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Gene expression profiling of *p53* and *c-myc* in HTLV-1 positive blood donors in Congo

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Abstract
Objectives. The HTLV-1 infection persists for life, remaining as asymptomatic viral reservoirs in most patients, ensuring the chain of transmission, but around 4% develop adult T-cell leukemia/lymphoma (ATLL). HTLV-1 is an oncogenic retrovirus that transforms $CD4^+$ T lymphocytes and deregulates the lymphoproliferative pathways that contribute to the development of ATLL. To achieve cell transformation, most oncogenic retroviruses use proto-oncogene capture transduction, with proviral integration disrupting the expression of tumor suppressors or proto-oncogenes.
The aim. We conducted this study on the prevalence of HTLV-1 infection in blood donors to expand the HTLV-1 database, assess the risk of transmission via blood products, as well as evaluate the risk of persistent infection or development of neoplastic diseases in HTLV-1 carriers.
Materials and methods. This is a cross-sectional study of blood donors of all categories. For this study, 265 blood donors were recruited at the Centre National de Transfusion Sanguine in Brazzaville. After testing for HTLV-1 antibodies by ELISA, proviral DNA was extracted from all ELISA-positive samples for detection by nested PCR, followed by RT qPCR using specific primers p53 and c-myc for gene expression.
Results. 20/265 were positive for anti-HTLV-1 antibody, 5 donors were positive for proviral DNA. The prevalence of HTLV-1 was 1.8%. All HTLV-1-positive donors were male (1.8%), with a positive correlation ($p = 0.05$); the 1.1% of positive donors were regular, with the majority aged between 31 and 45 years (1.5%), and concubine donors were the most frequent (1.1%). All samples showed normal expression of the *p53* and *c-myc* genes.
Conclusion. The prevalence, though low, remains a serious problem. No abnormal *p53* or *c-myc* gene expression was detected in HTLV-1-positive donors, which could mean that none of the T lymphocytes in these donors had been transformed by HTLV-1.

Keywords: *c-myc; Congo; Blood donors; Gene expression; HTLV-1; p53*

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Профилирование экспрессии генов *p53* и *c-myc* у HTLV-1-инфицированных доноров крови в Конго

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Резюме
Введение. Инфекция HTLV-1 сохраняется в течение всей жизни человека, обусловливая бессимптомное вирусное носительство у большинства пациентов и обеспечивая цепь передачи. Однако примерно у 4% инфицированных развивается Т-клеточная лейкемия/лимфома взрослых (ATLL). HTLV-1 – это онкоретровирус, который трансформирует CD4$^+$-Т-лимфоциты и дерегулирует лимфопролиферативные пути, что способствует развитию ATLL. Для достижения трансформации клеток большинство онкогенных ретровирусов используют трансдукцию с захватом протоонкогенов, при этом провирусная интеграция нарушает экспрессию опухолевых супрессоров или протоонкогенов.
Цель исследования. Мы провели исследование по определению распространенности HTLV-1-инфекции среди доноров крови с целью расширения базы данных HTLV-1, оценки риска передачи вируса через компоненты крови, а также оценки риска персистенции инфекции или развития неопластических заболеваний у носителей HTLV-1.
Материалы и методы. Настоящая научная работа – перекрестное исследование доноров крови всех категорий. В исследовании участвовали 265 доноров крови из Национального центра переливания крови в Браззавиле. После тестирования на антитела к HTLV-1 методом ИФА во всех положительных в ИФА-образцах проводили определение провирусной ДНК методом «вложенной» ПЦР, а затем методом количественной ОТ-ПЦР с использованием специфических праймеров p53 и c-myc оценивали экспрессию генов.
Результаты. Из 265 доноров 20 человек были положительны по анти-HTLV-1-антителам, у 5 доноров была выявлена провирусная ДНК. Распространенность HTLV-1 составила 1,8%. Все HTLV-1-инфицированные доноры были мужчинами (1,8%), с положительной корреляцией между наличием инфекции и мужским полом (p = 0,05); 1,1% положительных доноров были регулярными, большинство в возрасте от 31 до 45 лет (1,5%), и наиболее часто встречались доноры-совместители (1,1%). Во всех образцах наблюдалась нормальная экспрессия генов *p53* и *c-myc*.
Заключение. Распространенность HTLV-1, хотя и низкая, остается значительной проблемой. У HTLV-1-инфицированных доноров не было обнаружено аномальной экспрессии генов *p53* или *c-myc*, что может означать, что ни один из Т-лимфоцитов доноров не был трансформирован HTLV-1.

Ключевые слова: *c-myc; Конго; доноры крови; экспрессия генов; HTLV-1; p53*

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Финансирование. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.
Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.
Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Работа проведена в соответствии с этическими стандартами исследований в области здравоохранения (N0: 62/UMNG.FSSA.V-DOY). Протокол исследования одобрен Internal Committee of the National Blood Transfusion Center (CNTS)

From the collection of a person's blood to its distribution to the recipient, there are numerous safety measures in place to validate the blood supply [1]. With the introduction of serological and molecular tests, at least in high-income countries, detecting several pathogens other than human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), transfusion-associated infections have become extremely rare. However, in the city of Congo, for untested pathogens, especially those causing emerging infectious diseases such as HTLV-1, it seems that complete control of infectious diseases has not been achieved. Transfusion safety relies

on several measures: education of donors with risk factors for infectious diseases and deferral of their donation, blood testing, interventions to reduce pathogens and patient blood management [2].

Human T lymphoid virus type 1 (HTLV-1) was the first oncogenic retrovirus discovered in humans in 1980 by Americans using CD4+ T cell cultures [3]. These cells were derived from the peripheral blood of a patient suffering from a hematological malignancy, initially thought to be a cutaneous T-cell lymphoma associated with leukemia [4–6]. The HTLV-1 virus is the cause of two serious diseases: adult T-cell leukemia/lymphoma (ATLL) and HTLV-1-associated myelopathy, also known as tropical spastic paraparesis (HAM/TSP) [7].

Once contracted, HTLV-1 infection persists throughout life and remains an asymptomatic viral reservoir in most patients, ensuring the chain of transmission. But around 4% develop adult T-cell leukemia/lymphoma (ATLL), a highly aggressive CD4+ T-cell malignancy. HTLV-1 transforms CD4+ T lymphocytes, deregulating the lymphoproliferative pathways that contribute to ATLL development [8]. Interestingly, steady-state p53 protein expression is elevated in HTLV-1-transformed human T cells [9]. Hematological malignancies, such as adult T-cell leukemia/lymphoma (ATLL), frequently show overexpression of wild-type (unmutated) p53 and c-myc, as p53 is a downstream target of c-myc.

The main routes of HTLV-1 transmission are: vertical transmission, unprotected sexual intercourse, transfusion of blood or blood products, as well as needle sharing [10]. In particular, a number of factors are thought to be linked to the sexual transmission of HTLV-1 infection, such as the presence of ulcers on the genitals, unprotected sexual intercourse and high blood pressure. Sexual transmission of HTLV-1 infection occurs through ongoing contact with an HTLV-1-infected sexual partner [11, 12].

HTLV-1 is not a ubiquitous virus. It is present throughout the world, with foci of high endemicity often located close to areas where the virus is virtually absent [13, 14] In these foci, HTLV-1 seroprevalence in adults is estimated to be at least 1–2%, but can also reach 20–40% in people aged over 50 in specific groups. According to the 2014 international estimate the prevalence was 1–2% [15], in 2018 a study reveals 0.07% in southern Italy [16].

Sub-Saharan Africa is considered one of the largest endemicity areas for HTLV-1 infection, with around 2 to 4 million individuals infected with HTLV-1.

Consequently, very few data have been reported to date for large blood donor populations in West or Central Africa [4, 17, 18]. Other studies have revealed a prevalence of 3.6% in Nigeria [19], 0.5% in Uganda [20], 0.16% in Senegal and 1.02% in Sudan [21].

In Congo, very few studies have been carried out on HTLV-1 in terms of transfusion safety, particularly in the donor community. Seven cases were described among Pointe-Noire blood donors in a previous study [22]. Although these data suggest the presence of HTLV-1 in Congo, they do not yet allow systematic screening for antibodies against HTLV-1 at the Centre National de Transfusion (CNTS) in Brazzaville. However, we conducted this study with the objectives of expanding the HTLV-1 database, assessing the risk of transmission via blood products, as well as evaluating the risk of persistent infection or development of neoplastic disease in HTLV-1 carriers.

Materials and methods

– *Study sites:* The study was carried out at the Centre National de Transfusion Sanguine (CNTS) after approval by the center's director.

– *Participants:* In this cross-sectional study, the sampling method was a simple random draw among unscreened of 265 blood donors.

◦ *Inclusion criteria:*

Have given consent

Must be between 18 and 60 years of age;

Physically fit for clinical examination;

Weight greater than or equal to 55 kg;

◦ *Did not give consent*

Be on medication;

Have a chronic disease;

Have had a blood transfusion;

Sexually transmitted disease (STD) risk behavior;

Menstruating, breast-feeding or pregnant women;

We analyzed 265 samples from blood donors of both sexes and aged 18 to 60 years in accordance with national guidelines on blood donation, from the blood bank of the CNTS of Congo in Brazzaville. All donors undergo routine screening in the blood bank, including HBsAg testing, antibodies against HIV-1/2, HCV and VDRL.

1. Serological screening: Anti-HTLV-1 antibodies were first determined by ELISA. Four milliliters of blood were collected in additive-free tubes from the blood bag tubing at the time of collection. Results were interpreted according to the manufacturer's instructions.

2. Genomic DNA extraction: Only data from blood donors with a negative routine screening test were analyzed for DNA. After routine screening of the blood bank, high-molecular-weight DNA was extracted from frozen peripheral blood leukocytes of all selected, according to the manufacturer's instructions. After extraction, DNA concentration was measured to assess the quality of DNA extracts.

3. Internal control: A first PCR was carried out on the DNA extracts as a qualitative test, amplifying the human beta globin gene (268 Pb) GH20: 5'GAA GAG CCA AGG ACA GGT AC 3' and PC04: 5' CAA CTT CAT CCA CGT TCA CC 3' to check the quality of our samples. After amplification on the thermocycler, visualization was carried out on a 2% agarose gel. All our samples were positive for beta globin, so they were reliable and of good quality.

4. HTLV-1 detection: HTLV-1 infection was determined by nested PCR of the Pol gene (**Table 1**), using 2 µl of DNA in a complete volume of 25 µl containing 12.5 µl of Green Taq Mix, 6.5 µl of ultrapure water, 2 µl of sense primer and 2µl of antisense primer.

Both reactions were performed under the following cycling conditions:

For the first nested PCR (**Nid-1: Pol EF + Pol ER**), initial denaturation at 94 °C for 3 min, followed by denaturation, 35 cycles at 94 °C-15s, hybridization at 54 °C for 20s, elongation at 72 °C for 5 min and followed by the final step which is cooling at 4 °C for an indeterminate time.

For the second nested PCR (**Nid-2: Pol IF + Pol IR**), conditions were the same, except for the hybridization step, which this time took place at 52 °C for 20s.

After amplification in a thermal cycler, they were visualized on a 2% agarose gel.

5. RNA extraction and detection of p53 and c-myc messenger RNA (mRNA) expression

One microgram of total RNA was collected from each group after extraction. Expression of p53 and c-myc mRNA, was detected by Quantitative Reverse Transcription PCR (RT-qPCR), combining the effects of reverse transcription and quantitative PCR. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the internal control. The *c-myc* and *p53* genes were amplified using a one-step kit enabling reverse transcription and PCR to be performed in the same tube.

For a total volume of 20µl in each tube, the reaction mixture contained 10µl of master mix, 2µl of extracted RNA, 0.8µl of each primer (10µM), 1µl of specific probes p53 and c-myc (4µM) (**Table 2**), 4.4µl of RNase-free ddH_2O, 1µl of 20x ROX Reference Dye. Temperature conditions included a first reverse transcription (RT) step; incubation was performed at 42 °C for 30 minutes in one cycle. The second step was the PCR stage, which included an initial pre-denaturation phase at 95 °C for 5 minutes, followed by a first cycle of 45 cycles comprising denaturation at 95 °C for 10 seconds and a hybridization step at 60 °C and 62 °C for c-myc and p53 for 20 seconds. Ultrapure PCR water was included in each assay as a negative control.

Table 1. Primers

Round	Name	5'–3' oligonucleotide sequence
Nest-1 PCR	PolEF	TTTAGGTGCCCAAACTGGAG
Nest-1 PCR	PolER	GCAGGATATTGGAAGCCTCAG
Nest-2 PCR	PolIF	GCCCTCATGCCAGTGTTTAC
Nest-2 PCR	PolIR	CCTGGAGATGGGATCAGGTAG

Calculation of gene expression fold change: For expression data, the p53 Ct target gene was normalized with GAPDH Ct. The mean Ct values of the *p53* gene and the mean Ct values of the beta-actin gene were compared. The calculation was based on the ΔΔCt method. The fold change values of all donor samples were calculated relative to the reference gene and also relative to the normal sample.

6. Statistical analysis: The association between the PCR Result variable and the variables gender, age range and type of honorifics and marital status was performed using Pearson's Chi-squared test or Fisher exact test using RStudio 2023.06.0+421 «Mountain Hydrangea» Release with R version 4.2.2. The significance threshold was set at 0.05.

The study was conducted with the informed consent of the patients. The study was carried out in compliance with the ethical standards for research in the health sciences (N0 : 62/UMNG.FSSA.V-DOY). The research protocol was approved by the Internal Committee of the National Blood Transfusion Center (CNTS).

Results

Socio-demographic characteristics of donors: A socio-demographic analysis of the donors revealed a predominance of males, with a sex ratio (M/F) of 1.4. The age range 31 to 45 was the most represented. The average age was 35, with extremes ranging from 18 to 60. Voluntary donors made up the majority of the donor group. According to marital status, the most represented status was single (**Table 3**).

HTLV-1 serological results for blood donors: Of the 265 plasma samples tested by ELISA, 20 were found to be seropositive for HTLV-1.

HTLV-1 molecular detection results: Molecular amplification was performed on DNA extracted from the peripheral blood blast cells (PBBC) of 20 ELISA-positive individuals. The results showed that 5 were positive by nested PCR (for the Pol gene).

Prevalence of HTLV-1 in the study population: On the basis of the serological and molecular results, 5 individuals were considered to be infected with HTLV-1 (Table 3). Thus, 5 HTLV-1-infected individuals were included in the epidemiological analysis. This gives an overall prevalence of HTLV-1 infection of 1.8%.

Table 2. Primers and Probes

Genes	Name	5'–3' oligonucleotide sequence
c-myc	F	GA GGA GAC ACC GCCCAC
	R	CAA CAT CGA TTTCTT CCT CAT CTT
	Probe	FAM-CCA GCA GCG ACT CTG AGG AGG AAC A-TAMRA
p53	F	CTG CTC AGA TAG CCG ATG GTC TG
	R	TTG TAG TGG ATG TGG TAC AGT CA
	Probe	FAM-CCC CTC CTC AGC AT CTT ATC CGA GTG G-TAMRA
GAPDH	F	TCC TGC ACC ACC AAC TGC TTA G
	R	CAT CAC RCC ACA GYT TYC CAG AG
	Probe	FAM-AGG TCA TCC ATG ACA ACT TTG GYA TCG-BHQ

Distribution of variables according to PCR results: All HTLV-1-positive donors were male with a positive correlation (p = 0.05). Among positive donor types, regular donors were the most affected, with the majority aged between 31 and 45. Living common-law donors were the most common (Table 3).

Gene expression of p53 and c-myc in positive cases: Expression of the *p53* gene and the *c-myc* gene in positive donors was performed. The Ct value of each case was normalized by the Ct value of the *GAPDH* gene. The results were compared with those of healthy donor samples, and variations in Ct compared with normal samples were obtained. A factor changes greater than 1 indicates up-regulation, and a factor change less than 1 is considered down-regulation. All samples showed normal gene expression.

Table 3. Distribution of variables according to PCR results

	PCR result			
	N/265(%)	Négative (%)	Positive (%)	P-value
Age				0.3
18–30	113 (42.6)	112 (42.3)	1 (0.3)	
31–45	127 (47.9)	123 (46.4)	4 (1.5)	
46–60	25 (9.5)	25 (9.5)	0 (0.0)	
Sex				0.05
Male	155 (58.5)	150 (56.7)	5 (1.8)	
Female	110 (41.5)	110 (41.5)	0 (0.0)	
Marital status				0.2
Single	141 (53.3)	140 (52.8)	1 (0.4)	
Married	43 (16.2)	42 (15.9)	1 (0.3)	
Cohabiting	81 (30.6)	78 (29.5)	3 (1.1)	
Type of donor				0.2
Voluntary	125 (47.2)	123 (46.4)	2 (0.7)	
Family replacement	59 (22.3)	59 (22.4)	0 (0.0)	
Regular	81 (30.5)	78 (29.4)	3 (1.1)	

Discussion

The aim of the present study was to investigate *p53* and *c-myc* gene expression profiles by real-time PCR in HTLV-1 positive blood donors, as no such study has been performed previously. The present study concluded that *p53* and *c-myc* gene expression was normal in all HTLV-1 positive blood donors.

The majority of blood donors were young people aged between 31 and 45, i.e. 47.9%, as observed in most studies carried out at the CNTS, reflecting the Congolese population. Age is unlikely to be a factor affecting the quality of the results, as our study focuses on blood donors, and blood donors are only permitted between the ages of 18 and 60.

The study population was generally made up of male donors. This male superiority, is explained by the many contraindications to blood donation in women (breastfeeding, pregnancy and menstruation). This is in line with several studies carried out at the CNTS [21–24].

The majority of blood donors in the study were volunteers. The latter are considered to represent the general population and present a high risk of transmission. In our study, bachelors made up a large proportion of the study population.

The results obtained from this study show that the prevalence of HTLV-1 was 1.8%, which is similar to that found among blood donors in Sudan, where HTLV-1 seroprevalence was 1.02% [21]. HTLV-1 seroprevalence in this study was relatively comparable to the 2014 international estimate of 1–2% [15]. However, our results are 3.6% lower than those obtained in Nigeria in 2011 [19]. And higher than the level obtained in Uganda and southern Italy, which is 0.5% and 0.07% respectively [16, 20]. This difference can be explained by the difference in the study population.

All HTLV-1-positive donors were male (1.8%), with a positive correlation (p = 0.05). This is inconsistent with studies showing a higher prevalence of HTLV-1 in women than in men, explained by more efficient male-to-female transmission during sexual intercourse [25]. However, it is consistent with studies also showing a higher prevalence of HTLV-1 in men than in women [21]. The high presence of HTLV-1 in male blood donors, compared with female blood donors, is probably due to the higher proportion of men than women in blood collection.

The age range most affected by HTLV-1 in our study was 31 to 45 years. A study carried out in Congo also revealed a high prevalence among blood donors in the same age range [22]. This may be explained by the fact that this age range represents the most active population for blood donation in our study.

Cohabiting living donors were the most common, at 1.1%, contrary to other studies showing a high prevalence among bachelors [19–21, 26].

Among donor types, three (3) out of the 5 were regular donors, in contrast to a study showing a high prevalence among voluntary donors [20].

All PCR-positive HTLV-1 samples showed normal p53 and Cmyc gene expression. This may be explained by the fact that T lymphocytes from HTLV-1-positive donors are not yet transformed by the virus. It should be noted that steady-state p53 protein expression is elevated in HTLV-1-transformed human T lymphocytes [9].

Conclusion

Although HTLV-1 is rare in the city of Congo, according to the present and previous studies, it should not be ignored and, given its transmissibility by blood and sexual transmission, could be a major public health problem in the years to come. No abnormal *p53* or *c-myc* gene expression was detected in HTLV-1-positive donors, which could mean that none of the T cells in these donors had been transformed by HTLV-1. It was also noted that the frequency of HTLV-1 transformation is very low, estimated to be less than 5% [10].

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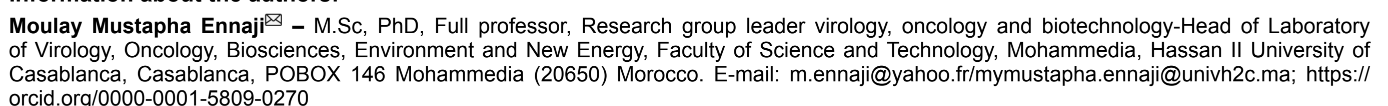

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