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Performance Certification of Gelatin Particle Agglutination Assay for Anti-HTLV-1 Antibody: Inconclusive Positive Results

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In order to test the performance of particle agglutination assay (PA), 800 preselected PA-positive sera at 8 blood centers in the Kyushu area were tested in various assays. Most blood centers should improve their PA technique, since a third of the samples were PA-negative in our hands. A third of our PA high-titer sera were negative in indirect immunofluorescence and enzyme immunoassay, the results of which were consistent with each other. Western blots did not detect every positive serum. PA inhibition positivity was not consistent with PA titer. Most IgM antibody-positive sera also contained IgG antibody. PA should be used in combination with other methods before notifying the results to positive testees.

Key words: Human T-cell leukemia virus — HTLV infection — Immunoassay — Viral proteins

Human T-lymphotropic virus type-1 (HTLV-1) carriers are at risk for adult T-cell leukemia (ATL),[1,2] HTLV-1 associated myelopathy,[3,4] and some other clinical entities.[5–8] Kyushu, the southwesternmost area of Japan, is one of the most strongly endemic foci of HTLV-1 in the world,[2] and has 2–5% HTLV-1 carriers among the adult population.[9,10] The carriers are diagnosed on the basis of anti-HTLV-1, and the screening of seropositive carriers is essential to stop blood-[9,11] and milk-borne[10,12,13] infections.

Most of the essential evidence indicating the association of HTLV-1 carriers and ATL was obtained by the indirect immunofluorescence (IF) method.[1,2,9,10] Other methods were adopted afterwards for the sake of simplicity, higher sensitivity, and/or capability to analyze antibodies directed against each viral protein, including a gelatin particle agglutination (PA),[14] an enzyme linked immunoassay (EIA),[15] western blotting (WB), PA-inhibition test (PAI),[16] etc. PA (Fuji-Rebio, Tokyo) is widely used for screening of HTLV-1 carriers, especially at blood banks in Japan. However, discrepancy of results with other assays has been noted. Yano *et al.* reported that as many as 76% of PA(+) were IF(−) and EIA(−), although 78% of the discrepant cases were PAI(+).[16] Kamihira *et al.* reported that the discrepancy between PA and EIA was more prominent in low-titered sera in PA, which could be explained in part by the presence of IgM antibodies using EIA and WB.[17]

We tested samples from blood donors in Kyushu comparatively with PA, IF, EIA (Eisai, Tokyo), PAI and WB. The result suggested that PA is usable as a primary screening of blood donors, but a PA-positive result requires confirmation before notification of the result to donors. None of the assay systems we tested was perfect by itself. At present, a combination of at least 2 assays seems to be necessary for reports.

Each of 8 blood centers in Kyushu was requested to supply 100 blood samples with low anti-HTLV-1 titers at an end-point dilution of 1/16 to 1/64 in PA [PA-(low+)]. These samples were selected from $5–10 \times 10^4$ blood donors. All of these had been rejected for use in blood transfusions. At blood centers the PA has been modified for automated processors, and positive sera have been retested by the standard manual PA. We repeated the PA as indicated by the supplier.[14] IF was performed as described previously using MT-1 cells as targets.[1] EIA was performed as indicated by the supplier.[15] WB slips were obtained from Fuji-Rebio, Tokyo and we followed the method recommended by the supplier using the biotin, avidin and peroxidase method.

Table I shows the results of PA at each blood center and our laboratory. The blood centers C and G were good for selecting positive bloods with PA(low+), since less than 5% of their PA(low+) sera were PA(−) in our test. However, 57 and 80% of PA(low+) samples from the blood centers E and F, respectively, and 21 to 39% of PA(low+) samples from other centers were PA(−) in our test. In total, a third of the PA(low+) sera selected

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Table I. Correlation of Gelatin Particle Agglutination Assays at Blood Centers and Our Laboratory on Blood Samples Collected by 8 Blood Centers in Kyushu

Blood centers	PA test at blood center		Our PA test			Our negative among blood center's low+
	low+[a]	high+[a]	-ve	low+	high+	
	(No. samples)		(No. samples)			(%)
A	100	0	34	62	4	34
B	95	5	29	68	3	31
C	100	0	4	83	13	4
D	58	42	12	45	43	21
E	94	6	54	39	7	57
F	100	0	78	21	1	78
G	100	0	2	85	13	2
H	100	0	39	56	5	39
Total	747	53	252	459	89	34

a) The PA(low+) sera in this report represent those with PA titers of 1/16 to 1/64, and the PA(high+) sera with 1/128 or over.

Table II. Correlation of PA Titers with IF and EIA

PA titers	No. samples tested	IF(−) EIA(−)	IF(−) EIA(+)	IF(+) EIA(−)	IF(+) EIA(+)
		(per cent)			
Negative	252	100	0	0	0
1/16	200	95	1.0	1.0	3.0
1/32	208	93.7	0.5	0	5.8
1/64	51	78.4	0	2.0	19.6
Subtotal low+[a]	459	92.6	0.4	0.9	6.1
High+[a]	89	34.8	0	1.1	64.1
Total	800	88.5	0.4	0.5	10.6

a) The PA(low+) sera represent those with PA titers of 1/16 to 1/64, and the PA(high+) sera with 1/128 or over.

by the blood centers were found by us to be PA(−). Most of these blood centers should improve their performance in PA. The results suggest that as much as 0.5% of total donated blood is discarded owing to inaccurate procedures or readings, and that blood center statistics will not give reliable data for estimation of prevalence of HTLV-1 carriers in Japan. However, since the current system in blood centers does not inform donors of the results of anti-HTLV-1 test, a confirmation test at the blood center level might not be economically viable if each blood center is processing less than 10 PA(+) units per day.

We tested these sera in IF and EIA to analyze the specificity of assays (Table II). The IF(−)EIA(+) and IF(+)EIA(−) sera were 0.4 and 0.9%, respectively,

Table III. Correlation of PAI with PA in IF(−)EIA(−)

PA titers	No. samples tested	PAI(−)	PAI(?)	PAI(+)
		(per cent)		
1/16	190	27.3	3.2	69.4
1/32	195	24.1	4.1	71.8
1/64	41	22.0	0	78.0
Subtotal low+	426	25.4	3.3	71.4
High+	31	6.5	3.2	90.3
Total	457	24.0	3.3	72.6

indicating that the results of IF and EIA were reasonably consistent each other even with the PA(low+) sera. No PA(−) serum was either IF(+) or EIA(+). The fraction of IF(+)EIA(+) sera increased with increasing PA titers: 3, 6, and 20% of sera were IF(+)EIA(+) at PA titers of 1/16, 1/32 and 1/64, respectively. PA(high+) sera were 35% IF(−)EIA(−). These indicated that IF and EIA are basically reliable for screening of seropositive donors, since every IF(+) or EIA(+) sample was picked up. The cut-off of PA at 1/16 is still valid, since 3% of sera with the PA titer of 1/16 were IF(+)EIA(+). On the other hand, there were significant numbers of IF(−)EIA(−) samples among PA(+) ones; 90% of PA(low+) and 35% of PA(high+), respectively. Although these PA(high+) are prescreened, a third of PA(high+) in the child-bearing female population was also IF(−) (SH, unpublished).

Yano *et al.*[16] reported that PAI with crude cell extracts of HTLV-1 producer and non-producer cells is specific to anti-HTLV-1. We tested PA(+) sera in the PAI (Table III). The results indicated that more than 70% of PA(+)IF(−)EIA(−) sera were PAI(+), which was consistent with the report of Yano *et al.* However, the PAI(+) ratio among PAI(+)IF(−)EIA(−) sera was not significantly correlated with PA titers, chi square = $8.49 < 12.59$ ($P=0.05$, $f=6$). Furthermore, PAI(+) sera did not tend to be WB(+) (data not shown). Our results did not support the idea that PAI activity in IF(−)EIA(−) sera is due to the lower sensitivity of IF and EIA; it is probably related with reactivity unrelated to HTLV-1, as indicated by the fact that many of PA(+) are IF(−)EIA(−).

PAI uses crude cell extract as the competitor. Any competition assay should include a pure component, at least in one competitor, antigen or antibody, but this is not the case in PAI. Even "purified" HTLV-1 preparation used for coating PA particles usually contains a significant amount of contaminants including cellular components. In this context, PAI may detect many other reactants.

Table IV. Correlation of WB with PA, IF, EIA and PAI

PA titers	Results of IF/EIA/PAI	No. tested	WB at criteria[a] A	B	C	D
			(per cent)			
Total PA(+)	+/+	15	60	80	87	40
1/16–1/64	+/+	8	38	75	100	13
	+/−	4	0	25	75	0
	−/−/+	50	8	28	86	2
	−/−/−	19	5	53	79	5
	−/−(subtotal)	69	7	35	84	3
Negative	−/−	19	0	16	79	0

a) Criteria for positivity were the presence of (A) all of the p19, p24 and p28 bands, (B) 2 of the 3 bands, (C) one of the 3 bands, and (D) p53 and one of the 3 bands.

Table V. Frequency of IgM Antibody-positive Cases

PA titers	IF/EIA /PAI	IF[IgM] (No.(+)/tested)	WB[IgM] at criterion A[a]	B	C	D	WB[IgG] at A (No.(+)/tested)
			(No.(+) cases)				
Total PA(+)	+/+	8/51	1(1)[b]	1	1	1(1)	1/4
1/16–1/64	+/+	0/7	0	0	0	0	1/3
	−/−/+	0/95	3(3)	25	43	2(2)	5/60
	−/−/−	1(1)/26	1(1)	8	17	3(2)	2/28
Negative	−/−	1/14	0	10	14	0	0/20

a) Criteria to diagnose positives in WB, see the footnote to Table IV.
b) No. of positive cases (No. of cases also positive in WB[IgG] assay at criterion A, if tested).

Although WB is popular in serodiagnosis of anti-HTLV-1, the criterion to take WB positive is still controversial. We examined 4 criteria, the presence of (A) all of p19, p24 and p28 bands, (B) 2 of the 3 bands (irrespective of combination), (C) one of the 3 bands, and (D) p53 and one of the 3 bands (Table IV). The rates of WB(+) among PA(+)IF(+)EIA(+) sera were 87, 80, 60, and 40%, and those among PA(−)IF(−)EIA(−) sera were 79, 16, 0 and 0%, by criteria C, B, A and D, respectively. We prefer the criterion A, the simultaneous detection of p19, p24 and p28, because it fits best with the results of IF and EIA. The criterion A picked up 7% of PA(+)IF(−)EIA(−) sera. WB picked up less than 10% of PA(+)IF(−)EIA(−) sera as positive, but on the other hand, WB even with the criterion C did not detect all IF(+)EIA(+) sera. Thus, WB alone is not satisfactory for secreening.

PA detects IgM antibodies as well as IgG antibodies, and this may account for the discrepant results obtained by IF and EIA.[14, 16, 17] Careful follow-up of patients after blood transfusions revealed a phase of IgM(+)IgG(−).[9, 14] However, if most HTLV-1 infections take place early in life,[10, 12, 13] most adults carriers should have been infected many years ago, so that IgM(+) cases should also be IgG(+), i.e IgM(+)IgG(−) cases should be minor.

We tested IgM antibodies in IF and WB with anti-human μ-chain as the second antibody (Table V). There were 8 IF[IgM](+) cases, but each case was also IF-[IgG](+)EIA(+). A case of PA(low+)IF[IgG](−)-EIA(−) was IF[IgM](+) but it was also positive for p19, p24, p28 and p53 in both WB[IgG] and WB[IgM]. A PA(−)IF[IgG](−)EIA(−) case was IF[IgM](+), which might have been a false-positive since it showed only 2 bands in each of WB[IgG] and WB[IgM].

In the case of WB[IgM], the criterion for WB[IgM]-(+) is again an important question. We employed the same 4 criteria as for WB[IgG]. Since it is hard to believe that as much as a half of PA(−)IF(−)EIA(−) sera contain specific IgM antibodies, the criterion A seems to be a suitable choice. All of the WB[IgM](+) sera, 5/5, were WB[IgG](+) even if some of them were IF(−)EIA(−). Moreover, WB[IgM](+) sera amounted to 8% of PA(+)IF(−)EIA(−) sera tested,

which hardly explains the discrepancy between PA and IF/EIA. Our data are consistent with the conjecture that most cases with IgM antibodies are likely to show IgG antibody as well in the respective assays. Thus, the contribution of IgM(+)IgG(−) sera should be minor in normal healthy populations, and it is not likely to be the cause of major discrepancies between PA, IF and EIA.

There still is a possibility that PA is more sensitive than IF and EIA as suggested by the minor fraction of PA(+)IF(−)EIA(−) sera that were WB(+). We must read WB carefully, since a less stringent standard will pick as many as 79% of IF(−)EIA(−) sera. We believe that most PA(+)IF(−)EIA(−) sera are false-positives, since an early study using IF for screening virtually stopped blood-borne transmissions.[9] PA should be used in combination with other methods for confirmation (PIA is not a good candidate for this purpose).

Not all laboratories may have this degree of discrepancy, since minor changes in conditions or techniques as well as in reading standard may change the profile. However, our data are basically consistent with previous reports by others.[16, 17] This type of work would be better supported if we use blood samples defined by a specific and sensitive method, such as polymerase chain reaction (PCR), to detect true carriers. However, even theoretically very sensitive PCR may not detect every seropositive carrier.[18] Although we could not define a method to select true carriers, we hope our data will provide useful background information for scientists and doctors working on identifying HTLV-1 carriers.

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