results were calculated by cut-off and Standard Binding Ratio (SBR). In the cut-off method of results calculation a serum was considered positive for IgG antibodies, if the mean of the test serum OD exceeded by more than three standard deviations the mean OD given by 12 replicate test of a negative control serum. Whereas in the SBR method of results calculation a serum was regarded positive if the SBR (mean test serum OD with viral antigen/mean test serum OD with cell control antigen) was greater than 2 (Klapper *et al*., 1990).

Calculation of IgG avidity: Once the O.D of the samples from different plates was known as mentioned above IgG avidity was measured by following equation.

IgG avidity (%)
= [(average OD of wells with denaturant)×100]÷ (Average OD of wells without denaturant)

As soon as the standardization of in-house ELISA for detection of HHV-6 IgG antibody was complete, 210 sera from pediatric renal transplant and 213 samples from leukaemic children were assayed by in-house ELISA. In the next step HHV-6 IgG avidity indices were too calculated by 20 and 70 mM Diethylamine (DEA).

RESULTS

Intra-assay reproducibility of optimized HHV-6 IgG ELISA: This was calculated by the following equation:

Coefficient of variation (CV%)
$= \text{Standard deviation (SD)} \div \text{Mean } (X_{n-1}) \times 100$

Thus, calculation of CVs revealed that the intra-assay CVs of the optimized HHV-6 ELISA were 19, 16 and 19% with strong positive (S +ve), weak positive (W+ve) and negative (-ve) sera (8 replicates).

Screening and IgG avidity measurement of sera from pediatric renal transplant: The serial renal transplant sera were subjected to selective screening and avidity measurement, as the Table 1 indicates the samples were obtained sequentially and kept in serum bank before usage therefore, the date of receipt has been pointed out (Table 1).

Patient PMG (34 Months observation period): All sera were seropositive for HHV-6 by cut-off and most by ratios (low but consistent positive ratios with four negative ratios between 1 and 2). As the first sample was negative for HHV-6 antibodies, this patient possibly seroconverted to this virus. Of the HHV-6 avidities, all but one was low (<30%). The HHV-6 ELISA SBRs showed a decrease in prevalence of ratios of >4.1 [7/12 (58%) versus 1/11 (9%), $p<0.05$] before and after 7.9.92.

Patient PRS (36 months observation period): All sera were positive for HHV-6 antibodies by cut-off, but most [16/24 (67%)] were negative by SBR though all the negative ratios were≥1.1. Also, 14 (88%) of 16 negative ratios were≥1.5. All the positive HHV-6 ratios were≥5.4 and most [7/8 (88%)] were 2.1-4; therefore the ratios were generally close to 2. HHV-6 IgG avidity was usually high (>50%) with 20 mM DEA [18/25 (72%) or 70 mM DEA [17/25 (68%)].

Patient NN (23 months observation period): The HHV-6 ELISA gave readings positive by cut-off negative by ratio

Table 1: Frequency of HHV-6 IgG and avidity assay results from pediatric renal transplant recipients

Date	Ratio	Cut-off	20 mM (%)	70mM (%)
Patient's initial:PMG				
2.3.90	-ve	-ve	<10	<10
1.10.90	NC	+ve	12	<10
4.2.91	NC	+ve	14	<10
4.3.91	2.6	+ve	12	<10
8.4.91	4.7	+ve	10	11
5.8.91	8	+ve	<10	17
2.9.91	NC	+ve	<10	<10
4.11.91	4.8	+ve	11	14
21.12.91	4.6	+ve	15	<10
6.1.92	2	+ve	13	33
3.2.92	4.5	+ve	<10	21
26.2.92	NC	+ve	<10	<10
2.4.92	6	+ve	<10	<10
27.4.92	2.8	+ve	31	14
1.6.92	NC	+ve	<10	22
6.7.92	5.4	+ve	<10	10
3.8.92	2.2	+ve	12	14
7.12.92	1.5,-ve	+ve	24	29
11.1.93	1.9,-ve	+ve	16	25
9.2.93	3.7	+ve	13	12
11.2.93	5.9	+ve	10	<10
6.4.93	2.1	+ve	18	21
16.5.93	3.2	+ve	<10	23
11.6.93	1.8, -ve	+ve	17	22
1.9.93	2	+ve	18	18
4.10.93	3	+ve	18	21
1.11.93	1.6,-ve	+ve	12	18
2.1.90	1.8,-ve	+ve	74	67
29.1.90	NC,+ve	+ve	55	71
5.3.90	3.2	+ve	42	20
30.4.90	2.2	+ve	53	18
11.6.90	1.6,-ve	+ve	63	61
2.7.90	1.7,-ve	+ve	68	68
6.8.90	1.6,-ve	+ve	40	75
5.11.90	1.1,-ve	+ve	36	56
4.2.91	2	+ve	54	85
4.3.91	1.9,-ve	+ve	48	70
8.4.91	1.9,-ve	+ve	77	62
8.5.91	1.6,-ve	+ve	63	47
5.8.91	2.2	+ve	79	64
30.9.91	1.7,-ve	+ve	44	63
2.3.92	1.5,-ve	+ve	48	44
6.4.92	2.6	+ve	63	30
27.4.92	2.1	+ve	88	59
1.6.92	1.4,-ve	+ve	64	51
6.7.92	3	+ve	55	50
7.9.92	1.6,-ve	+ve	87	52
5.10.92	1.7,-ve	+ve	76	59
2.11.92	1.7,-ve	+ve	47	38
30.11.92	1.7,-ve	+ve	43	32
4.1.93	2.6	+ve	57	69
Patient's initial: NN				
4.11.91	1.6,-ve	+ve	58	38
2.12.91	1.9,-ve	+ve	67	66
3.2.92	1.6,-ve	+ve	92	62
6.5.92	1.8, -ve	+ve	67	70
3.8.92	1.0,-ve	+ve	67	65
1.2.93	1.4,-ve	+ve	73	61
5.4.93	1.4,-ve	+ve	66	48
5.5.93	1.9,-ve	+ve	55	49
2.6.93	1.4,-ve	+ve	63	53
6.7.93	2.4	+ve	100	46
3.8.93	1.9,-ve	+ve	73	60
7.9.93	3.4	+ve	85	85
Patient's initial: KW				
12.10.87	1.6,-ve	+ve	48	58
27.1.88	2.3	+ve	39	37
2.3.88	1.8,-ve	+ve	47	44

Table 1: Continued

Date	Ratio	Cut-off	20 mM (%)	70mM (%)
15.4.88	1.8,-ve	+ve	51	49
20.4.88	2.5	+ve	39	31
26.7.88	2	+ve	69	80
25.10.88	1.9,-ve	+ve	67	58
22.11.88	2.5	+ve	84	42
21.3.89	2.5	+ve	46	52
3.10.89	2.0	+ve	62	64
10.7.90	4.9	+ve	59	54
27.3.91	16	+ve	100	39
10.4.91	2.5	+ve	57	45
25.11.92	0.9,-ve	+ve	98	43
26.2.93	1.6,-ve	+ve	52	27
25.8.93	4.2	+ve	76	37
Patient's initial: CB				
21.1.93	4.1	+ve	42	68
1.2.93	NC	+ve	74	74
1.3.93	NC	+ve	68	61
14.4.93	1.6, -ve	+ve	87	97
1.6.93	NC	-ve	ND	ND
24.8.93	NC	-ve	ND	ND
8.9.93	10	+ve	84	50
6.10.93	NC	+ve	90	34
2.11.93	-ve	-ve	ND	ND
Patient's initial: AB				
20.10.87	NC,+ve	+ve	100	71
12.1.88	NC,+ve	+ve	80	80
15.3.88	NC,+ve	+ve	100	80
18.10.88	1.5, -ve	+ve	82	36
13.12.88	NC,-ve	-ve	ND	ND
10.1.89	NC,-ve	-ve	ND	ND
21.2.89	2.1	+ve	89	72
2.5.89	NC,-ve	-ve	ND	ND
13.6.89	2.3	+ve	88	90
25.7.89	NC,+ve	+ve	60	64
3.10.89	NC,-ve	-ve	ND	ND
2.1.90	4.2	+ve	42	90
19.6.91	3.5	+ve	69	79
13.12.92	1.3, -ve	+ve	100	67
1.9.93	1.5, -ve	+ve	92	61
Patient's initial: BH				
30.6.87	NC,-ve	-ve	<10	<10
28.7.87	NC,-ve	-ve	<10	<10
1.9.87	1.6, -ve	-ve	<10	<10
5.10.87	NC,-ve	-ve	<10	<10
2.11.87	NC,-ve	-ve	<10	<10
30.11.87	NC,-ve	-ve	<10	<10
4.1.88	NC,-ve	-ve	<10	<10
1.2.88	NC,-ve	-ve	<10	<10
29.2.88	NC,-ve	-ve	<10	<10
3.5.88	4.8	+ve	65	85
1.8.88	1.8	+ve	50	53
5.9.88	1.6, -ve	+ve	66	52
3.10.88	1.4, -ve	+ve	82	85
31.10.88	1.7, -ve	+ve	54	69
5.12.88	2.5	+ve	61	49
4.1.89	8.6	+ve	49	59
30.1.89	2.0	+ve	53	53
6.3.89	NC,-ve	+ve	44	75
3.4.89	2.3	+ve	89	62
3.5.89	1.5, -ve	+ve	84	37
5.6.89	2.4	+ve	82	52
3.7.89	2.6	+ve	59	<10
31.7.89	3.9	+ve	68	30
7.11.89	1.5, -ve	+ve	81	58
4.12.89	NC,-ve	+ve	81	70
2.1.90	1.8, -ve	+ve	83	66
30.1.90	3.3	+ve	64	68
6.3.90	6.4	+ve	66	65
29.5.90	2.1	+ve	68	87

Table 1: Continued

Date	Ratio	Cut-off	20 mM (%)	70mM (%)
6.6.90	NC,-ve	+ve	56	42
27.3.91	1.8, -ve	+ve	52	25
19.6.91	3.3	+ve	79	75
27.11.91	1.8, -ve	+ve	100	54
29.8.92	1.4, -ve	+ve	65	78
21.12.92	2.1	+ve	41	41
Patient's initial: RS				
21.11.89	2.4	+ve	78	69
2.11.90	1.9, -ve	+ve	89	58
1.10.90	3.4	+ve	65	36
5.11.90	2.1	+ve	30	<10
4.2.91	2.6	+ve	43	<10
4.3.91	NC,-ve	+ve	17	19
8.4.91	3.0	+ve	82	50
8.5.91	10	+ve	62	30
9.10.91	3.4	+ve	76	30
19.8.92	3.2	+ve	83	67
30.9.92	NC,-ve	+ve	61	36
6.1.93	17	+ve	100	66
Patient's initial: MS				
10.12.93	1.3, -ve	+ve	<10	<10
14.1.93	1.6, -ve	+ve	15	10
18.3.93	1.0, -ve	+ve	18	21
13.4.93	1.1, -ve	+ve	10	11
12.5.93	1.6, -ve	+ve	18	15
13.5.93	NC,-ve	+ve	19	<10
7.7.93	1.1, -ve	+ve	10	11
Patient's initial: JR				
17.9.92	1.6, -ve	+ve	18	10
10.1.92	1.5, -ve	+ve	19	16
19.2.92	1.4, -ve	+ve	15	20
9.9.92	2.1	+ve	17	27
17.9.92	1.4, -ve	+ve	32	50
19.9.92	1.5, -ve	+ve	30	29
4.11.92	1.6, -ve	+ve	15	23
3.12.92	1.2, -ve	+ve	13	14
2.2.93	1.5, -ve	+ve	14	24
1.3.93	1.2, -ve	+ve	15	24
9.3.93	1.4, -ve	+ve	19	30
2.5.93	NC,-ve	+ve	27	30
2.6.93	1.3, -ve	+ve	16	27
12.6.93	NC,-ve	+ve	28	29
Patient's initial: JW				
12.3.92	NC,-ve	+ve	62	89
8.4.92	1.3, -ve	+ve	61	47
5.5.92	1.3, -ve	+ve	69	73
16.6.92	1.5, -ve	+ve	20	39
27.10.92	1.8, -ve	+ve	22	50
24.11.92	1.5, -ve	+ve	21	53
23.12.92	1.9, -ve	+ve	20	40
19.1.93	9.3	+ve	75	35
6.2.93	2.2	+ve	14	42
9.2.93	1.9, -ve	+ve	18	34
11.2.93	0.2, -ve	+ve	50	40
15.2.93	2.4	+ve	49	60
18.2.93	2.3	+ve	48	71
17.3.93	2.1	+ve	50	48
24.3.93	2.6	+ve	45	40
6.5.93	1.5, -ve	+ve	48	45
Patient's initial: SB				
8.2.92	1.9, -ve	+ve	74	39
15.10.92	1.2, -ve	+ve	100	81
4.1.93	0.8 -ve	+ve	74	72
10.2.93	1.9, -ve	+ve	39	27
19.2.93	1.2, -ve	+ve	44	37
1.3.93	1.3, -ve	+ve	42	42
17.3.93	1.0, -ve	+ve	53	50
5.4.93	1.3, -ve	+ve	50	36

NC (Not Calculable), ND (Not Detectable), +ve (Positive), -ve (Negative)

Table 2: Age distribution of HHV-6 IgG ELISA results for children with leukaemia

Result	Up to 5 years	5<10 years	10<15 years	Unknown age
+ +	45 (49%)	20 (51%)	10 (48%)	5 (20%)
+ -	0	0	0	0
- +	29 (32%)	9 (23%)	7 (33%)	16 (64%)
- -	17 (19%)	10 (26%)	4 (19%)	4 (16%)
Total	90	42	23	29

+ + positive by SBR and cut-off, + - positive by SBR and negative by cut-off, - + negative by SBR and positive by cut-off, - - negative by SBR and cut-off

in 12 of 14 instances; however all 12 negative ratios were 1-2. Also, one third [4/12 (33%)] of the negative HHV-6 ratios were =1.5 and both positive ratios with this virus were 2.1-4. The HHV-6 avidities at 20 mM DEA were all high (>50%).

Patient KW (70 months observation period): All the HHV-6 cut-off readings were positive with the ratios positive (mostly low) in half (9/18) the sera. All but one negative ratio was =1.5. The HHV-6 avidities with 20 mM DEA were mostly high (>50%) [12/18 (67%)] and increased before and after 1.8.89 [for >50%, 4/10 (40%) versus 8/8 (100%), p<0.05].

Patient CB (10 months observation period): The HHV-6 ELISA ratios were mostly not calculable but the cut-off readings were generally [7/10 (70%)] positive. The avidity indices for HHV-6 with 20 mM or 70 mM DEA were largely >50% [6/7 (86% or 5/7 (71%)].

Patient AB (71 months observation period): The HHV-6 ELISA, the cut-off and ratio readings were mostly both positive [11/15 (73%)] and the antibody avidity appeared high [for 20 mM or 70 mM DEA 10/11 (91%) and 9/10 (90%) were >50%].

Patient BH (77 months observation period): The HHV-6 IgG ELISA gave largely positive readings by cut-off (29/35 (83%) and half the calculable ratios were also positive [12/24 (50%)]. The antibody avidity indices for HHV-6 were mostly >50% [24/29 (83%)] and even>70% [21/29 (72%)] at 20 mM DEA.

Patient RS (38 months observation period): The HHV-6 IgG ELISA readings were doubly positive in 9 (75%) of 12 sera with all three remaining samples being positive by cut-off and the SBRs being either not calculable (n = 2) or negative but close to 2 (1.9). The HHV-6 avidities indices were >50% at 20 mM DEA in 10 (83%) of 12 specimens.

Patient MS (9 months observation period): The HHV-6 results were mostly cut-off positive but ratio negative (5/6) though one sample was cut-off positive with no calculable SBR (all five ratios were =1.0). The HHV-6 avidities values were all low (<30%).

Patient JR (9 months observation period): The HHV-6 cut-off readings were all positive (14/14) but the calculable ratios with only one exception negative (11/12 negative, all negatives≥1.0; solo positive 2.1). The HHV-6 avidities virtually exclusively <30% at 20 mM DEA [13/14 (93%)] but less often so low at the higher denaturant concentration [3/14 (21%)].

Patient JW (16 months observation period): The HHV-6 ELISA cut-off readings were all positive (n = 16), but approximately one third [6/16 (38%)] of the SBRs were positive. The ratios were largely close to 2 [6/9 (67%) negatives≥1.5; 5/6 (83%) positives 2.1-4]. Approximately half of the HHV-6 avidities indices at 20 mM DEA were >50% [7/15 (47%)].

Patient SB (14 months observation period): All eight sera tested for HHV-6 antibodies gave cut-off positive SBR negative readings and all ratios were =1.0. The HHV-6 avidity values were in >50% in 4 (50%) of eight instances with 20 mM DEA.

Application of optimized HHV-6 ELISA in screening and IgG avidity of leukaemic children sera: Sera from children with leukaemia collected and stored in blood bank during several years were also tested in the newly developed HHV-6 ELISA (Table 2). Subsequently, the same batch of sera was subjected onto avidity assay by use of 2 dilutions of 20 and 70 mM DEA (Table 3).

Most children with leukaemia showed a high prevalence of HHV-6 antibodies (about 80%) irrespective of age if all sera positive by either ratio or cut-off were considered truly positive.

Doubly positive sera showed higher avidity with 20 mM rather than 70 mM DEA (increase in 30-50%, decrease in <30%, little change in >50%; p<0.05) Table 3.

Samples negative by SBR but positive by cut-off had higher avidity antibody with 20 mM rather than 70 mM DEA (p<0.001, affecting all avidities although, doubly negative specimens revealed no change in avidity indices with different DEA concentrations. Furthermore, all samples positive by ratio had a similar distribution of avidity indices whatever the cut-off result (p>0.1) and doubly positive sera had much higher avidities than those doubly negative at 20 mM DEA (p<0.001) but the difference was smaller at 70 mM DEA (p>0.1), in part because virtually all the avidity indices were low with the latter denaturant conditions (Table 3).

Table 3: Distribution of HHV-6 IgG avidity indices in sera from leukaemic children

ELISA Results	20 mM DEA			70 mM DEA		
	≤30%	31-50%	≥51%	≤30%	31-50%	≥51%
+ +n = 80	43 (54%)	28 (35%)	9 (11%)	59 (74%)	15 (19%)	6 (7%)
+ -n = 0	0	0	0	0	0	0
-+n = 61	29 (48%)	19 (31%)	13 (21%)	51 (84%)	9 (15%)	1 (1%)
- -n = 35	30 (86%)	2 (5.7%)	3 (8.6%)	30 (86%)	3 (8.5%)	2 (5.7%)

Footnote: each value is number of sera having the given ELISA ratio and cut-off results and an IgG avidity index in the specified range. Results in order ratio then cut-off

DISCUSSION

Optimization of In-house ELISA: For this purpose i.e., ELISA for HHV-6 IgG antibodies every step of the protocol had to be standardized and the best way of was checkerboarding. In order to do this, three sets of sera were chosen for every step of optimization process; namely strong positive, weak positive and negative. Confirmation of the reactions of these sera was obtained from diagnostic laboratory for HHV-6. Therefore, checkerboarding was set up for every step including: antigen, serum, anti-IgG monoclonal antibody and enzyme conjugate dilutions. Besides, washing times both after coating the plates and after antibody binding were investigated however, the optimization of ELISA might have been further improved by using smaller dilution steps in checkerboards. The optimal protocol was chosen after careful consideration of the results of every checkerboarding and in particular where the best discrimination was observed between strong positive, weak positive and negative sera.

Choice of ELISA protocol: In previous studies, indirect ELISA has been proved efficient for the detection of antibodies to human herpes viruses (Shen *et al*., 1992, Yamanaka *et al*., 1992). The ELISA in this project was therefore indirect and avidine-biotin based to increase its sensitivity. After adding biotinylated monoclonal antibody the plates were washed and avidine peroxidase was then added to the appropriate wells. By doing this unbound antigen and antibody were washed away before conjugate and subsequently substrate addition. The latter reduced the chances of false positivity and increased specificity and sensitivity.

Interpretation of results: As described earlier two methods of interpretation were employed for HHV-6 IgG ELISA cut-off and SBR (Klapper *et al*., 1990) therefore both modes for the calculation of results were already mentioned. However, these two methods occasionally generated discrepant readings on the same serum or serial sera from the same patient. One cannot easily claim that one method is more acceptable than the other. The cut-off method had the theoretical advantage that the range for positive results theoretically excludes all but 0.05% of negative sera (Klapper *et al*., 1990). The choice of negative control sera was clearly crucial, a large number of sera (say 100) from persons never infected with the virus in question would have been ideal. These samples could have been tested repeatedly to establish the negative range of the test.

IgG avidity and choice of denaturant: In this project, DEA that was already employed by other workers (Thomas and Morgan-Capner, 1991; Thomas *et al*., 1993) was evaluated at 20 and 70 mM concentrations. The avidity indices for sera from patients with recent primary or recurrent infection declined with increasing DEA concentration because of the effects of more powerful denaturation on antigen-antibody bonds of a given avidity.

In the presentation of avidity index results, they were mostly divided into 3 groups: ≤30% (band 1), 31-50% (band 2) and ≥51% (band 3). Samples with an avidity index of ≤30% were considered to indicate low avidity, samples with indices of 31-50% were regarded as showing intermediate avidity and samples with high avidity indices were those with indices ≥51% (Thomas and Morgan-Capner, 1991). In addition avidity of <10% or >70% were considered to represent very low or very high antibody avidity.

Sensitivity of HHV-6 IgG ELISA: No formal attempt was made to assess sensitivity of the in-house HHV-6 IgG ELISA, because sera previously tested for HHV-6 IgG antibodies were not available locally. However, if all sera positive by cut-off or SBR in the HHV-6 ELISA were considered to contain HHV-6 antibodies, the prevalence of these antibodies in serum samples from children with leukaemia aged less than 15 years was approximately 80% for all age groups. The sensitivity of the in-house HHV-6 IgG ELISA was probably high, because other studies indicated HHV-6 prevalence rates of 80-90% in infants and children (Okuno *et al*., 1989; Asano *et al*., 1990).

HHV-6 IgG ELISA and avidity assay reproducibility: Formal assessment to find out reproducibility of these tests was not achieved but the data obtained for serial sera from pediatric renal transplant recipients (as

revealed in results section) showed largely consistent cut-off, SBR readings and avidity indices for each individual subject. Indirectly, these observations provided evidence of excellent assay reproducibility.

HHV-6 and sera from children with leukaemia: The data in Table 2 and 3 confirmed for HHV-6 the conclusion that sera giving ratio negative cut-off positive or uniformly positive HHV-6 ELISA readings all came from HHV-6 seropositive children. This was because the two groups of samples had similar distribution of avidity indices.

Primary HHV-6 infection and resultant seroconversion are reported most commonly between 6 and 18 months of age (Asano *et al*., 1990; Frenkel and Wyatt 1992; Ward *et al*., 1993). Among children older than 3 years most subjects are seropositive and antibody titers are virtually stable, though declining seropositivity rates have been reported in adults (Brown *et al*., 1988; Briggs *et al*., 1988). The age grouping of the sera from leukaemic children did not allow detection of the expected seroconversion in infants and young children aged 6 months to 3 years. Nonetheless, a high prevalence of HHV-6 IgG antibodies was documented in all children if all SBR and/or cut-off positive HHV-6 IgG ELISA readings were considered to represent seropositivity. No evidence of a decline in HHV-6 antibody positivity was seen with increasing age amongst children, but that phenomenon might be seen in adults. Alternatively, the decline among adults in the prevalence of HHV-6 IgG antibodies as assayed by IIF (Briggs *et al*., 1988; Brown *et al*., 1988) might no longer been apparent using a more sensitive avidin-biotin amplified ELISA. The HHV-6 IgG antibody avidity indices were stable throughout childhood in patients with leukaemia, also indicating primary infections with this virus were not seen among the age groups studied but were presumably restricted to infancy.

CONCLUSIONS

The data obtained in this study supported the potential role of HHV-6 IgG ELISA and avidity index tests in differentiation of seropositivity from seronegativity. Differentiation o f seropositivity from seronegativity to this virus was problematic probably because antibody levels declined to very low levels (approaching seronegativity) with age. Commercial indirect IgG ELISAs which use only viral antigens might allow equivocal differentiation of positivity from negativity for herpesvirus antibodies as was illustrated if the in-house ELISAs were interpreted solely on the basis of cut-off readings. Nonetheless, failure to include a cell control antigen carried a risk of generating false positive results the latter by inclusion of SBR calculation has been ruled out.

REFERENCES

Ablashi, D.V., N. Balachandran, S.Z. Salahuddin and S.F. Josephs, 1991. Genomic polymorphism, growth properties and immunogenic variations in human herpesvirus-6 isolates. Virology, 184: 545-552.

Asano, Y., Yoshikawa and A. Suga, 1990. Enzyme linked immunosorbant assay for detection of IgG antibody to human herpesvirus 6. J. Med. Virol., 32: 119-123.

Bernerman, Z.N. and D.V. Ablashi, 1992. Molecular Biology of HHV-6. In: Ablashi, Dv., G.R.F Krueger, and S.Z. Salahuddin, (Eds.), Human Herpesvirus-6. Elsevier Sci. Publishers B.V., pp: 81-96.

Briggs, M., J. Fox and RS. Tedder, 1988. Age prevalence of antibody to HHV-6. The Lancet I: 1058-1059.

Brown, N.A., C.V. Sumaya *et al*., 1988. Fall in human herpesvirus-6 serpositivity with age. The Lancet II: 396.

Clark, D.A., 2000. Human herpesvirus-6. Rev. Med. Virol., 10: 155-173.

Dewhurst, S., K. McIntyre, K. Schnabel and C.B. Hall, 1993. Human herpesvirus-6 (HHV-6) variant B accounts for the majority of symptomatic primary HHV-6 infections in a population of U.S infants. J. Clin. Microbiol., 31: 414-418.

Fox, J.D., R.S. Tedder and E.M. Briggs, 1995. Human Herpesvirus 6 and 7. In: Principles and Practice of Clinical Virology. Zuckerman, AJ., Je. Bantavala and Jr. Pattison, (Eds.), 3rd Edn. John Wiley and Sons Ltd., pp: 135-151.

Frenkel, N. and L.S. Wyatt, 1992. HHV-6 and HHV-7 as exogenous agents in human lymphocytes. Develop Biol. Standard, 76: 259-265.

Green, N.M., 1964. Avidin. The use of [^{14}C] biotin for kinetic studies and assay. Biochem. J., 89: 585-589.

Gutierrez, J., M.J. Vergan, M. Guerrero, O. Fernandez, G. Piedrola, P. Morales and M.C. Maroto, 2002. Multiple sclerosis and human herpesvirus-6. Infection, 30: 145-149.

Klapper, P.E., G.M. Cleator, D. Prinja-Wilks, D.J. Morris and G. Morrel, 1990. Immunoradiometric assay for cytomegalovirus specific IgG antibodies; assay development and evaluation in blood transfusion practice. J. Virol. Methods, 27: 327-340.

Kruger, G.R.F., D.V. Ablashi, S.F. Josephs and N. Balachandaran, 1992. HHV-6 in Atypical Polyclonal Lymphoproloferation (APL) and Malignant Lymphomas. In: Human herpesvirus-6. D.V., Ablashi, Krueger, G.R.F. Salahuddin, S.Z., (Eds). Elsevier Science Publishers, B.V., pp: 185-208.

Leach, C.T., C.V. Sumaya and N.A. Brown, 1992. HHV-6 and clinical implications of a recently discovered ubiquitous agent. J. Pediatr., 121: 173-181.

Lopez, C., 1993. HHV-6 and HHV-7 (Molecular biology and clinical aspects). In: Roizman, B., RJ. Whitley, C. Lopez,, (Eds). *The human herpesviruses.* 1st Edn, Raven Press, New York, pp: 309-316.

Nash, P.J., R.K. Avery, W.H. Tang, R.C. Starling, AJ. Taege and M.H. Yamani, 2004. Encephalitis owing to HHV-6 after cardiac transplant. Am. J. Transplant, 4: 1200-1203.

Neilsen, L and B.F. Vestergaard, 1996. Competitive ELISA for detection of HHV-6 antibody; seroprevalence in Danish population. J. Virol. Methods, 56: 221-230.

Neilsen, L. and B.F. Vestergaard, 2002. A mu-capture immunoassay for detection of HHV-6 IgM antibodies in human serum. J. Clin. Virol., 25: 145-154.

Okuno, T., K. Takahashi, K. Balachandara, K. Shiraki and K. Yamanishi *et al.*, 1989. Seroepidemiology of HHV-6 infection in normal children and adults. J. Clin. Microbiol., 27: 651-653.

Salahuddin, S.Z. and D.V. Ablashi *et al.,* 1986. Isolation of a new virus (HBLV) in patients with lymphoproliferative disorders. Science, 234: 596-600.

Shen, S.Y., W.W. Chang, S.F. Chang, M.F. Chao, E.S. Huang and C.W. Wu, 1992. Seroepidemiology of CMV infection among children between ages of 4 and 12 years in Taiwan. J. Med. Virol., 37: 72-75.

Sloots, T.P., J.P. K apeleris, I.M. Mackay, M. Batham, P.L. Devine, 1996. Evaluation of a commercial ELISA for detection of serum IgG response to HHV-6. J. Clin. Microbiol., 34: 675-679.

Stiller, I., R. Pusztai, E. Sombor, L. Orosz, A. Pal and B. Tarodi, 2006. Prevalence and avidity of HHV-6 specific IgG antibodies in pregnant women in Hungary. Acta Microbiol. Immunol. Hung., 53: 25-34.

Thomas, H.I.J and P. Morgan-Capner, 1991. Rubella-specific IgG1 avidity: A comparison of methods. J. Virol. Methods., 31: 219-228.

Thomas, H.I.J., P. Morgan-Capner and N.S. Connor, 1993. Adaptation of a commercial rubella-specific IgG kit to assess specific IgG avidity. Serodiagn. Immuno. Ther. Infect. Dis., 1: 13-16.

Ward, K.N., J.J. Gray, M.W. Fotheringham and M.J. Sheldon, 1993. IgG antibodies to HHV-6 in young children: Change in avidity of antibody correlate with time after infection. J. Med. Virol., 39: 131-138.

Ward, K.N., 2005a. The natural history and laboratory diagnosis of HHV-6 and HHV-7 infections in the immunocompetent. J. Clin. Virol., 32: 183-193.

Ward, K.N., 2005b. Human herpesvirus 6 and 7 infections. Curr. Opin. Infect. Dis., 18: 247-252.

Yamanaka, T. and K. Kiuoyani *et al.*, 1992. Detection of cytomegalovirus in urine samples by an enzyme linked immunosorbent assay using a monoclonal antibody against the viral 150 kilo Dalton protein. J. Clin. Microbiol., 30: 685-690.

Yamanishi, K., T. Okuno, K. Shiraki, K. Takahashi, T. Kondo, Y. Asano and T. Kurata, 1988. Identification of human herpesvirus-6 as casual agent for exanthema subitum. The Lancet, 1: 1065-1067.

Zerr, D.M., 2006. Human herpesvirus 6: A clinical update. Herpes, 13: 20-24.