Use of dried blood spots for the detection and confirmation of HTLV-I specific antibodies for epidemiological purposes

S P Parker, M B Taylor, A E Ades, W D Cubitt, C Peckham

Abstract

Aims—To modify and evaluate a gelatin particle agglutination test that could provide a sensitive, specific and inexpensive method for the detection of HTLV-I antibody in dried blood spot samples (DBS) collected on filter paper.

Methods—A set of 26 reference samples confirmed as HTLV-I antibody positive were assembled from patients with tropical spastic paraparesis or adult T cell leukaemia and blood donors. Serum samples and simulated antibody positive dried blood spot eluates were tested using the Serodia assay together with two confirmatory tests: HTLV BLOT 2.3, a western blot, and Select-HTLV, an enzyme immunoassay (EIA). Both confirmatory tests use synthetic peptides to differentiate between antibodies to HTLV-I and -II. The modified Serodia assay was then used to test anonymously 10 135 DBS collected from neonates from London. Samples reactive in the modified Serodia test producing a positive result were titrated to an end point and confirmed as before.

Results—All 26 eluates made from simulated DBS derived from positive reference samples were identified as positive by the modified Serodia HTLV-I test and were confirmed as anti-HTLV-I positive by EIA. Two eluates derived from relatively low titre reference samples gave indeterminate results on western blotting. Screening of the 10 135 neonatal DBS resulted in six repeat reactives, five of which were confirmed. The remaining reactive sample gave an indeterminate result on western blotting and there was insufficient eluate for testing by EIA. The overall seroprevalence of HTLV-I in this population was 0.05% (five of 10 135).

Conclusion—The modified Serodia HTLV-I assay provides a sensitive, specific and inexpensive (10 pence/test) method for screening large numbers of DBS. The format of the assay makes it ideally suited for simultaneous screening of antibodies to HIV-1, HIV-2 and HTLV-I using semi-automated equipment.

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Human T cell lymphotropic virus type I (HTLV-I) has been recognised as the causal agent of several diseases, adult T cell leukaemia (ATL),¹ tropical spastic paraparesis (TSP),² also known as HTLV-I associated myelopathy

(HAM) in Japan,³ and, more recently, HTLV-I uveitis.⁴ Transmission may occur through the transfer of infected lymphocytes⁵ by sexual transmission,⁶ during transplantation (blood or organ), intravenous drug abuse, breast feeding,⁷ and possibly saliva.⁸

HTLV-I is endemic in Japan (where over 700 cases of ATL are diagnosed every year),⁹ the Caribbean,¹⁰ South America,¹¹ central and southern Africa,¹²⁻¹⁴ and occurs in Australian aborigines,¹⁵ and in intravenous drug users in the USA.¹⁶

Many assays have been developed for the detection of HTLV-I antibody and antigen, including antigen capture, immunofluorescence, the polymerase chain reaction (PCR), and in situ hybridisation.^{17,18} The Serodia test, a gelatin particle agglutination assay, is a highly sensitive test introduced in 1987 for the detection of antibodies to HTLV-I.^{19,20} However, following concerns about the test's specificity in comparison with other assays such as the radioimmunoassay (RIA) and western blot,²⁰⁻²² particularly when it was used to test serum samples from Africans,²³ the test was modified by increasing the relative proportion of env to gag proteins.²⁴ Subsequent evaluation of the assay in comparison with other commercial tests showed it to have superior specificity, 97.6%, and sensitivity, 100%.²⁵ In spite of improvements in the screening assay confirmatory testing remains essential as cross reactivity with HTLV-II has been reported.²⁶ The most reliable technique for the differentiation of HTLV-I and HTLV-II is PCR and the use of type specific recombinant proteins in enzyme immunoassays (EIAs) and western blots have improved the differentiation of antibodies to the two viruses.^{27,28}

The improved Serodia test has been used extensively for epidemiological surveys to estimate the seroprevalence of HTLV-I in blood donors in the United Kingdom,²⁶ France,²⁹ Japan,⁹ and Australia.³⁰ In this paper we describe the use of a modified version of the Serodia test to screen for the presence of antibody to HTLV-I in dried blood spot samples from anonymous neonatal Guthrie cards followed by confirmatory testing.

Methods

REFERENCE SAMPLES

Twenty six samples were obtained from patients with confirmed HTLV-I infection, from three sources. Seven plasma samples originating from the Japanese Red Cross (ADP 507, MRC AIDS Reagent Project) were provided by the National Institute of Biological Standards, UK. Serum samples from four

Department of Virology, Institute of Child Health, 30 Guilford Street, London WC1 1EH
S P Parker

Department of Epidemiology and Biostatistics
A E Ades
C Peckham

Department of Virology, Great Ormond Street Hospital for Sick Children NHS Trust, Great Ormond Street, London WC1N 3JH
W D Cubitt

Department of Medical Virology, University of Pretoria, PO Box 2034, 0001 Pretoria, Republic of South Africa
M B Taylor

Correspondence to: Mr S P Parker.

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patients with TSP associated symptoms diagnosed as HTLV-I positive at St Bartholomew's Hospital, London, were included together with 15 samples from African patients with symptoms of TSP or ATL referred to the University of Pretoria, Republic of South Africa. All samples had been confirmed as anti-HTLV-I positive by other laboratories.

SIMULATED HTLV-I POSITIVE DRIED BLOOD SPOT SAMPLES

A 1 in 2 dilution of each anti-HTLV-I positive sample, or serial dilutions, were made in anti-HTLV-I negative human "O" cells and a 60 µl aliquot spotted out onto absorbent filter paper (Guthrie card). Cards were allowed to dry at room temperature overnight before storage at 4°C in gas permeable bags.

SOURCE OF NEONATAL DRIED BLOOD SPOT SAMPLES

Dried blood spot samples punched from 10 135 Guthrie cards that had been routinely collected for universal newborn metabolic screening were obtained from health districts in inner London with a relatively high proportion of Caribbean and African ethnic populations. All samples were rendered anonymous prior to receipt in the laboratory.

ASSAY FOR THE DETECTION OF HTLV-I ANTIBODY IN SERUM SAMPLES

Serodia HTLV-I gelatin particle agglutination tests (Fujirebio Inc, Tokyo, Japan) were obtained from Mast Diagnostics, Bootle, Merseyside, UK.

Serum and plasma samples were tested according to the manufacturer's instructions. Briefly, samples were titrated in a series of doubling dilutions from 1 in 16 across a U well microtitre plate; 25 µl of sensitised particle suspension was added to each well. The contents of each well were thoroughly mixed using a plate shaker, and left to incubate at room temperature for two hours on a level, vibration free surface.

Positive results were indicated by the formation of an agglutination pattern and negative results were indicated by a smooth compact button of particles.

DRIED BLOOD SPOT SAMPLE ASSAY

A dried blood spot sample, 5.5 mm in diameter, was punched out of each Guthrie card and eluted, as described previously,³¹ into flat bottomed microtitre plates containing 100 µl buffer (phosphate buffered saline (pH 7.2) containing 0.05% Tween 80 and 0.005% sodium azide) overnight at 4°C. A 1 in 6 dilution of each eluate was made in TPHA buffer (catalog number 9224, Mast Diagnostics), in a corresponding V well plate using a 96 pin automatic replicator calibrated to transfer 5 µl aliquots, (Wellrepp, Denley Instruments, Billingham, Sussex, UK). HTLV-I gelatin particles were reconstituted according to the manufacturer's instructions and then diluted 10-fold in TPHA buffer immediately before use. A 25 µl aliquot of diluted particles was

added to each well using an automated programmable dispenser (Wellfill, Denley Instruments). The plates were incubated for 15 minutes at room temperature on a flat, vibration free surface and were then centrifuged for three minutes at 200 × g, sloped on a light-box at an angle of 70°, and the results read after 10 minutes. A positive result could be distinguished by a tight discrete agglutination pattern whereas negative samples formed a teardrop.

All eluates found to be reactive in the initial screen were titrated in a series of doubling dilutions from 1 in 5 to 1 in 10 240 or 1 in 4 to 1 in 16 384 in TPHA buffer, and reacted against both antigen coated particles and unsensitised control particles and the endpoint recorded.

CONFIRMATORY TESTING

A repeat reactive screening result was confirmed using western blot analysis (HTLV-BLOT 2.3, Diagnostic Biotechnology, Singapore) and an EIA (Select-HTLV, IFCI Clonesystems, Biostat Diagnostics, Cheshire, UK).

The HTLV BLOT 2.3 contains viral lysate, recombinant rgp21 (env), and incorporates MTA-1 and K55 recombinant peptides corresponding to sequences specific to gp46^{env} of HTLV-I and -II, respectively. A 1 in 100 dilution of serum (20 µl in 2.0 ml) was tested according to the manufacturer's instructions. The presence of bands to the envelope proteins rgp21 and rgp46-I and core proteins p19^{gag} or p24^{gag} was regarded as the minimum criterion indicative of antibody to HTLV-I.

The Select-HTLV kit differentiates between antibodies to HTLV-I and -II using synthetic peptides representing type specific epitopes derived from p19^{gag} and gp52^{env}, respectively. Serum samples were tested at a 1 in 50 (5 µl in 250 µl) dilution and results interpreted according to the manufacturer's instructions. Optical densities (OD) of samples greater than the cut off value (mean of three negative controls ((supplied) + 0.15) were considered positive. When the cut off value was exceeded on both the HTLV-I and -II plates, a binding ratio (OD HTLV-II/OD HTLV-I) was calculated and a ratio of ≤ 0.5 was considered indicative of reactivity to HTLV-I.

Minor modifications were required when applying the confirmatory tests to dried blood spot samples in that a volume of 40 µl eluate was used in the western blot assay and 20 µl in the EIA. Simulated dried blood spot samples derived from serially diluted reference antibody positive and antibody negative eluate controls were used to provide an accurate cut off point in the EIA.

Results

REFERENCE PANEL

The results of tests on HTLV-I positive reference serum and plasma samples and eluates from simulated dried blood spot samples are shown in table 1. Use of the standard Serodia HTLV-I test and confirmatory assays on the reference samples gave the expected results.

Table 1 Results of screening and confirmatory tests on 26 reference samples and simulated dried blood spot samples (DBS)

Sample No.	Diagnosis	Reference sample			Simulated DBS eluate		
		Serodia titre	HTLV BLOT 2-3	Select-HTLV*	Serodia titre	HTLV BLOT 2-3	Select-HTLV*
1	TSP	80 000	+	>3.0	16 384	+	2.84
2	TSP	80 000	+	2.88	8192	+	2.84
3	TSP	80 000	+	2.84	8192	+	2.91
4	TSP	64 000	+	2.79	8192	+	2.84
5	TSP	64 000	+	2.84	4096	+	2.77
6	TSP	64 000	+	>3.0	2048	+	2.84
7	TSP	64 000	+	>3.0	1024	+	2.24
8	TSP	32 000	+	2.59	4096	+	1.38
9	TSP	32 000	+	2.69	2048	+	2.44
10	TSP	32 000	+	2.89	2048	+	2.15
11	TSP	32 000	+	2.74	1024	+	2.06
12	TSP	32 000	+	2.77	512	+	1.78
13	TSP	16 000	+	>3.0	512	+	2.70
14	ATL	16 000	+	>3.0	160	+	1.72
15	TSP	8000	+	2.52	256	+	0.39
16	TSP	8000	+	>3.0	32	+	0.68
17	TSP	2000	+	2.23	128	+	0.32
18	TSP	2000	+	2.80	64	+	0.93
19	ATL	2000	+	2.88	80	+	0.83
20	ATL	1000	+	1.90	64	p24, rgp41	0.17
21	ATL	1000	+	2.58	80	+	0.74
22	ATL	1000	+	2.82	40	+	0.47
23	ATL	500	+	2.67	20	+	0.65
24	ATL	400	+	2.44	80	+	0.47
25	ATL	200	+	1.65	16	p24, rgp41	0.19
26	ATL	100	+	1.50	10	+	0.17

TSP=tropical spastic paraparesis; ATL=adult T cell leukaemia.
* OD of HTLV-I plate.

All tests on eluates from simulated dried blood spot samples gave positive results using the modified Serodia agglutination test (table 1). When eluates were tested against un-sensitised particles provided in the Serodia test there was no evidence of non-specific agglutination.

Confirmatory testing by western blotting produced two indeterminate results (samples 20 and 25), suggesting that two of 26 eluates were on or just below the limit of sensitivity of

the assay. All eluates derived from serum or plasma reference samples were positive in the EIA.

In order to set a cut off value for the EIA when confirming Serodia reactive eluates, simulated dried blood spot samples derived from serially diluted samples from the reference panel were tested in the modified Serodia HTLV-I and the EIA (figure). The figure shows that eluates with a Serodia titre greater than 1 in 10 can be reliably detected by the EIA using a cut off OD of 0.1. The mean and median ODs of Serodia negative (<1 in 5) eluates were less than 0.03.

Comparison of the absorbance values obtained in the EIA and the titre observed in the modified Serodia test. n=number of samples; median value; closed bars denote range.

Table 2 Results of screening and confirmatory testing of repeat reactive eluates derived from anonymous neonatal dried blood spot samples

Sample No.	Serodia eluate titre	HTLV BLOT 2-3	Select-HTLV		
			HTLV-I plate	HTLV-II plate	Binding ratio*
1	≤20	p19, rgp21	INS	INS	INS
2	40	HTLV-I+	1.15	0.20	0.17
3	40	HTLV-I+	0.35	0.07	0.20
4	80	HTLV-I+	1.38	0.18	0.13
5	160	HTLV-I+	0.37	0.07	0.19
6	160	HTLV-I+	0.58	0.06	0.10

* Binding ratio OD=HTLV-II/OD HTLV-I. A binding ratio of ≤0.5 was indicative of reactivity to HTLV-I.
INS=insufficient sample to test.

NEONATAL PILOT STUDY RESULTS

Twelve dried blood spot samples were initially reactive in the modified Serodia assay, of which six were negative when repeated. Five (0.05%) of 10 135 of the remaining six dried blood spot samples were confirmed as HTLV-I antibody positive by western blot and EIA (table 2). Sample 1, which had an end point titre of ≤20, showed weak banding on western blotting to p19^{gag} and rgp21 but was insufficient to test in the EIA.

Discussion

The modified Serodia HTLV-I assay was found to be sensitive when used to test simulated dried blood spot samples prepared from reference samples from patients in Japan, Africa and the UK. Comparison of the results of tests on serum and plasma samples and eluates showed that the modified Serodia test was more sensitive than the confirmatory assays when testing eluates from dried blood spot samples. Loss of sensitivity of the western blot with low titre eluates could be overcome by increasing the volumes of eluate. Although it has been suggested that the absence of a rgp21 positive band should be regarded as western blot negative in this assay,³² this would have resulted in two of the 26 eluates from the reference panel being recorded as negative.

The Select-HTLV EIA confirmed all Serodia HTLV-I antibody positive eluates that had a titre greater than 1 in 10. Only one of 26 eluates derived from a reference serum sample had an eluate titre below this threshold, indicating that very few neonatal samples would be likely to have such low titres. However, it must be noted that patients with TSP, some of whose serum samples make up the reference panel, will in general have higher titres than those diagnosed with ATL or who are asymptomatic.³³ Furthermore, studies have shown that premature infants have decreased levels of maternal IgG.³⁴ In a London based neonatal screening survey approximately 1.5% of dried blood spot samples would be derived from neonates born seven to 12 weeks prematurely³⁵ and would be expected to show a two- to fourfold loss of IgG.

Despite a potential problem in confirming very low positive eluates, the Select-HTLV would be the confirmatory test of choice for dried blood spot samples because of its adaptability when using limited volumes of eluate.

Five of six repeat reactive eluates were confirmed as positive from 10 135 neonatal dried blood spot samples: a seroprevalence of 0.05% in pregnant women. This indicates that the modified Serodia assay has a very high specificity when testing samples obtained from neonates in the general population. The six initial reactives that failed to repeat were deemed technical failures perhaps due to dust contaminated wells as seen with similar frequency in other agglutination assays. The prevalence was lower than that reported (0.26% and 0.21%) in antenatal surveys from other London health districts,^{36,37} but higher than the rates reported (0.005% and 0.004%) in extensive surveys of blood donors in the UK.^{21,26}

The use of the modified Serodia HTLV-I test on dried blood spot samples collected from heel, finger or ear prick could provide a reliable, specific and sensitive test for economic large scale seroepidemiological studies. The stability of the samples on filter papers allows them to be collected without the need for refrigeration and tested centrally in laboratories with the appropriate equipment.

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