Clinical and Laboratory Evaluation of the new Argene EBV DNA quantitative real-time PCR assay (R-gene)

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Abstract

Epstein - Barr virus (EBV) is a lymphotropic member of the herpes family. It is responsible for a variety of clinical conditions ranging from infectious mononucleosis (IM) to post transplant lymphoproliferative disease (PTLD). Increasingly, molecular techniques are used in the diagnosis of PTLD following transplantation, where the detection of DNA in blood samples has been shown to accurately predict or diagnose PTLD depending on the EBV load. In this assay evaluation study we have compared the new commercial Argene EBV R-gene quantitative real time PCR assay on the ABI Prism 7500 against our routine assay on both whole blood and plasma samples. A total of 243 samples from 58 individuals were tested using both methods on whole blood and plasma. We found that both assays had a higher positivity rate when whole blood was tested rather than plasma, as 42 samples were found positive in at least one assay when using DNA extracts taken from whole blood. Using the Argene whole blood assay, 17 additional positive samples were detected in comparison to the in-house whole blood assay. This coupled with results obtained from sequential samples from one patient would lead us to believe that the Argene commercial assay is more sensitive than our in-house method. The Argene assay was easy to use and could be used by laboratories with limited molecular testing experience. As all the reagents required are ready to use the need for PCR clean areas and the chance of contamination is reduced. Using a commercial method also facilitates stringent quality control within the laboratory and enables greater consistency between testing centres to provide a standard from which meaningful viral loads can be given.

Introduction:

Epstein - Barr virus (EBV) is a lymphotropic member of the herpes family. It is responsible for a variety of clinical conditions ranging from infectious mononucleosis (IM) to post transplant lymphoproliferative disease (PTLD) (1). PTLD results from the uncontrolled expansion of EBV infected B cells, in the absence of an effective CD8 T cell response; this proliferation can result in the development of clonal malignancy. The incidence following transplantation ranges from <1%-33% and is dependant on type of transplant (haematopoietic stem cell or solid organ), level of immunosuppression and whether the recipient has ever been exposed to EBV in the past (2). Increasingly, molecular techniques are used to predict the risk of developing PTLD following transplantation where the detection of rising EBV DNA load in blood samples has been shown to correlate with the development of PTLD (3, 4) and in some cases these results are being used to guide pre-emptive therapy in stem cell transplant recipients. The detection of EBV DNA in blood is also useful in diagnosing or excluding PTLD as a cause of a patient's symptoms, as the clinical picture may not always be clear-cut. As up to 50% of patients are only positive in one or 2 samples, some centres advocate delaying treatment until the development of symptoms (5). PCR may also be useful when the serological results are difficult to interpret.

In this study we compared the new commercial Argene EBV R-gene whole blood assay on the ABI prism 7500 against our in house test, on samples submitted to the routine laboratory service. Currently, we only test patients plasma samples for EBV DNA, but were able to adapt this format to also test whole blood.

Materials and Methods:

A total of 243 EDTA samples from 58 individuals were submitted for EBV PCR testing between 20.07.05 and 21.10.05. These samples were not part of a study and were tested at the request of the clinical team responsible for the patient. Each sample was divided into 2 aliquots (plasma and whole blood) and the DNA was extracted.

Patients and samples:

The majority of the samples were from patients who were immunocompromised either as a result of transplantation (36 bone marrow transplant, 14 solid organ (13 cardiac and 1 renal) or chemotherapy (6 haematological malignancies), and others (2 patients with suspected acute EBV infection and 1 HIV positive patient). All of the 36 bone marrow transplant patients had undergone transplantation within the last year (8 siblings, 28 volunteer unrelated donors - VUD).

Sample Preparation:

All samples were extracted using the Qiagen QIAamp DNA mini blood kit or the Argene EBV R-gene kit (whole blood), which is the Qiagen QIAamp DNA mini blood kit as supplied by Argene. DNA was eluted in 100µl. Plasma aliquots were also extracted using the QIAamp Virus BioRobot 9604 kit and tested in the next routine run in the laboratory.

Real time PCR assays:

DNA extracts from whole blood were tested using both the in-house and the R-gene assays. DNA extracts from plasma were only tested with the in-house assay. All assays were performed in 96 well plates on ABI 7500 SDS equipment using 10µl of extracted DNA.

Argene EBV R-gene kit (ref 69002):

The EBV R-gene kit is a real time 5' nuclease PCR based assay for the detection and quantification of EBV. The amplification target is in the thymidine kinase gene. The probes are dual-labelled FAM and TAMRA. The kit includes 4 quantification standards (equating to a starting concentration of 5 x 103 to 5 x 106 copies/ml in whole blood), a sensitivity control (500 copies/ml equivalent in whole blood), amplification premix (contains EBV probe) and inhibition control premix (contains inhibition control probe). In addition to the kit controls, negative and positive extraction controls were included in order to compare the two assays. PCR reactions were performed in 25µl final volumes. The Ct was calculated by placing the threshold at the start of the exponential phase of the curves. A sample was considered as inhibitory if the Ct value observed in the inhibition control well for that sample was more than 2 Cts greater the Ct value of the water control (included in the kit).

In-house real time PCR:

The in house assay is based on the method described by Niesters et al (6). The primers and probe used in this assay target a region in the gene encoding the nonglycosylated membrane protein BNRF1 (p143). The forward (5' GGA ACC TGG TCA TCC TTG C) and reverse (5' ACG TGC ATG GAC CGG TTA AT) primers (Operon, Cologne, Germany) generate a product of 74bp detected using a dual-labelled probe (FAM- CGC AGG CAC TCG TAC TGC TCG CT-BHQ). PCR reactions were performed in 25µl final volumes containing Invitrogen Platinum Quantitative PCR supermix-UDG, 1000nm forward and reverse primer, 200nm probe and 10µl of extracted DNA. The cycling conditions were altered to 95 oC for 2 minutes followed by 40 cycles of 95 oC for 8 seconds and 60 oC for 34 seconds. The standards used were extracted Raji DNA (7.5 x 107 – 7.5 x 10 3 copies/ ml). Positive and negative extraction controls and a no template control were also included in the test. Following completion of the PCR run the Ct was then calculated using a predetermined threshold of 0.1.

Results and discussion:

There was a good correlation between the results obtained with the different assays. Of the 243 samples tested, 183 were negative in all of the assays and 18 were positive (81% agreement). As expected the whole blood assays detected more positive samples than the plasma assay and this has been seen in previous studies (7). This reflects the development of the infection: first in the B-lymphocyte fraction, which then spills over into the plasma fraction as the amount of virus increases. The distribution of positive and negative results in the 3 assays is shown in the Venn diagram (Fig 1).

Fig 1- Venn Diagram showing the relationship between the 3 assays

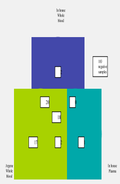

Table 1- All results of samples positive in one or more assay.

Study No	Argene plate- whole blood			in house plate- whole blood			in house plate- plasma			routine result		
	RESULT	CT VALUE	COPIES	RESULT	CT VALUE	COPIES	RESULT	CT VALUE	COPIES	RESULT	CT VALUE	COPIES
122	P	38.06	56	n			n			n		
159	P	37.18	111	P	39.42	33	n			n		
30	P	31.77	4050	P	31.49	5259	P	35	406	P	36.84	297
51	P	33.46	4000	P	32.11	8197	n			P	35.19	602
109	P	34.55	785	P	33.56	1546	n			n		
151	P	34.17	1352	P	34.84	805	n			n		
170	P	35.89	262	n			n			n		
214	P	37.45	81	n			n			n		
186	P	34.64	540	n			n			n		
13	n			P	36.75	271	n			n		
16	P	34.45	678	P	34.17	671	n			n		
143	P	29.18	39421	P	27.74	129389	P	31.43	9399	P	29.5	27260
53	P	38.3	150	P	36.26	442	n			n		
155	P	37.97	103	n			n			n		
208	P	37.47	80	n			n			n		
41	P	38.84	104	n			n			n		
1	P	31.27	4833	P	31.2	16784	n			n		
40	P	32.16	9653	P	30.83	20193	P	32.66	5582	P	34.85	982
47	P	32.17	9614	P	32.04	8649	n			P	34.08	1258
61	P	32.64	6994	P	32.06	8501	n			P	35	1506
77	P	33.92	2521	P	32.2	4275	n			n		
102	P	33.36	1911	P	32.3	3737	n			P	37.15	87
121	P	34.04	1149	P	34.09	1072	n			n		
131	P	35.1	721	P	34.82	813	n			n		
154	P	36.04	380	n			n			n		
195	P	34.88	460	n			n			n		
210	P	32.41	2423	P	33.26	2192	n			n		
237	P	33.8	934	P	31.62	8901	n			n		
238	P	34.18	715	P	35.83	580	n			n		
25	P	31.83	3912	P	31.22	6478	n			n		

Two samples were inhibitory in the Argene assay, but as no internal control was included in the in-house assays we were not able to confirm whether the negative result obtained was due to inhibition. A sample of all positive results are shown in table 1 (see handout for all results).

As can be seen from the results, 17 samples were positive in the Argene assay only. These samples came from 12 patients and as 4 out of the 12 patients had previous positive results, the isolated Argene positives are unlikely to be false positives. These findings suggest that the Argene test is the most sensitive of the three assays in our comparison. This is further supported by the findings in Table 2, where sequential samples from one patient show how the Argene result was positive before either of the other two assays and remained positive when the other two had undetectable loads.

Table 2- Whole blood and plasma results for samples taken from one patient over 2 months.

Date of Sample	in house plate- whole blood			Argene plate- whole blood			routine result- plasma		
	RESULT	CT VALUE	COPIES	RESULT	CT VALUE	COPIES	RESULT	CT VALUE	COPIES
18.07.05	P	31.2	16784	P	31.27	4833	n		
27.07.05	P	30.83	20193	P	32.16	9653	P	34.85	982
01.08.05	P	32.04	8649	P	32.17	9614	P	34.08	1258
04.08.05	P	32.06	8501	P	32.64	6994	P	35	1506
08.08.05	P	32.2	4275	P	33.92	2521	n		
15.08.05	P	32.3	3737	P	33.36	1911	P	37.15	87
18.08.05	P	34.09	1072	P	34.04	1149	n		
22.08.05	P	34.82	813	P	35.1	721	n		
29.08.05	n			P	36.04	380	n		
05.09.05	n			P	34.88	460	n		
12.09.05	P	33.26	2192	P	32.41	2423	n		
19.09.05	P	31.62	8901	P	33.8	934	n		
19.09.05	P	35.83	580	P	34.18	715	n		

The clinical significance of earlier detection offered by using whole blood or the Argene assay is still not clear as a number of patients have transient EBV reactivation, which does not result in disease. However the earlier detection offers the clinicians the opportunity to monitor patients more frequently and perhaps identify those at greatest risk as a result of anti rejection therapy for example.

Only one of the bone marrow transplant patients developed PTLD during the study period. This was a 46-year-old male who had received an unmatched transplant about 3 months before EBV was first detected. Within 6 weeks of his transplant he had developed non-specific symptoms including nausea, vomiting and flu like illness. CMV was detected and he commenced a course of ganciclovir. Two months later he became EBV DNA positive and subsequently developed lymphadenopathy. The test results and clinical features are shown in Table 3. As expected, EBV DNA was detected in the whole blood fraction first. Unfortunately as only one patient developed PTLD during the study period, it is difficult to draw conclusions on what is a significant load. Our preliminary data would suggest, using the median loads in Table 4 that a load greater than 10,000 copies in either of the whole blood assays is associated with an increased risk of PTLD, however this would need to be confirmed with more sample/patients and more frequent sampling to monitor the rate of increase in load.

Table 3- Test results and clinical features for patient with clinical evidence of PTLD

Date of Sample	In house plate- whole blood			Argene plate- whole blood			Routine result			Clinical Status
	RESULT	CT VALUE	COPIES	RESULT	CT VALUE	COPIES	RESULT	CT VALUE	COPIES	
08.09.05	n			n			n			Patient well
12.09.05	P	31.96	5273	P	31.68	3965	n			Patient well
19.09.05	P	26.2	298236	P	26.07	231668	P	30.04	3340	Patient well
22.09.05	P	24.1	1.16E+06	P	24.37	784229	P	30.01	29658	LN noted
30.09.05	not tested			not tested			P	26.38	264556	Fever, renal failure, N/V
										Rituximab given.
01.10.05	P	25.84	375641	P	27.5	83752	P	23.78	1.92E+06	Rituximab given.
06.10.05	P	27.29	147600	P	29.82	15978	P	25.05	1.09E+06	Rituximab given.

Table 4: Comparison of median EBV loads In patients with and without PTLD

Clinical	Median load (copies/ml)		
	In house whole blood	Argene whole blood	In house plasma
No PTLD (57 patients)	8515	8636	2055
PTLD (1 patient)	580000	394097	4871

Although we only had one patient with PTLD during this time period, our results confirm the relationship between load and clinical disease, i.e. clinically significant disease was not seen until high loads were reached. In this particular patient the lag between the whole blood and plasma assays was about 7 days, although we did not receive any samples during the intervening period to confirm this. The load in plasma was about 100 fold less compared to whole blood and in our patient was first detected at about the same time as the onset of symptoms. We also confirmed response to Rituximab with a fall in viral load.

Advantages of the Argene test:

- Greater sensitivity as uses whole blood.
- Simple to use, could be used easily by laboratories with limited experience in molecular techniques.
- All reagents are pre-prepared so there is no need for PCR clean room facilities as the mastermix simply needs added to the plate.
- Includes an internal control for identification of inhibitory samples.
- Using a commercial method also facilitates stringent quality control within the laboratory and enables greater consistency between testing centres to provide a standard from which meaningful viral loads can be given.
- The Argene assay is CE marked.

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