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Human Herpes-8 virus copy to cell ratio: A diagnostic tool in primary effusion lymphoma

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ABSTRACT

Primary effusion lymphoma (PEL) is a serious sequel to Human Herpes Virus 8 (HHV8) infection in the immunosuppressed host. Usually requiring a cytological diagnosis, body cavity effusions are often referred for investigation for possible PEL. Although absence of HHV8 effectively refutes this, the presence of HHV8 DNA, though indicative is not diagnostic. Referred effusion and plasma samples from 10 patients with HHV8-related pleural and pericardial effusions were submitted for quantitative investigations. HHV8 DNA and human DNA from unseparated effusion extracts have been quantified allowing estimation of virus-to-cell ratios in effusion fluid. These ratios varied widely between 0.003 and 700. Five fluids had in excess of 10^6 HHV-8 DNA genome equivalents per ML (GEq/ML), ranging between 18 and 300 million GEq/ML. Four of these five effusions were from patients with cytologically proven PEL and had virus to cell (V:C) ratios between 100 and 700 to 1. The remaining high load effusion exhibited a ratio of 1.6 to 1 and came from a patient with extensive thoracic Kaposi's sarcoma. Five effusion fluids with low viral loads exhibited virus to cell ratios between 0.003 and 0.5. High effusion HHV8 load, though supportive of a diagnosis of PEL is less accurate than using virus to cell ratios.

1. Background

Human herpes Virus 8 (HHV-8), also known as Kaposi's Sarcoma Herpes Virus (KSHV), is a gamma herpes virus [1] linked to various diseases, primarily in HIV-1 infected patients but also in other immunosuppressive conditions and more rarely in immunocompetent individuals. First described in 1994 [1], the associated various diseases include Kaposi's Sarcoma (KS) [2], multicentric Castleman's disease [3] and other lymphoproliferative disorders, including lymphomas, haemophagocytic [4,5] syndromes and Primary Effusion Lymphoma (PEL) [6]. The relationship between HHV-8 and associated oncogeneses, inflammatory and lymphoproliferative disorders have recently been reviewed and referenced in some detail [7]. PEL is a rare B cell lineage non-Hodgkin's Lymphoma linked to HHV-8 as the causative agent. It presents with a malignant effusion in body cavities without a solid tumour mass. This condition has historically had a poor outcome, with a median survival rate of < 1 year and only a 36% likelihood of achieving complete remission [8]. Prompt diagnosis and treatment is required to maximise patient remission rates.

In 2010 it was observed that levels of HHV-8 in the pleural effusion fluid of 2 kidney transplant patients with PEL was much higher than levels in plasma [9]. High copy numbers of cell associated virus genomes in clonal PEL B cells have been observed [10], a high virus to cell ratio in PEL effusion fluid has also been described [11,12]. Estimation of this ratio in addition to HHV-8 loads in the effusion has the potential to assist in diagnosing PEL and differentiating it from KS-related HHV-8 containing effusions. The value of using viral genome copies per cell as a diagnostic aid has already been shown in the case of cytomegalovirus

Abbreviations: HHV-8, Human Herpes Virus 8; KSHV, Kaposi's Sarcoma Herpes Virus; KS, Kaposi's Sarcoma; PEL, Primary Effusion Lymphoma; GEq, genome equivalents; ml, millilitre; V:C, virus to cell; CMV, cytomegalovirus; DNA, deoxyribonucleic acid; PDH, Pyruvate de-Hydrogenase; µl, microlitre; PBMC, Peripheral blood mononuclear cell

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(CMV) associated intestinal disease [13]. Body fluids are often referred for investigation of HHV8 markers. We have previously shown that HHV8 plasma viraemia, potentially indicating escape from control is a good predictor of risk for developing clinical KS [14] and this marker is used commonly for diagnostic and therapy monitoring. It is particularly useful in presumptive Castleman's disease [14]. Effusion fluids, often of unknown storage provenance, are received for investigation of potential aetiological roles for HHV8 or its exclusion. The absence of detectable HHV8 DNA in an effusion extract renders HHV8 involvement unlikely. However the interpretation of detection of HHV8 DNA in the whole lysate of an effusion, often the only available diagnostic modality is yet to be determined.

2. Objectives

A review has been undertaken of HHV-8 molecular testing of effusion samples over a period of six years. The correlation between HHV-8 viral loads in whole effusion fluid extracts and plasma, the virus:cell (V:C) ratio in such effusion fluids and a correlation with the clinical features leading to referral for the investigation of potential PEL were investigated for potential diagnostic correlation.

3. Study design

3.1. Patients and samples

Samples of recurrent effusion were received from 22 patients. Ten specimens, nine of pleural and one of pericardial origin, contained detectable HHV8 DNA and were further investigated for HHV8 and cell DNA quantification. Clinical and diagnostic information was available and their clinical status and clinical management linked to the HHV8 markers. All but patient one were male, (mean age 35, range 23–63), patient 7 was a female aged 25. All were HIV 1 infected except patient 10 who was a renal transplant recipient. Patients 1 to 4 were subsequently considered to have a cytologically-substantiated diagnosis of PEL on follow up. Patient 5 was found to have extensive intrathoracic KS. The cause of the persistent effusion in patient 6–10 was considered to be secondary to other causes or remained unknown at the time of review. Three of the five remaining patients (patients 6, 8 and 9) had evidence of Kaposi sarcoma within or adjacent to the lungs. No additional investigations were undertaken in the two remaining patients (patient 7 and 10).

3.2. Molecular markers

HHV-8 viral loads were estimated from taqman assays based on dilutions of cultured HHV-8 virus quantified against target containing plasmids as previously described [15]. Cell numbers were estimated by measuring the Pyruvate de-Hydrogenase gene (PDH) against a quantified set of dilutions derived from commercially available human genomic DNA.

3.3. Nucleic acid extraction

DNA was extracted from 200 µl, of plasma and of unfractionated body cavity effusion fluids on the Roche MagNAPure 96, (Roche DNA and Viral NA small volume kit, (Viral NA Universal SV protocol, version 3.1) and eluted in 50 µl. HHV-8 standards were included for viral load estimation and an internal control of murine Cytomegalovirus (mCMV) was spiked into every sample prior to extraction.

3.4. Taqman PCR

The detection and measurement of HHV-8 in all samples was as previously described [15]. HHV-8 primers and probes were as described in Bourboulia et al. [16] and mCMV primers and probes were as described in Garson et al. [17]

Human cell numbers in the effusion fluid were calculated by a separate taqman PCR based on the Pyruvate de-Hydrogenase gene (PDH), using human DNA standards of known cell equivalents. The primers and probes for this assay were as follows:

PDH Forward Primer:

5′-TGAAAGTTATACAAAATTGAGGTCACTGTT-3′

PDH Reverse Primer:

5′- TCCACAGCCCTCGACTAACC -3′

PDH Probe:

5′ VIC – CCCCCAGATACACTTAAGGGATCAACTCTTAATTGT-3′TAMRA

All amplifications were carried out in 50 µl reaction volumes, including 20 µl of sample eluate, using the ABsolute QPCR ROX Mix with a final concentration of 400nMol for each primer and 200nMol for each probe. All taqman PCRs were carried out on an ABI 7500 real time platform with the following cycling conditions: 1 cycle; 95 °C for 15 min, then 45 cycles; 95 °C for 15 s, 60 °C for 60 s with data acquisition.

4. Results

Between November 2009 and June 2016, body cavity effusion fluid samples from 22 patients were sent to the Virus Reference Department for HHV-8 DNA detection and quantification. Samples from 12 patients, including both effusion fluids and plasma, did not contain detectable HHV8 DNA. These samples were therefore excluded from further consideration. Multiple plasma samples and effusion samples were available on the remaining HHV-8 study subset of 10 patients. Four patients were considered on a cytological basis to have PEL (patients 1–4). Four of the six remaining patients (patients 5, 6, 8 and 9) had evidence of Kaposi sarcoma within or adjacent to the lungs. No additional investigations were undertaken in the two remaining patients (patient 7 and 10).

Paired plasma and effusion samples taken within 20 days of each other were available for all 10 patients. Five effusion fluid samples (patients 1–5) contained high levels of detectable HHV8 DNA ranging between 1.8×10^7 and 3×10 [8] GEq/ml of fluid. The v:c ratio ranged between 1.6 and 700 (Fig. 1). Five other effusion samples (patients 6–10) also contained HHV8 DNA but at lower levels, ranging from 130 to 1.4×10^4 GEq/ml of fluid. The V:C ratio ranged between 0.003 and 0.5 (Fig. 1). The relationship between the effusion fluid HHV8 viral load and the virus to cell ratio in the effusion fluid is displayed for all patients (Fig. 1).

In the five individuals with high HHV-8 DNA levels the viral load was at least three orders of magnitude lower in the plasma in comparison to that in the effusion fluid (Table 1). Paired plasma and effusion samples were available from the five patients with low effusion HHV8 levels (patients 6–10). The plasma viral load ranged between 0.05 and 8.2 times that in the effusion.

5. Discussion

Patients investigated for PEL are almost exclusively immunosuppressed, with detectable HHV-8 DNA circulating in the plasma as cell free viraemia [18]. The use of cell-free plasma viraemia is widely used in clinical practice for alpha, beta and gamma herpes viruses [19–24] and HHV-8 is no exception [25–29]. All patients with PEL will have clinical evidence of body cavity fluid (exudation/transudation effusion fluid), often recurrent after drainage or dispersal. This may occur in any body cavity but is most commonly associated with the pleural space and the pericardium. This fluid, best considered as a transudate, will contain a significant element of plasma which may also contain plasma derived virus as one source of the effusion HHV8 load. Cell free virus may also be released in transudates from active KS tissue and therefore cell free virus can also be a marker of KS presence. In the

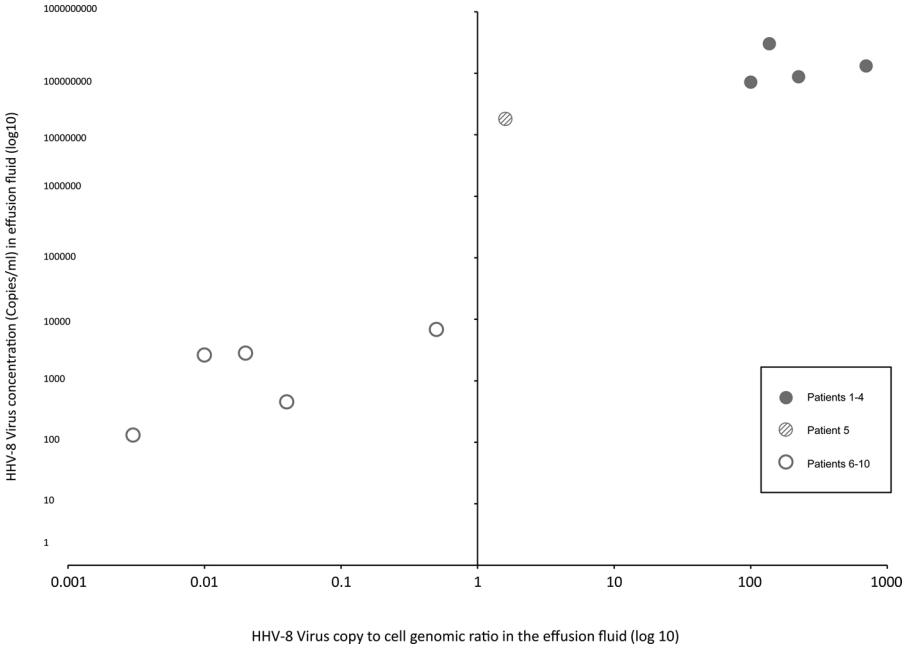

Fig. 1. HHV-8 concentration in effusion fluid vs virus copy to cell genome ratio in the effusion fluid.
The viral load (log_{10} copies per ml) in the effusion fluid is plotted against the virus copy to cell genomic ratio in the effusion fluid (log_{10}). Effusion fluids cluster in two distinct groups. Those from patients 6–10 (open circles) are separate from a second group (patients 1–4, solid circles). A single effusion fluid associates with neither group (patient 5, hatched circle).

Table 1
HHV-8 viral loads in effusion fluid, plasma and virus to cell genome ratio in effusion fluid from ten patients with detectable HHV-8 DNA.

Patient Number	HHV-8 viral load in effusion fluid (GEq/ml)	HHV-8 viral load in plasma (GEq/ml)	Ratio of effusion fluid viral load to Plasma viral load	HHV-8 virus copy/cell genomic ratio in effusion fluid
1*	3 × 10E8	3.3 × 10E4	9100	137
2*	1.3 × 10E8	3.3 × 10E4	3900	700
3*	8.7 × 10E7	2.3 × 10E3	38000	224
4*	7.1 × 10E7	2.8 × 10E4	2500	100
5	**1.8 × 10E7**	**9.2 × 10E3**	**2000**	**1.6**
6	6.8 × 10E3	1 × 10E3	6.8	0.5
7	2.8 × 10E3	2.3 × 10E4	0.12	0.02
8	2.6 × 10E3	1.2 × 10E2	22	0.01
9	4.5 × 10E2	9 × 10E2	0.5	0.04
10	1.3 × 10E2	4.2 × 10E2	0.31	0.003

Bold: Patient for whom the assignment of PEL status using the virus to cell ratio in the effusion fluid was uncertain.
* Patients subsequently diagnosed with PEL.

effusion of PEL, cell free and cell associated HHV-8 will be constituents of the effusion viral load. An initial review of the transmittal of the reference samples confirmed earlier findings and showed that plasma and effusion fluids were referred, often several days to weeks after the initial sample was taken. For this reason it was deemed that reliable PBMC analysis, cytology or separation and analysis of the cellular component of the effusion fluid was simply impossible in any of these cases. In clinical practice it is usual to confirm partitioning of virus across a potentially leaky barrier and this was the rational for determining cell free HHV8 DNA in plasma and exudate/effusion fluid. As shown in this study, high levels of HHV8 DNA detected in effusions are however not pathognomonic for PEL (See Table 1 and Fig. 1). Determination of the HHV-8 V:C ratio in the effusion fluid appears to be a better predictor of a diagnosis of PEL, as determined by cytology for all of these patients. This study also confirms previous findings that measurement of virus levels in the circulating plasma alone does not contribute to making a diagnosis of PEL. In both PEL and non-PEL cases circulating HHV-8 DNA levels in plasma were broadly similar, varying from 1.2 × 10E2-3.3 × 10E4GEq/ml (Table 1), confirming that the differentiation between PEL and non-PEL cases on the basis of plasma viral load is not possible. There seems no reason to suspect that a cellular measure of HHV8 in the peripheral blood would be any more accurate.

In 5 out of 6 of the non-PEL samples (patients 5 to 10 inclusive) HHV-8 levels in the effusion fluid ranged from 1.3 × 10E2-6.8 × 10E3 GEq/ml. These values broadly correlated with HHV-8 DNA levels in plasma, ranging in between 0.3 and 20 fold difference between matched effusion and plasma samples. For the remaining non-PEL high viral load sample (patient 5), HHV-8 DNA levels were 2000 fold higher in the effusion than in the matched plasma sample, reflecting a final diagnosis of very extensive intra-thoracic KS tissue.

In the four individuals in whom PEL had been diagnosed (patients 1–4), HHV-8 DNA levels in the effusion fluid were between 2500 and 40,000 times higher than matched plasma samples. On the basis of the evidence presented here, any effusion fluid sample which contains HHV-8 detectable DNA and exhibits a virus to cell genome ratio ≥ 100 would be highly likely to be indicative of PEL, whilst any sample with a virus to cell ratio ≤ 1 would be highly unlikely to be indicative of PEL as evidenced (Fig. 1). An effusion sample with a genomic ratio falling between these two figures should lead to further investigation to confirm or exclude a diagnosis of PEL, with the probability of PEL increasing as the virus to cell ratio increases. Further study of HHV-8 DNA positive body cavity effusions may help to refine this range further but given the rarity of this condition, the data from these 10 cases with 4 being confirmed as PEL, the estimation of virus copy to cell genome ratios in pleural effusions remains an important aid in the confirmation

of HHV-8 related effusion lymphomas. To facilitate the uptake of this methodology development of standards for the measurement of HHV-8 virus copy numbers would facilitate direct comparisons between differing HHV-8 quantification methods.

In summary, the determination of the circulating levels of HHV-8 viral DNA in plasma or effusion fluid alone is neither sufficient for the delineation of PEL, nor as a predictive value for the probability of PEL. In contrast the virus copy to cell genomic ratio in the relevant effusion fluid in conjunction with circulating virus levels in both plasma and effusion fluid should prove a diagnostically more accurate tool in the diagnosis of HHV-8 linked PEL.

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Competing interests

None declared.

Ethical approval

Informed consent was obtained from all patients for the testing undertaken.

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