Human T-lymphotropic virus type 1 (HTLV-1) prevalence and quantitative detection of DNA proviral load in a group of individuals originating from endemic areas and living in Italy.

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Running title: HTLV-1 in individuals originating from endemic areas.
Abstract

**Background:** HTLV-1 infection is currently restricted to endemic areas. To define the prevalence of HTLV-1 infection in patients living in Italy, we carried out a retrospective analysis in a group of people originating from African countries referred to our hospital from January 2003 to February 2005.

**Methods:** all the sera were firstly analysed by ELISA and Western Blotting and peripheral blood mononuclear cells from subjects with positive or inconclusive serological results were analyzed for the presence of proviral DNA by a novel SYBR real time PCR.

**Results:** Serological results disclosed serum reactivity by ELISA (absorbance values equal or greater than the cut-off value) in 9 out of 3408 individuals attending the Sexually Transmitted Diseases Clinic and/or Oncology Department, and 2 out 534 blood donors enrolled as a control population. Irrespective of positive or inconclusive serological results, all these subjects were analyzed for the presence of proviral DNA in peripheral blood mononuclear cells by SYBR real time PCR. A clear-cut positive result for the presence of HTLV-1 DNA was obtained in two subjects from endemic areas.

**Conclusions:** The assay is rapid and inexpensive, with excellent linear dynamic range, specificity and reproducibility readily revealing and quantifying the presence of virus in PBMCs. Our results highlight the need to monitor the presence of HTLV-1 also in countries, which have seen a large influx of immigrants in recent few years. Epidemiological surveillance and correct diagnosis are recommended to verify the prevalence and incidence of a new undesirable phenomenon.

Key words: HTLV-1 antibody, virus quantification, immigrants, Italy.
Background

HTLV-1 (Human T-cell lymphotropic virus type 1) is etiologically linked with adult T-cell leukemia (ATL) [1,2,3]. HTLV-I infection is geographically confined in specific areas such as Japan, the Caribbean basin, South America, Sub-Saharan Africa, Melanesia and the Middle East [4]. Japanese area-related studies estimated about one million people are currently infected by HTLV-I with 1-5% of infected patients showing developing ATL [5]. Therefore, the majority of HTLV-I infected subjects remain asymptomatic throughout their lives even though few individuals showed a chronic inflammatory neurological disease represented by HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1,3,6,7]. The relative percentage of malignant lymphoid proliferation varies widely depending on geographical location [4]. Although peripheral T cell lymphoma is relatively uncommon in the Caucasian population, the transmission routes (such as sexual intercourse, blood transfusion, tissue transplantation and prolonged breastfeeding) [8,9,10,11] and the increasing number of individuals emigrated from endemic areas suggest blood and tissue donors should be screened to reduce the spread of infection [Re 12,13,14]. However the epidemiology of HTLV-1 infection could change in the near future [15] in the wake of immigration. European countries, Italy in particular, represent the main destination for immigrants from Middle East and Africa, making epidemiological surveillance highly recommended to ascertain the prevalence and incidence of HTLV-1 infection [13,16,17]. In the recent years, a number of countries, including USA, Canada and France, have introduced screening for blood donors to avoid a possible spread of HTLV-1 infection by blood transfusion [18].

To date, blood screening for HTLV-I has not been mandatory in Italy, but a more careful screening of the population might be justified by several literature reports [19,20,21]. Screening tests are usually based on antibody detection by ELISA and western blot, even though the relatively large number of indeterminate results needs to be confirmed by highly sensitive molecular techniques [14,22]. In addition, to establish the presence of the genome and its
modulation over time and/or in the presence of specific therapy, PCR methods (commercially available and in-house modified tests) represent the gold standard useful to give results a high level of specificity and reproducibility, in short time [14,22,23,24].

Considering the need to update information on HTLV-1 incidence in Italy, we investigated the presence of HTLV-1 infection in a selected group of patients originating from endemic areas using serological methods and a novel real time PCR technique able to verify and quantify the HTLV-1 proviral load.
METHODS

Patients: We enrolled in the study a group of HIV-1/2 negative 3408 recent immigrants from African countries referred to the Sexually Transmitted Diseases Clinic and/or Oncology Department (group 1) and a group of 534 blood donors (group 2) undergoing laboratory analysis for serological diagnosis of other infectious diseases, from January 2003 to February 2005. Recent immigrants were defined as people stating they had lived in Italy for less than five years at the time of HTLV-I serological analysis. All patients, after informed consent, were screened for HTLV-1 antibodies by ELISA assay (Vironostika HTLV-I/II, BioMerieux, Boxtel, The Netherlands) as described by the manufacturer. In addition, all ELISA borderline or positive samples and some randomly selected ELISA negative samples were analyzed by Western-blot technique (Diagnostic Biotechnology HTLV WB, version 2.3, Genelabs Diagnostic, Singapore) following the manufacturers’ procedure.

Cell lines and PBMC DNA extraction and purification: T-lymphoblastoid cell line MT2 (obtained from American Type Culture Collection, Manassas, VA) the most commonly used cell line for HTLV-I production, previously characterized as having 2.1 copies of virus per cell [25] and Jurkat T lymphoblastoid cell line were used as HTLV-1 infected cells and control cells respectively. MT2 and Jurkat cells were kept in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% of foetal calf serum (FCS; Gibco). Peripheral blood mononuclear cells (PBMCs) from HTLV-1 negative blood donors and patients with indeterminate or positive immunoenzymatic assay were isolated from whole blood by Ficoll-Paque gradient separation (Amersham Pharmacia). As previously described [26], DNA was extracted and purified from MT2, Jurkat cells and PBMCs by DNAeasy tissue kit (Qiagen) following the manufacturer’s instructions. The DNA content of each sample was determined by spectrophotometric analysis at 260/280 nm and stored at -80 °C until use. PBMC, MT2 and Jurkat cell pellets, corresponding to $5 \times 10^6$, were prepared and stored at –80 °C until use.
Genomic DNA concentration and purity were determined by spectrophotometric analysis at 260/280 nm.

**Determination of HTLV-I proviral DNA by SYBR green real time PCR:** SYBR green real time PCR assay was performed in 20 µl PCR mixture volume consisting in 2X Quantitect SYBR green PCR Master Mix (Quiagen) containing HotStarTaq DNA polymerase, 0.5 µl of 25nM of each oligonucleotide primer and 5 µl of DNA extracted from scalar dilution of MT2 cell line (from $10^5$ to 1 copies ) and clinical samples.

HTLV-1 pol gene amplification was carried out as follows: one cycle of 15 min at 95°C (hot-start PCR) and 45 cycles in four step each (95°C for 5s, 60 °C for 30 s, 72 °C for 30 s, 76 °C for 5 s). At the end of amplification cycles, melting temperature was analyzed by a slow increase in temperature (0.1 °C/s) up 95 °C. The property elicited an accurate analysis of the melting temperature curve of the amplified fragments generated by real time PCR to determine the detection and quantification of specific products [27]. Real time target amplification profile demonstrated a specific main peak with a melting temperature at 79,35 °C.

HTLV-I pol primer sequence was: 5’ GTG GTG GAT TTG CCA TCG GGT TTT 3’ and: 5’ GTA CTT TAC TGA CAA ACC CGA CCT AC 3’. The amplification with this pair of oligonucleotides yielded a 117 bp pol fragment. Three replicates were done for each scalar dilution for intra-assay validation whereas four experiments were performed in triplicate for inter-assay analysis. All cell scalar dilutions (MT-2 and PBMCs) were equalized at 600 ng of total DNA by Jurkat cell DNA addition in all experiments. All patients’ samples were also analysed by SYBR Green real-time PCR for globin gene in a parallel run to check the equal amount in all samples determined by spectrophotometric data as described [28]. Electrophoresis agarose gel of PCR amplicons was carried out as previously described [27].
RESULTS

Serological analysis of patients’ sera for specific HTLV-I antibodies: We analyzed the sera from 3408 immigrant African individuals HIV-1/2 negative, attending the Sexually Transmitted Diseases Clinic and/or Oncology Department (Group 1), and 534 blood donors by ELISA assay. Among the patients selected, serum reactivity (absorbance values equal or greater than the cut-off value) was disclosed in 9 out of 3408 patients (0.26%) and 2 out of 534 blood donors (Group 2). Hence, we assayed the reactive serum of these 11 individuals by HTLV-I specific Western-blot. As shown in Table 1, Western Blot analysis performed on these samples showed a clear serum reactivity to rgd21, p19, p24, p32, p36, gp46, p53 and rgp46-I proteins in patient n° 3 and a serum reactivity to rgd21, p19, p24, gp46, p53 and rgp46-I proteins in patient n° 7, both belonging to Group 1. Both samples (n° 3 and n° 7) were classified as positive on the basis of current guidelines [18]. In addition, a serum reactivity to p19 alone or in the presence of p26 or p30 was observed in three (patients n° 2, 6 and 9 of Group 1) out of the eleven and the absence of serum reactivity to any viral proteins in six (1, 4, 5, 8 of Group 1; 10 and 11 of Group 2) out of the eleven (Table 1).

Western-blot assay results demonstrated that only 2 samples out of 11 (18.2%) were confirmed serologically positive for HTLV-1 antibodies whereas 3 out of 11 (27.3%) were indeterminate and 6 out of 11 (54.5%) were negative.

In particular the two positive samples with a clear antibody pattern reacting to all HTLV-1 proteins belonged to patients originating from Nigeria (samples n°3) and Ghana (sample n°7) respectively. Interestingly, none of blood donors tested were Western-blot reactive or indeterminate for HTLV-I.

SYBR Green-based real time PCR analysis of HTLV-I indeterminate and reactive Western-blot patients: We evaluated indeterminate and reactive Western-blot patients by in house SYBR green based Real time PCR technique to determine the presence of HTLV-I proviral genome in the peripheral blood mononuclear cells. This technique was validated on scalar dilutions (from
$10^5$ to $10^7$ HTLV-I genome equivalent copies) of MT2 cell line genome containing 2.1 copies of proviral copies per cell by using an oligonucleotide specific pair able to amplify a 117 bp conserved region of HTLV-I *pol* gene. The assay encompasses at least five orders of magnitude with a high linear relationship ($r^2 > 0.99$) between the Ct values and the cell line input copies. The specificity of amplified products was assessed by melting curve analysis: all specific PCR amplicons showed the same dissociation temperature (79.35 C°).

The sensitivity of SYBR Green real time PCR on MT-2 DNA scalar dilutions displayed a limit of $10^5$ HTLV-I equivalent genome copies (*Table 2*), whereas parallel scalar dilution of Jurkat DNA cell line did not show any positive signal (data not shown). This method shows a good intra- and inter assay reproducibility determined by scalar dilutions of HTLV-I-positive MT-2 cell DNA equivalent genome analysis. In particular, *intra-assay* reproducibility was evaluated using three replicates of each point of scalar dilutions between $10^5$ and $10^7$ HTLV-I genome equivalent copies. The coefficient of variation (CV) of Ct was <3.8 % for all scalar dilutions tested. The CV of copy number was <35% for 10 copies and <25% for all more-concentrated dilutions (from $10^5$ to $10^2$ copy/sample) (*Table 2*). The *inter-assay* reproducibility was obtained by analysis of three different experiments, performed in triplicate, showing a CV for Ct <4.1% for all MT-2 DNA dilutions analysed. The CV of copy number was <40% for 10 copies and <25 % for all more-concentrated dilutions (*Table 2*).

Hence, we applied this sensitive and specific technique on PBMCs DNA from the eleven subjects previously selected for Western-blot indeterminate or positive results. SYBR green based real time PCR technique disclosed the HTLV-I genome in the two Western-blot positive samples whereas the ELISA positive but Western blot indeterminate or negative samples did not display any significant positive fluorescent signal. In particular, the two real time PCR positive samples show a HTLV-I genome copy number of $1.2 \times 10^5$ (n°3 patient) and $5.9 \times 10^2$ (n°7 patient) per $10^6$ PBMC genomes. Electrophoresis agarose gel analysis of amplicons indicated the presence of a specific band at 117 bp (*Figure 1*).
Oncologic clinical investigation of patient n°3 revealed that the positive sample belonged to a 27-year-old Nigeria-born female prostitute living in Italy since 2001. History-taking disclosed ATL disease with diffuse exfoliative dermatitis followed by a rapid deterioration of the patient’s general condition, high white blood cell (WBC) count (total WBCs: 54,070/mm$^3$), a high number of lymphocytes (35,684/mm$^3$), severe hypercalcemia (12.7 mEq/L; normal values: 4.2–5.2 mEq/L) and elevated LDH plasma values (5,630 U/L; normal values: 230-450 U/L). Hematological findings showing a monoclonal T-cell lymphocytosis (95% of peripheral blood lymphocytes were CD3+/CD4+/CD8+/TCR $\alpha/\beta$) confirmed the diagnosis of ATL.

Patient n°7, a 29-year-old Ghana-born female prostitute living in Italy since 2000, monitored by the Sexually Transmitted Diseases Clinic, showed normal white blood cell count and number of lymphocytes, whereas no clinical information was accessible because the patient, when identified as HTLV-1 positive, refused any further control and/or hematological monitoring. Neither patient displayed any serological reactivity for human immunodeficiency virus types 1 and 2, human hepatitis B virus or human hepatitis C virus infection.
DISCUSSION

The increasing rate of immigration towards European countries and the global tourism has determined a new approach by national health committees to control the spread of some infectious diseases previously confined to specific endemic areas. In particular, this new global situation elicits European countries to monitor the local epidemiology of these emerging diseases. In Italy, HTLV-I infection is still sporadic and is confined to immigrants arriving from endemic areas. In particular, sexual transmission, HIV-1 seropositivity and intravenous drug abuse are the preferential infection routes that may lead an increase in HTLV-I infection incidence in the next years. Our study, focused on disclosing the presence of HTLV-1 in Italy, revealed two HTLV-1 infections in a selected group of individuals originating from African countries. The specific presence of infection in Africa-born patients demonstrated that the infection is noticeable in individuals coming from endemic areas whereas no positive cases were found in the in Italy-born patient group. These data are in accordance with several studies on the HTLV-I epidemiology in Italy [15,29,30] even though a high prevalence of HTLV-I infection was more frequent in subjects co-infected with human immunodeficiency virus [16]. Our results disclosed HTLV-1 infection in two HIV-1 negative women, living in Italy from several years. Even though we found a low prevalence (0.058%: two out the 3408 individuals enrolled in our study) of HTLV-1 infection, up-to-date information is necessary in non endemic countries to gain a more knowledge on the spread of this virus.

In our study, both serological techniques used (ELISA and WB) revealed clear-cut positive results in two samples but Western blot did not rule out inconclusive results in three serum samples from high risk individuals. However, the finding of inconclusive results both by ELISA and immunoblotting analysis suggest the need to confirm virus presence by molecular methods. The identification of specific viral sequences in infected cells is essential to confirm serological diagnosis in subjects with positive or indeterminate results [13,18,31]. In addition to
determining the distribution of virus in the organism, amplification techniques also document
the pathogenesis of infection and the effectiveness of antiviral therapy [32,33].

The clinical interest in molecular biology assays for HTLV diagnosis is increasing since proviral
DNA levels represent a measure of integrated genome [34] and a surrogate marker of HTLV-I
viral replication [35,36,37].

Our study also shows a novel SYBR Green based real time PCR committed to HTLV-I provirus
detection and quantitation. We optimised the conditions of SYBR Green real time PCR for
HTLV-I DNA proviral detection with a high level of specificity (no healthy blood donors’
samples showed any detectable fluorescent signal). Moreover, the assay has excellent dynamic
range from $10^5$ to $10^1$ copies with a detection limit established at ten copies: a sensitivity
comparable to other PCR formats for HTLV-I. SYBR Green was chosen instead of different real
time approaches such as TaqMan or beacons to generate fluorescence signals, for several
reasons. In particular, SYBR Green is less expensive than labelled probes that could also
determine PCR artefacts beyond the 30th cycle during the amplification. In addition, probe
selected sequences may be prone to specific mutations [26,27].

Proviral HTLV-I DNA detection in two clinical samples, and the lack of any signal in all the
other samples with inconclusive serological results demonstrated that SYBR green real-time
PCR is a highly sensitive and specific novel approach to detect HTLV-I proviral DNA.
Moreover, the speed, an increased throughput over conventional PCR and the quality
performance must be taken into consideration for a wide application of this technique.

Since our SYBR green based real time PCR technique is a specific and simple assay for
quantitative detection of HTLV-I proviral DNA, its application to monitor disease progression
and verify the effectiveness of therapy offers interesting option not only for first level diagnosis,
but also for ongoing epidemiological surveillance.

HTLV-I proviral DNA quantification opens interesting prospects. Cell-free viremia in plasma is
not a prominent aspect of HTLV-associated diseases, unlike HIV infection, where the
quantitative determination of RNA copies, the main prognostic parameter for disease evolution, directly mirrors viral replication [31]. Therefore, although the relationship between proviral load DNA and pathogenesis for HTLV-I is not well understood, the determination of proviral load, in combination with other biomarkers, could be an important step in the pathogenesis of HTLV-associated disease.

Our method may also be useful for screening blood donors. Blood donor screening for HTLV was introduced in Japan in the mid 1980s, in the United States and Canada in 1988 and finally in France in 1991 [18], and even though the probability of collecting blood products from a viremic donor is extremely low, it is not negligible.

**Conclusions**

In conclusion, even though our data demonstrated that HTLV-I infection mainly is confined to African immigrants, the feasibility of a simple and effective real time approach like our SYBR Green real time PCR suggests a possible application and screening in the diagnosis of HTLV-1 infection and to monitor proviral and viral load during the course of infection and the efficacy antiviral therapy.

**Competing interest:** The authors declare that they have not competing interests

**Authors’ Contributions Section:** MCR and DG conceived and designed the study. FV, PS and IB developed the HIV-1 DNA real time and performed all the experimental work. AD and LG provided blood samples and clinical information on the patients enrolled in this study. MCR drafted the manuscript and DG reviewed it. All authors contributed to the final version of manuscript, read and approved it.
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We thank Ms Anne Collins for editing the manuscript.
Table 1. Western blot results in the eleven serum samples with different level of reactivity by immnoenzymatic assay (ELISA).

<table>
<thead>
<tr>
<th>WB results</th>
<th>ELISA results</th>
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<tbody>
<tr>
<td>Pt. n°1</td>
<td>negative</td>
</tr>
<tr>
<td>Pt. n°2</td>
<td>p19</td>
</tr>
<tr>
<td>Pt. n°3</td>
<td>gd21, p19, p24, p32, p36, gp46, p53, rgp46I</td>
</tr>
<tr>
<td>Pt. n°4</td>
<td>negative</td>
</tr>
<tr>
<td>Pt. n°5</td>
<td>negative</td>
</tr>
<tr>
<td>Pt. n°6</td>
<td>p19, 26</td>
</tr>
<tr>
<td>Pt. n°7</td>
<td>gd21, p19, p24, gp46, p53 and rgp46-I</td>
</tr>
<tr>
<td>Pt. n°8</td>
<td>negative</td>
</tr>
<tr>
<td>Pt. n°9</td>
<td>p19, 30</td>
</tr>
<tr>
<td>Pt. n°10</td>
<td>negative</td>
</tr>
<tr>
<td>Pt. n°11</td>
<td>negative</td>
</tr>
</tbody>
</table>

WB positive: serum reactivity to envelope and gag proteins.
WB indeterminate: serum reactivity to one or two HTLV-1 proteins.
WB negative: lack of reactivity to viral proteins.
ELISA borderline: absorbance value equal to or greater (ranging from 0.1 to 0.5 optical density) than the cut-off value
ELISA positive: absorbance value greater (ranging from 0.6 to >2.00 optical density) than the cut-off value
Table 2: Intra-assay and inter-assay analysis of Ct mean value of standard curves obtained with scalar dilution of MT2 cell line (from $10^5$ to 1).

<table>
<thead>
<tr>
<th>MT$_2$ cell line N° copies/reaction</th>
<th>Ct mean values ±SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-assay</strong>$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^5$</td>
<td>23,35 ± 0,7</td>
<td>3,0</td>
</tr>
<tr>
<td>$10^4$</td>
<td>25,91 ± 0,8</td>
<td>3,1</td>
</tr>
<tr>
<td>$10^3$</td>
<td>28,10 ± 1,0</td>
<td>3,5</td>
</tr>
<tr>
<td>$10^2$</td>
<td>31,78 ± 1,2</td>
<td>3,7</td>
</tr>
<tr>
<td>10</td>
<td>36,18 ± 1,3</td>
<td>3,5</td>
</tr>
<tr>
<td>1</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td><strong>Inter-assay</strong>$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^5$</td>
<td>23,90 ± 0,7</td>
<td>2,9</td>
</tr>
<tr>
<td>$10^4$</td>
<td>26,12 ± 0,9</td>
<td>3,4</td>
</tr>
<tr>
<td>$10^3$</td>
<td>28,30 ± 1,1</td>
<td>3,8</td>
</tr>
<tr>
<td>$10^2$</td>
<td>32,10 ± 1,3</td>
<td>4,0</td>
</tr>
<tr>
<td>10</td>
<td>36,50 ± 1,4</td>
<td>3,8</td>
</tr>
<tr>
<td>1</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

S.D. : Standard Deviation; C.V.: coefficient of variation; ND: not detectable

$^a$For each sample, the CV value is the average of results from three replicates.

$^b$For each sample, the CV value is the average of results from three different experiments performed in triplicate.
REFERENCES


Legend to figure

Figure 1: Agarose gel electrophoresis.
Lanes 1, 2, 3: HTLV-1 negative samples
Lane 4, 5, 6: MT2 representative cells dilutions (from $10^2$ to $10^4$)
Lane 7: HTLV-1 positive sample (N°3 of Table 1)
Lane 8: molecular weight markers
Additional files provided with this submission:

Additional file 1: HTLV BMC.doc: 573Kb
http://www.biomedcentral.com/imedia/1462776052789679/sup1.DOC