Relevance of molecular tests for HTLV-1 infection as confirmatory tests after the first sero-screening

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Abstract

The diagnosis of human T-cell leukemia virus type-1 (HTLV-1) infection has been widely examined by serologics. In the first screening tests, serological false negative and positive samples have been reduced thanks to advances in assay techniques that apply new emission agents and sensors. On the other hand, western blot (WB) remains problematic. For example, WB analysis yields many samples equivalent to antibody positive ones. To reduce the need for WB, an alternative testing strategy is required to detect HTLV-1 infection. Polymerase chain reaction (PCR) for the HTLV-1 provirus has recently been recommended for a final diagnosis of infection. However, although PCR is thought to be one element, the validation of detection performance for HTLV-1 infection between serological and molecular testing is not always clear. Thus, this study aimed to evaluate the accuracy and test the validity of an improved methodology for serological detection of HTLV-infection, as well as that of PCR. In conclusion, the high values of kappa-statistics are expected to deliver high quality in chemiluminescent enzyme immunoassay (or chemiluminescent immunoassay), while the problems with WB assays remain to be elucidated. As an alternative to WB, a combination of real-time qPCR and nested PCR is proposed as a suitable confirmatory test.

Introduction

Recently, the diagnosis of human T-cell leukemia virus type-1 (HTLV-1) infection has been widely examined by serological tests[1-7]. Screening of serum by particle agglutinations (PA) and chemi-luminescent immune assays (Chemiluminescent Enzyme Immunoassay: CLEIA and chemiluminescent immunoassay: CLIA) and confirmation by western blot (WB) is a common testing strategy[8,9]. However, when this strategy is applied to low infection prevalence populations, false positive samples may increase markedly, because the first screening tests are usually highly sensitive and low specific. Furthermore, WB as a confirmatory test is known to yield many indeterminate results. In the first screening tests, serological false negative and positive samples have been reduced thanks to advances in assay techniques applying new emission agents and sensors. On the other hand, WB remains problematic. For example, WB analysis yields many samples equivalent to antibody positive ones. To reduce the need for WB, an alternative testing strategy is required to detect HTLV-1 infection.

Polymerase chain reaction (PCR) for the HTLV-1 provirus has recently been recommended for a final diagnosis of infection[10]. However, although PCR is thought to be one element, the validation of detection performance for HTLV-1 infection between serological and molecular testing is not always clear. Thus, this study aimed to evaluate the accuracy and test the validity of an improved methodology for serological detection of HTLV-infection, as well as that of PCR.

Material and Methods

Materials

A total of 105 pregnant blood samples collected from January 2011 to December 2011

were used. During this period, 9,718 samples were tested for screening. 105 samples excluding double negative in PA and CLEIA samples were used as the secondary samples, and were examined for infection by 4 methods as described below. Meanwhile, using 25 practical blood samples collected from hospitalized patients in complete remission from adult T-cell leukemia (ATL), the role of PCR in serological detection of HTLV-1 infection was investigated.

Next, we examined low titer samples or sero-negative converted samples selected from 350 ATL patients who underwent bone marrow transplantation (BMT) and/or chemotherapy.

Methods

Serological detection of HTLV-1 infection was done using commercially available assay kits according to the manufacturer's instructions, with Serodia-HTLV-I (PA; Fuji-Rebio, Tokyo), Lumipulse-HTLV-I (CLEIA; Fuji-Rebio, Tokyo, Japan), Architect-HTLV (CLIA; Abbott, Illinois, USA) and problot-HTLV-I (WB; Fuji-Rebio, Tokyo, Japan). The positivity of WB analysis was decided according to the WHO criteria[9].

HTLV-1 proviral load (PVL):

After separation of peripheral blood mononuclear cells (PBMC) in the Conray manner, genomic DNA was extracted using Qiagen kits (Qiagen, Crawley, UK. Quantitative PCR (qPCR) and quantitative nested PCR (nPCR) detection for HTLV-1 were performed as described previously[11-13]. Briefly, primers were set in the pX region, and the density of the template was 30ng per reaction. The PVL was normalized using β-globin and

represented as a percentage. The detection density level was in a linear range of ~ 0.5% and the lowest sensitivity was 0.1%. Furthermore, when presenting 0.1% or less, we conducted nPCR [14]and sub-classified these samples into two types of qPCR (-) and nPCR (+), and both were negative in un-infected cases.

Statistics:

To evaluate the test and diagnostic performance for the qualitative detection of anti-HTLV-1, Cohen's kappa-statistics were used. This is a more robust measure than a simple % agreement calculation.

Results

1. Various results with the 4 methods

The positive rates by PA, CLEIA, CLIA and WB were significantly different between the former 3 methods and WB (Table 1) using pregnant blood samples. The cause of the low positive rate in WB was likely high indeterminates (14.3%). The band patterns of WB and serological and molecular status for HTLV-1 positivity are compared in Table 2. The 15 indeterminate samples consisted of 6 with no only gp46 band pattern (group A) and 9 with no gp46 and gag band (p53, p24 or p19) pattern (group B). Samples of Group A were seropositive in all but case no 21. In addition, All 3 samples (case no. of 62, 60 and 100) available for molecular testing showed either a qPCR or nPCR positive reaction (Figure 1). When no amplicon was observed, the sample was re-evaluated using an increased dose of template (60ng). The amplicon curve in case 62 of Figure 1A emerged as a consequence of an increased template dose. These findings indicate that most samples with indeterminate WB patterns contain a very small number of

HTLV-1 provirus-integrated cells (Table 2). Thus, the condition of gp46 positivity in the WHO criteria may not be ideal.

The value of kappa-statistics concerning the validation of test performance in a combination of the two methods is summarized in Table 3. The two chemi-luminescent immune assays (CLEIA and CLIA) kits were evaluated to be excellent.

2. The role of PCR in the serological diagnostic strategy of HTLV-1 infection

About half the cases with sero-indeterminate WB patterns had negative or low titers in other assays. Accordingly, we examined whether the HTLV-1 provirus could be detected in low titer samples or sero-negative converted samples selected from 350 ATL patients who underwent bone marrow transplantation (BMT) and/or chemotherapy. As shown in Table 4, PVL was 0 to 0.5% in 25 of the 350 samples, consisting of 14 sero and molecular positive samples (group Ⅰ), 7 negative qPCR samples with or without sero-positivity (group II), and 4 sero-negative and qPCR(-) ·nPCR(+) or (-) samples (group III). This means that serological detection of HTLV-1 infection is better than qPCR for blood samples with a PVL of less than around 0.4%. However, if the detection sensitivity of qPCR was supplemented with nPCR, such molecular methods gave high detection rates. No sample was nPCR(-) and sero-positive. The value of kappa-statistics in the combination of qPCR and CLEIA was 0.545 and the combination of qPCR or nPCR and CLEIA was 0.744, respectively. These findings indicate we should use qPCR or nPCR and CLEIA when we test unnatural samples collected in the complete remission state after BMT and/or chemotherapy in patients with ATL. The discrepancy between the provirus by qPCR and anti-body by CLEIA seems to be related

to the detection sensitivity.

Discussion

Since the discovery that HTLV-1 is causative for ATL as well as HAM/TSP[15,16], about 30 years have passed. However, the number of HTLV-1 carriers and annual morbidity number of ATL patients are about 1 million and about 1,000 respectively in Japan[17]. Furthermore, there has been little advance in the treatment for ATL. If people are not infected with HTLV-1, no ATL develops, indicating that prevention of HTLV-1 transmission is important. The natural transmission route is known mainly to be mother to child via breast-feeding[18-21]. The preventive effect of refraining from breast-milk has been demonstrated through the ATL Prevention Program in Nagasaki (APP), which started in 1986 [18,22]. On the other hand, a nationwide prevention program similar to APP only started in Japan from 2011, including non-endemic areas for HTLV-1. The low prevalence of infection leads to a greater frequency of indeterminate or false positive results. In general, PA and CLEIA(or CLIA) methods are applied as the screening test. If HTLV-1 is positive, the result is confirmed by WB, as a confirmatory test. So the final indeterminate judgment may be undesirable for mental health of the mother. In fact, this strategy yields a high rate of inconclusive or equivalent results. Accordingly, it is now controversial as to whether or not real-time qPCR can be substituted for WB.

The present study revealed that although the positive rates of PA, CLEIA and CLIA were equally high, the values of kappa-statistics gave a more robust evaluation in terms of test performance. Both CLEIA and CLIA showed high proportional agreement, while

WB was the worst performer; no validity as a confirmatory test was found. The reason for this appears to result be presence of many sero-indeterminates (14.3%) in endemic erea(Nagasaki). Additionally, the present study indicated that 60 to 70% of WB indeterminates were probably positive, in particular in cases with no gp46 band. Taken together, the CLEIA (and/or CLIA) is probably suitable for the first screening, but there seems to be no appropriate serological test as a confirmatory test.

Moreover, when serological assays yield discrepant results, no one knows which result is correct. From biological and confirmatory points of view, the genetic approach is promising and attractive. Thus, we compared test and diagnostic performance of PCR and serological assays. Although the benefits of real-time qPCR are well understood, as summarized schematically in Figure 2, the detection limit of 0.01% is a disadvantage of this technology. However, since a combination with nPCR became sensitive at 0.001%, nPCR(-) may be thought of as un-infected. Currently, the pathological significance in such a very low number of infected cells remains to be elucidated. In general, the limit of detection of qPCR is thought to be 0.01 to 0.5%[13,23-27], the same as with our method. If we convert 0.01% of PVL into total infected cells per body, a total of $3 \times 10^{-4\sim-5}$ infected cells exist within the whole body, indicating that such an infected cell burden seems to be natural in the production of anti-HTLV-1 antibodies. Since even some samples with qPCR(-) and nPCR(+) were positive for the antibody, real-time qPCR may yield seropositive individuals carrying a small number of infected cells detected by only nPCR. This suggests that a combination of qPCR and nPCR is a better algorithm as a confirmatory test.

In conclusion, the high values of kappa-statistics are expected to deliver high quality in CLEIA (or CLIA), while the problems with WB assays remain to be

142 elucidated. As an alternative to WB, a combination of real-time qPCR and nested
143 PCR is proposed as a suitable confirmatory test.

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148

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242 Figure Legends

243 Figure1.

244 Amplicon curve in Real-time qPCR in 3 samples with indeterminate WB pattern.

245 (A) The amplicon curve incompletely elevated out of the dynamic range using 100 ng

246 templates.

247 (B) Western blot pattern of 3 sampled and that were not observed gp 46 band.

248 PC* means weak positive control.

249 In order to determine sero-positive, both gp46 and one or more gag band(p53, p24 or

250 p19) were positive.

251 (C) The results of triplicate nested PCR in case no. 60 were showed 3 images in left panel.

252 PCR was two times positive amplification reaction detecting agarose gel analysis.

253 PC means positive control and NC means negative control. PC have 2 different

254 concentrations 30 pg and 3 pg.

255

256 Figure 2.

257 The relationship between the HTLV-1 molecular proviral detection status by PCR

258 methodology and the HTLV-1 sero-positive status measured with the

259 CLEIA method.

260

261 Table 1 .

262 The HTLV-1 serological results of 105 pregnant sera by each method.

263 The positive rate was significantly lower in WB compared to other 3 methods (<.05) with

264 high indeterminates in 15 sera.

265

Table 2.

Comparison of results between 3sets of serological data for the CLEIA, CLIA and PA) and 19 cases showing 4 negative and 15 indeterminate WB. The cut of value for CLEIA and CLIA is 1.0.

qPCR and nested PCR gave a positive amplicon in all cases tested.

Table 3 .

The proportional agreement and kappa-statistic between the two methods.

The values of kappa-statistics were evaluated to be poor in less than 0.4, moderate in 0.4-0.59, good in 0.6 – 0.79, and excellent in 0.89 – 1.0.

Table 4.

Comparison of the serological and proviral status in hospitalized patients with ATL treated by either BMT or intensive chemotherapy.

The cut off value of CLEIA is 1.0. (+): positive, but no number data.

NT means nottested.1.0< means over 1.0 and below 1.2.

Figure 1 .

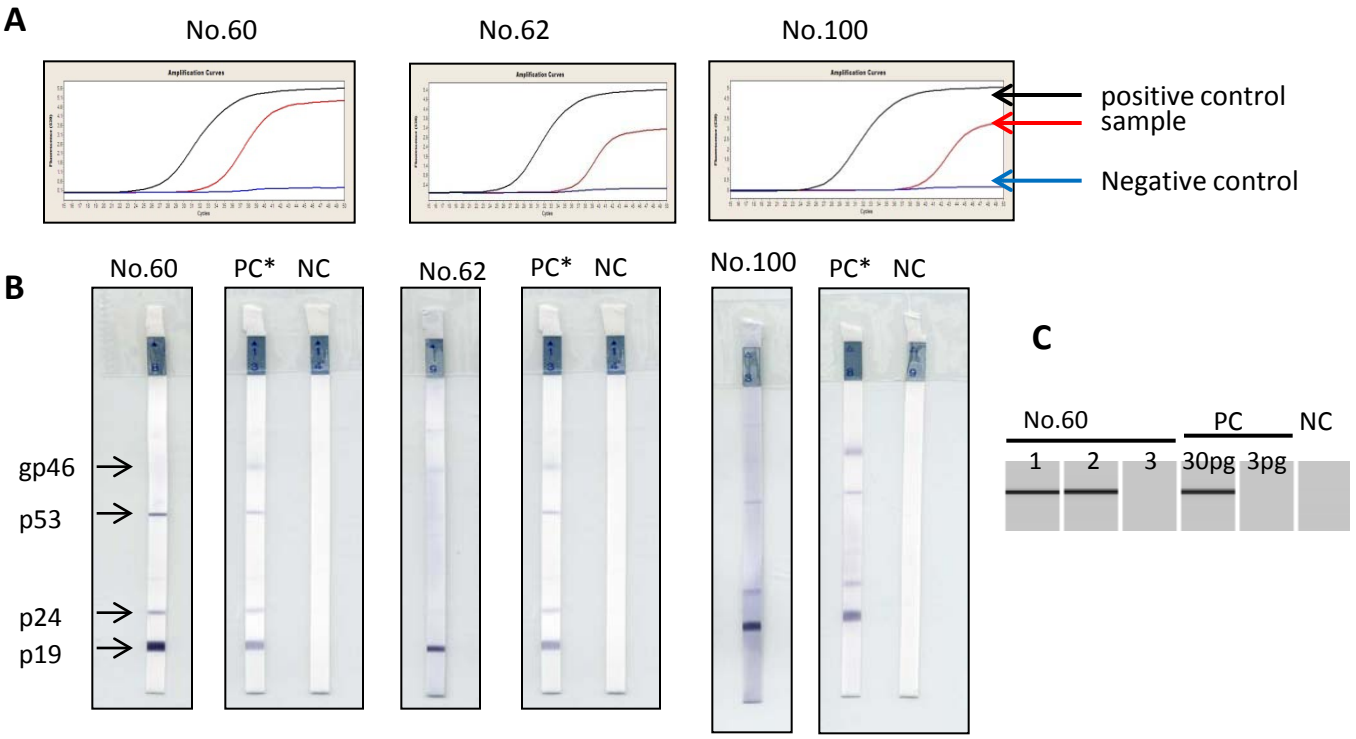
A
No.60
No.62
No.100
positive control
sample
Negative control
B
No.60
PC*
NC
No.62
PC*
NC
No.100
PC*
NC
gp46
p53
p24
p19
C
No.60
PC
NC
1
2
3
30pg
3pg

Figure 2

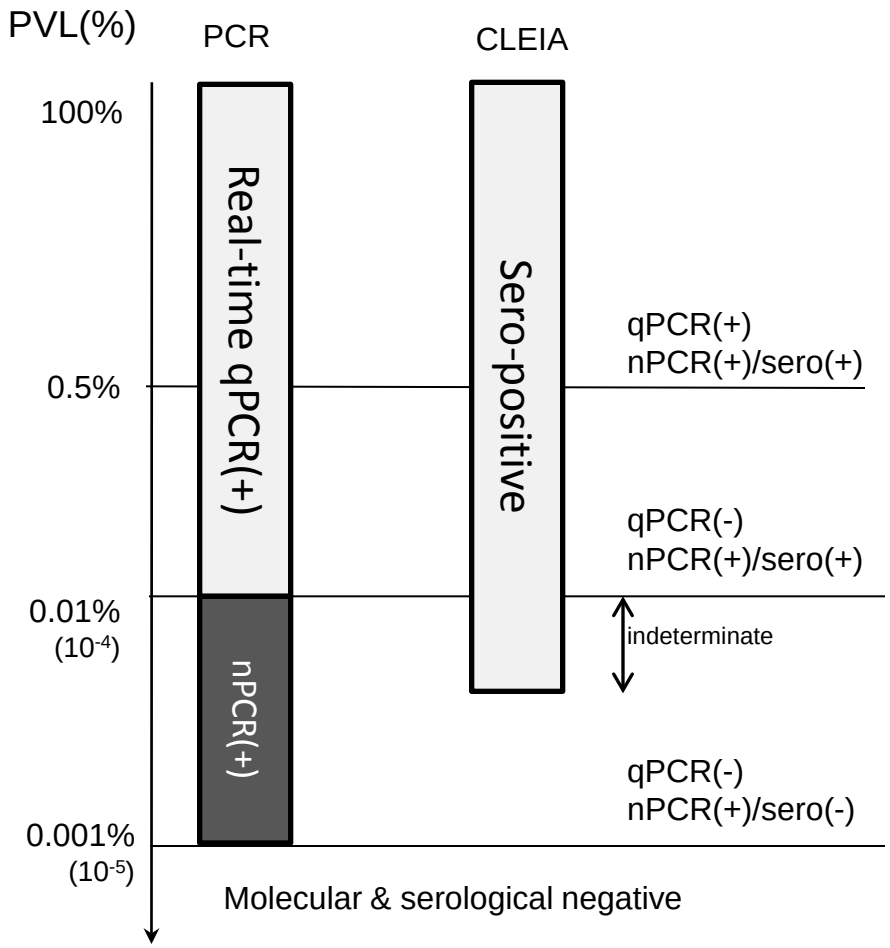
PVL(%)
PCR
CLEIA
100%
0.5%
0.01%
(10-4)
0.001%
(10-5)
Real-time qPCR(+)
nPCR(+)
Sero-positive
qPCR(+)
nPCR(+)/sero(+)
qPCR(-)
nPCR(+)/sero(+)
indeterminate
qPCR(-)
nPCR(+)/sero(-)
Molecular & serological negative

Table 1 .

	PA	**CLEIA**	**CLIA**	**WB（WHO)**
Positive	99(94.3%)	98(93.3%)	97(92.4%)	86(81.9%)
Indeterminate	0	0	0	15(14.3%)
Negative	6(5.7%)	7(6.7%)	8(7.6%)	4(3.89%)

Table 2.

	Case No.	CLEIA	CLIA	PA	WB results	band pattern				molecular test	
						env	gag			qPCR	nPCR
						gp46	p53	p24	P19		
group A	21	0.5	0.12	+	Indeterminate	-	+	+	+		
	60	25.9	44.15	+	Indeterminate	-	+	+	+	0.01%(+)	(+)
	50	14.3	90.7	+	Indeterminate	-	+	+	+		
	35	2.4	3.98	+	Indeterminate	-	+	+	+		
	100	8.3	53.12	+	Indeterminate	-	+	+	+	0.02%(+)	(+)
	3	34	90.4	+	Indeterminate	-	+	+	+		
group B	92	0.2	0.14	-	Indeterminate	-	-	+	-		
	64	0.1	0.27	-	Indeterminate	-	-	+	-		
	18	6.3	53.44	+	Indeterminate	-	-	+	-		
	58	0.1	0.13	-	Indeterminate	-	-	-	+		
	32	1.7	1.54	+	Indeterminate	-	-	-	+		
	65	1.2	7.55	-	Indeterminate	-	-	-	+		
	62	14.7	40.17	+	Indeterminate	-	-	-	+	(+)	(+)
	15	5.5	4.95	+	Indeterminate	-	+	-	+		
	33	8.7	24.45	+	Indeterminate	-	+	-	+		
group C	29	3.3	0.1	-	-	-	-	-	-		
	67	0.1	0.12	-	-	-	-	-	-		
	94	0.2	0.12	-	-	-	-	-	-		
	40	0.1	0.49	+	-	-	-	-	-		

Table 3 .

			proportion of agreement	kappa-statistics
PA	-	WB	0.88	0.488
CLEIA	-	WB	0.87	0.428
CLIA	-	WB	0.89	0.542
PA	-	CLEIA	0.96	0.693
PA	-	CLIA	0.96	0.846
CLEIA	-	CLIA	0.99	0.928

Table 4.

		Case No	PVL (%)	nestedPCR	amplified site of standard curve	CLEIA
group Ⅰ	qPCR (+)	1	0.50%	NT	border	1.3
		2	0.50%	NT	out of linear R	14.9
		3	0.50%	NT	out of linear R	10.5
		4	0.50%	NT	out of linear R	2.4
		5	0.40%	NT	out of linear R	38.1
		6	0.40%	NT	out of linear R	38.1
		7	0.40%	NT	out of linear R	1.0<
		8	0.40%	NT	out of linear R	1.0<
		9	0.40%	NT	out of linear R	2.4
		10	0.40%	NT	out of linear R	1.0<
		11	0.40%	NT	out of linear R	1.0<
		12	0.40%	NT	out of linear R	2.4
		13	0.40%	NT	out of linear R	39.5
		14	0.40%	NT	out of linear R	14.3
group Ⅱ	qPCR (-) nPCR (+)	15	0.00%	nPCR(+)	no amplicon curve	1.2
		16	0.00%	nPCR(+)	no amplicon curve	5.4
		17	0.00%	nPCR(+)	no amplicon curve	6.3
		18	0.00%	nPCR(+)	no amplicon curve	9.8
		19	0.00%	nPCR(+)	no amplicon curve	18.9
		20	0.00%	nPCR(+)	no amplicon curve	0.1
		21	0.00%	nPCR(+)	no amplicon curve	0.9
group Ⅲ	qPCR (-) nPCR (-)	22	0.00%	nPCR(-)	no amplicon curve	0.5
		23	0.00%	nPCR(-)	no amplicon curve	0.8
		24	0.00%	nPCR(-)	no amplicon curve	0.8
		25	0.00%	nPCR(-)	no amplicon curve	0.2