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Low-cost and simple PCR process for access to molecular diagnosis of HTLV-1/2 in low-resource countries

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ABSTRACT

Background: HTLV-1/2 exhibit a widespread distribution globally and are associated with severe clinical manifestations, necessitating precise viral identification for diagnosis. Currently, there are no official diagnostic guidelines, and a variety of published protocols exists. We introduce an enhanced nested real-time PCR technique followed by high-resolution melting (rtPCR-HRM), designed to offer a cost-effective and straightforward tool for the simultaneous identification of both viruses.

Methods: The technique was tested in a retrospective, blinded study, involving a total panel of 110 samples, of which 47 were positive for HTLV-1, 12 for HTLV-2, and 51 tested negatives. Additionally, we compared the performance of this technique with a line immunoassay (LIA).

Results: The results demonstrate 100 % sensitivity, specificity, and diagnostic accuracy for both viruses. Sensitivity analysis indicated that at least 1 viral copy of HTLV-1 and 14.4 viral copies of HTLV-2 could be reliably detected.

Conclusions: Our results indicate that rtPCR-HRM is effective in confirming HTLV-1 and HTLV-2 infection, important in Latin American countries where both viruses circulate. Furthermore, the proposed strategy provides a new tool that can be used to resolve indeterminate cases identified by Western blot, with the added advantage of being faster and simpler than n-PCR and more cost-effective than other probe-based RT-PCRs.

1. Introduction

Human T-cell lymphotropic viruses (HTLV) type 1 (HTLV-1) is a human retrovirus discovered in 1980 and is the most clinically significant of this family (Poiesz et al., 1980). HTLV-1 is the causative agent of adult T-cell leukemia/lymphoma (ATLL) and tropical spastic paraparesis/HTLV-1-associated myelopathy (HAM/TSP). Additionally, it is linked to various inflammatory disorders, including uveitis, respiratory conditions, strongyloidiasis, and infective dermatitis (Futsch et al., 2017; Schierhout et al., 2020). It is estimated that between 5 and 10 million people worldwide are infected with HTLV-1, and it is globally widespread, with notable prevalence in regions such as southern Japan, the Caribbean islands, tropical Africa, parts of Latin America, the Middle East, and Melanesia (de Mendoza et al., 2024; Gessain and Cassar, 2012). HTLV-2, isolated in 1982, (Kalyanaraman et al., 1982) has a prevalence 6 to 12 times lower than that of HTLV-1, with the highest rates among vulnerable population in South America (Abreu et al., 2022; Ishak et al., 2020; Murphy et al., 2015).

This virus has been associated with rare neurological syndromes exhibiting clinical similarities to HAM/TSP and although a definitive clinical correlation between the virus and diseases has yet to be established, clinical cases related to this retrovirus are constantly described (Biglione et al., 2003; Hall et al., 1996; Rosadas et al., 2014).

The HTLV retrovirus genome maintains stability, displaying genetic

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diversity among strains ranging from 0.1 % to 6.9 %. The regions of env and long terminal repeat (LTR) demonstrate the highest variability and serve as markers to classify virus strains into distinct geographic subtypes (Ratner et al., 1991). HTLV-1 and 2 can be vertically transmitted (such as during childbirth or breastfeeding), parenteral transmission (including transfusion of contaminated blood products, transplantation of infected organs, or intravenous drug use), and sexual transmission (de Mendoza et al., 2024; Gessain and Cassar, 2012; Toro et al., 2003).

Mandatory screening for HTLV-1/2 in blood banks and organ transplantation has been widely implemented in many countries to date. In the initial detection of HTLV-1/2 infection in blood banks or hospital services, screening techniques used are not confirmatory of the infection, not differentiating between HTLV-1 and HTLV-2, and often resulting in a high number of false positives (Glasser et al., 2013). Consequently, reactive samples undergo serological confirmation, such as Line Immunoassay (LIA) or Western Blot (WB), which are utilized to determine seronegativity or specific HTLV-type seropositivity (Okuma et al., 2020). The proportion of indeterminate results from Western blot varies widely, ranging from 0 to 68 %. Several factors associated with false positive results have been identified, including cross-reactivity with structural proteins from other types of HTLV, proteins from Plasmodium falciparum in areas where HTLV-1 and malaria are endemic, and nonspecific cross-reactivity with host proteins or other pathogenic proteins (Legrand et al., 2022). However, while LIA assays offer improved performance with the inclusion of recombinant proteins, their high cost and the occurrence of indeterminate results can pose challenges in diagnostic interpretation and patient counseling (Eusebio-Ponce et al., 2019; Sabino et al., 1999).

Therefore, a molecular diagnosis becomes necessary. Diverse Polymerase Chain Reaction (PCR) protocols with different sensibility have been established worldwide for the detection and identification of HTLV. It is worth noting that, for improved specificity and sensitivity in HTLV-1 detection, some studies advocate the use of the tax region rather than the pol region in PCR assays. This recommendation stems from documented instances of deletions within the pol gene, potentially undermining the precision of HTLV-1 diagnosis (Kamihira et al., 2005; Katsuya et al., 2019; Tamiya et al., 1996).

Over time, several working groups proposed various 'in-house design' protocols for molecular diagnosis to address gaps in current methods, with protocols evolving alongside advancements in equipment and techniques. Highly sensitive nested-PCR protocols of pol and tax genes were proposed for detection and identification of HTLV-1 and 2 ((Besson and Kazanji, 2009; Campos et al., 2017; Gomes et al., 2020; Heneine et al., 1992; Liu et al., 1999; Thorstensson et al., 2002; Tuke et al., 1992; Vandamme et al., 1997).

Particularly in South American countries, where both virus HTLV-1 and HTLV-2 are circulating, protocols have been devised to detect and differentiate between, utilizing the conserved pol and/or tax segments of the provirus (Gallego et al., 2004; Heneine et al., 1992). Some of them proposed a generic outer PCR followed by discriminatory inner PCRs (Heneine et al., 1992; Vandamme et al., 1997), whereas other used restriction enzyme digestion of nested PCR products for further typing of HTLV-1 and 2 (Tuke et al., 1992), P-endlabelled oligonucleotides (Heredia et al., 1996), Taqman (Waters et al., 2011) and sybergreen (Castro et al., 2013). Recently, real time PCR as loop-mediated isothermal nucleic acid amplification (LAMP) technology (Gomes et al., 2020) or other designs using probe –based real time PCR (Gonçalves et al., 2022; Ji et al., 2023). The technique choice will depend on the virus epidemiology, available equipment, costs, and sensitivity required.

Higher-income countries tend to use probe-based real-time PCR, which is simpler to perform but in resource-limited countries conventional PCR is still used (Miranda Ulloa et al., 2023).

In this context, we introduce an alternative optimized method that integrates nested PCR with real-time reaction followed by high-resolution melting (rtPCR-HRM), targeting the tax region. This approach enables precise and specific diagnosis of HTLV-1 and HTLV-2 infections in a simple and cost-effective manner.

2. Materials and methods

2.1. Clinical sample panel

A panel comprising 110 DNA samples, gathered between 2017 and 2022 at the Biomedical Research Institute on Retroviruses and AIDS (INBIRS) in Buenos Aires, Argentina, was utilized. In this sample panel, there were 47 positives for HTLV-1, 12 positives for HTLV-2, and 51 negatives. The positive samples for HTLV-1 and HTLV-2 originated from blood donors seeking confirmation of their HTLV diagnosis after initially testing positive in a screening assay at the blood banks. The confirmatory diagnosis of infection was conducted at INBIRS, a national referent for diagnosis in Argentina, using a nested polymerase chain reaction (n-PCR) targeting the pol gene of both HTLV-1 and HTLV-2 that has been set up years ago (Heneine et al., 1992). However, an ELISA assay from plasma to screen for anti-HTLV-1/2 antibodies (ELISA HTLV I&II Ab, version ULTRA, Diapro, Italy) was performed on all samples previously. All individuals provided informed consent, which was reviewed and approved by an Ethics Committee (Nexo Asociación Civil) under protocol number 3008/08/2012. This panel was employed for blinded analysis using the newly developed rtPCR-HRM.

2.2. Nested real-time PCR technique followed by high-resolution melting

The rtPCR-HRM assay used was based on a previously described assay targeting the *tax/rev* regions of the HTLV-1 and 2 reference sequences deposited in GenBank: J02029.1 (HTLV-1) and M10060.1 (HTLV-2) (Caputo et al., 2022). A target for *gapdh* as amplification control was added as an optimization in the first round of PCR, visualizing a melting temperature peak at ~82.4 °C; Ct max= 28. The first amplification round was performed using primers POF and POA for *tax* HTLV target and *gapdh* primers for amplification control (Table 1). Total genomic DNA quantification was conducted using a fluorimeter (Quantus™ Fluorometer, Promega Madison) to standardize the amount of DNA incorporated into the molecular techniques. Only samples with concentration >1 ng/μl were considered. PCR reaction mixture was: 5 ng of genomic DNA, 20pM of POF and POR (Table 1), 10pM of each gapdh primers, 0.16Mm dNTPs, 25 mM syto9, 1X Q-solution (Qiagen Cat. No. 210220), 1 U of Go Taq polymerase (Promega, Madison, WI, USA) and 5X Go Taq reaction buffer in a total volume of 25 μl. The cycling conditions were: 2 min denaturation at 95 °C; 30 cycles consisting of 30 s at 94 °C, 40 s at 55 °C and 20 s at 72 °C; followed by 2 min extension at 72 °C in a Real Time PCR equipment MIC (Corbet Inc., Australia). The second amplification round was performed using the inner/forward primer (PIF) and the same reverse primer of the first amplification round (POA) (Table 1), with a final PCR product of 94 base pairs (bp).

The PCR reaction mixture and cycling conditions were the same as those described above, except from the fact that 1 μl of the product of the

Table 1
List of *tax* HTLV sequence target and gadph control primers.

Reaction	Primers	Sequence 5′→3′
1°PCR	POF	CGGATACCCAGTCTACGTGT
1°PCR	POA	GAGCCGATAACGCGTCCATC
1°PCR	gadph F	GAGTCAACGGATTTGGTCGT
1°PCR	gadph R	TTGATTTTGGAGGGATCTCG
2°PCR	PIF	TATGTTCGGCCCGCCTACATCG
2°PCR	POA	GAGCCGATAACGCGTCCATC

First round included outer primers for HTLV tax region (POF-POA) and gadph as human internal control. Second round included an inner primer (PIF) and the same outer primer antisense (POA). Sequence target for tax region shows identical match with HTLV-1.

first round was added to the mixture and only 15 cycles were carried out in the cycling conditions. Finally, HRM was completed between 81 °C and 87 °C to distinguish between HTLV-1 and HTLV-2.

2.3. Standards for HTLV-1 and HTLV-2

Positive control for HTLV-1 was achieved using DNA extracted from the MT2 cell line, which is persistently infected by the virus. Demontis et al. conducted a characterization of the number of copies per cell and determined that it has 7 copies of tax per MT2 cell (Demontis et al., 2013). For HTLV-2, DNA from the MOT cell line infected by the virus was utilized. Characterization conducted by Kuramitsu et al. determined that the cell line has 5 copies of HTLV-2 gene coding region per cell (Kuramitsu et al., 2019). The MT2 and MoT cell lines were cultured in RPMI and IMDM media, respectively, supplemented with 10 % fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL).

2.4. Assessment of technique performance and statistical analysis

The sensitivity was evaluated using serial dilutions of the DNA extracted from cell lines infected with HTLV-1 (MT2) and HTLV-2 (MoT). To correlate genomic DNA quantity to virus copies, total human genomic DNA quantification was performed as described by Ginart et al., before/after dilutions were performed (Ginart et al., 2019). DNA-free samples and blanks were also included as controls.

Sensitivity was calculated as number of true positive/ (number of true positive + number of false negative); specificity was calculated as number of true negative/ (number of true negative + number of false positive) and test accuracy was calculated as (number of true positives + true negatives)/all subjects. A 95 % confidence interval (CI) -measured by https://www.medcalc.org/calc/diagnostic_test.php - and the Kappa index were calculated to measured correlation between molecular assays. (Table 2).

2.5. Comparison with a commercial immunoassay

Out of 110 cases analyzed, 74 plasma samples were tested using the commercial immunoassay recomLine HTLV-1 & HTLV-2 IgG (Mikrogen Diagnostik) to compare the performance of both molecular techniques.

2.6. Analysis of HTLV-2 sequences

A total of 60 HTLV-2 sequences from GenBank were compared to find similarities/differences between the different genotypes in the primer binding region. In this analysis, sequences of the HTLV-2a and HTLV-2b subtypes circulating in our country, as well as HTLV-2c sequences reported in Brazil and one HTLV-2d reported in Africa, were included.

Table 2
Test accuracy, sensitivity and specificity for HTLV-1 and HTLV-2 for rtPCR-HRM compared to n-PCR.

		rt-PCR-HRM
HTLV-1	Sensitivity	100 (92.45–100)
	Specificity	100 (93.02–100)
	Test accuracy	100
	Kappa Index	1 (0.80–1.20)
HTLV-2	Sensitivity	100 (73.54–100.00)
	Specificity	100 (93.02–100.00)
	Test accuracy	100
	Kappa Index	1 (0.63–1.37)

Results based in the comparison from 110 samples tested by the proposed assay, real-time PCR technique followed by high-resolution melting (rtPCR-HRM) and, the conventional nested PCR (n-PCR).
Sensitivity was calculated as number of true positive/ (number of true positive + number of false negative); specificity was calculated as number of true negative/ (number of true negative + number of false positive) and test accuracy was calculated as (number of true positives + true negatives)/all subjects. A 95 % confidence interval (CI) and the Kappa index were calculated to measure correlation between molecular techniques.

3. Results

The rtPCR-HRM technique described enables the detection of 1 viral copy per reaction of HTLV-1 tested in the MT2 cell line, and 14.4 viral copies per reaction of HTLV-2 tested in the MoT cell line. This estimation was calculated assuming the copies per cell of each cell line described in the methods section and that the genomic DNA content is 6 pg per cell (Fig. 1).

After analyzing the 60 HTLV-2 sequences, we found 7 sequences with additonal mismatch in the primer binding region. Mismatches were detected in 2 HTLV-2a sequences, 3 HTLV-2b sequences, 1 HTLV-2c sequence and 1 HTLV-2d (Supplementary Figure 1).

We achieved a 100 % concordance between the previously confirmed results obtained by nested PCR and those obtained using the new rtPCR-HRM technique on a panel of samples: from 110 samples, 47 were positive for HTLV-1, 12 for HTLV-2, and 51 tested negatives.

In Table 2, the results of sensitivity, specificity, test accuracy, and the kappa index for each virus comparing n-PCR and rt-PCR-HRM show an agreement between both techniques.

Subsequently, we compared the results of 74 samples analyzed via rtPCR-HRM with those obtained through a commercial immunoassay, aiming to juxtapose a molecular technique against a serological one (Table 3). The differences were found in two samples that were classified as indeterminate by the LIA. For these samples, one was confirmed as positive for HTLV-1 while the other was confirmed as negative by both molecular techniques.

4. Discussion

In March 2021, for the first time, the World Health Organization officially recognized HTLV-1 in a report with the aim to eliminate this retrovirus in 2030. However, there is a wide variety of alternatives to choose for the molecular detection of HTLV without a diagnosis guidelines available from a reference institution yet. This resulting in a wide array of available protocols published by different groups around the world with the absence of a gold standard for diagnosis and appropriate recommendations or policies. This diversity arises due to each country's circumstances, such as the circulating HTLV types and the available economic resources (Cassar and Gessain, 2017). Both HTLV-1 and HTLV-2 viruses circulate in Latin America with the presence of endemic areas for each one (Ishak et al., 2020). Therefore, it is necessary to develop confirmatory techniques for HTLV that distinguish between both viruses. Furthermore, the clinical presentations of HTLV-1 and HTLV-2 exhibit significant differences, necessitating a crucial differential diagnosis. It has been suggested that results from screening techniques should be confirmed through molecular techniques rather than relying solely on serological confirmation analysis (Ji et al., 2020). This recommendation is based on the high rates of indeterminate results in some regions and/or the considerable cost associated with these Western blot or line immunoassay tests (Costa et al., 2011). Additionally, molecular techniques are particularly valuable in areas where the prevalence of HTLV-1/2 is low, and only a limited number of cases need confirmation (Abrams et al., 2011; Jacob et al., 2008). There are no commercially available molecular tests nor a global consensus on how to diagnose the virus. We emphasize the importance of the available economic resources, as higher-income countries; for example, tend to use probe-based real-time PCR. However, this is not feasible to implement in countries with limited resources, and there is a need to adopt faster and more cost-effective PCR protocols.

This technique was developed in response to the need to streamline processing times and enhance result accuracy. We opted for the selection of primers designed within a conserved region of the *tax* gene. This

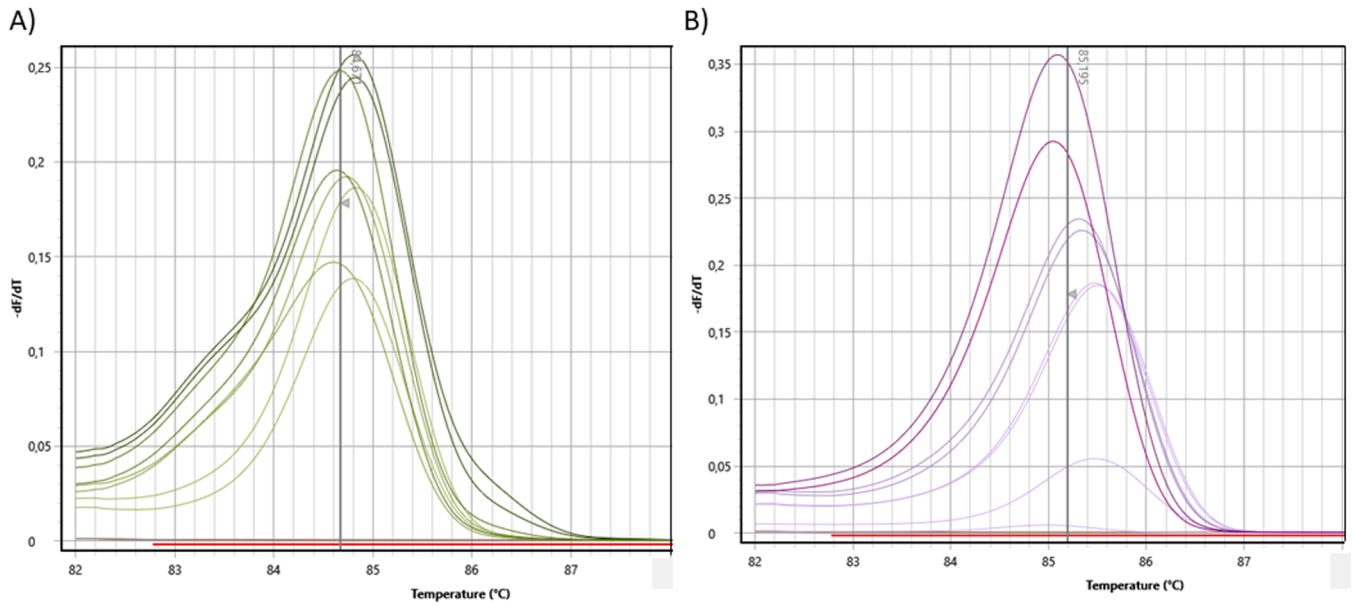

Fig. 1. High resolution melitng profile.
Legend: A) in green HTLV-1 dilution from MT2 cell line (1000, 100, 10 and 1 copies tax gene/per reaction) TM (melting temperature): 84.67. Each dilution was tested in duplicate; B) in purple HTLV-2 dilution from MoT cell line (1416.7, 141.6, 14.1 and 1.4 copies of virus/per reaction) TM: 85.20. Each dilution was tested in duplicate. Only 1 replicate of the 1.4 copies showed amplification.

Table 3
Comparison between molecular assays and a commercial immunoassay.

	rtPCR-HRM	n-PCR	LIA
HTLV-1	31	31	30
HTLV-2	6	6	6
NEGATIVE	37	37	36
INDETERMINATE	0	0	2
Total	74	74	74

Out of 110 cases analyzed, 74 plasma samples were tested using the commercial immunoassay recomLine HTLV-1 & HTLV-2 IgG (Mikrogen Diagnostik) (LIA) to compare the performance of both molecular techniques: real-time PCR technique followed by high-resolution melting (rtPCR-HRM) and conventional nested PCR (n-PCR). Correlation was obtained between molecular assays. The differences were found in two samples that were classified as indeterminate by the LIA: one was confirmed as positive for HTLV-1 while the other was confirmed as negative by both molecular techniques.

decision was based on reports suggesting deletions in the 5′region of the provirus (housing the gag and pol gene) may occur over time in patients, while the 3' region (tax region) remains stable (Kamihira et al., 2005; Katsuya et al., 2019; Tamiya et al., 1996). We evaluated the analytical sensitivity of the rtPCR-HRM assay by utilizing genomic DNA extracted from the MT2 and MoT cell lines. Dilution of these in-house standards resulted in detection sensitivities of at least 1 and 14.4 proviral copies per reaction of HTLV-1 and HTLV-2, respectively. We found a lower sensitivity in HTLV-2 detection. This is in line with studies from different countries which have consistently demonstrated a lower sensitivity, sometimes up to tenfold, for detecting HTLV-2 compared to HTLV-1 (Gomes et al., 2020; Gonçalves et al., 2022). This phenomenon appears to be independent of the target genes (tax, pol genes) or the reaction chemistry used (Besson and Kazanji, 2009). One possible explanation for the lower sensitivity in HTLV-2 detection in this strategy could be the mismatch between the primers and the HTLV-2 sequence. Among the 60 sequences analyzed, seven showed additional discrepancies in at least one primer-binding region. Only one sequence exhibited a critical mismatch at the 3′ position, corresponding to HTLV-2c, which is uncommon in our country (Caterino-de-Araujo et al., 2014). The rtPCR-HRM assay proved effective in accurately detecting samples of HTLV-1, HTLV-2, and negative confirming two indeterminate results by LIA reflecting the advantage of molecular techniques over serological ones.

Cost analysis of molecular diagnostics, starting from extracted DNA and considering the use of all reagents: Go Taq polimerasa (Part. Number: M7845 Promega Corp., Madison, WI, EE.UU.), primer (IDT technologies), dNTP's (Part. Number: U1240 Promega Corp., Madison, WI, EE.UU), syto9 (Part. Number: S34854 ThermoFisher Scientific, Waltham, MA), agarose (Part. Number: B030–100 Promega Corp., Madison, WI, EE.UU), showed the following: For rtPCR-HRM, the cost is approximately $1,1 per sample, while for n-PCR, it is about $3 per sample, not including the cost of controls. The rtPCR-HRM method involves performing two PCRs, whereas the n-PCR method requires four PCRs due to the need to test pol gen, with each design amplifying either HTLV-1 or HTLV-2, plus the cost of agarose electrophoresis.

If using probe-based real-time PCR such TaqMan approach (ThermoFisher Scientific, Waltham, MA), the cost of the probe must be considered, approximately $0.60 each. At least three probes are needed: one for HTLV-1, one for HTLV-2, and one for the reporter gene. Moreover, probe-based real-time PCR generally requires the use of specific reaction mixes, which are more expensive than routine ones.

In comparison, immunoassays, such as the recomLine HTLV-1 & HTLV-2 IgG (Mikrogen Diagnostik) used in this study, cost $25 per sample, or western blot as HTLV BLOT 2.4 (MP Diagnostics) cost U$S10 per sample which quite higher than the cost of molecular diagnostics."

In conclusion, the proposed rtPCR-HRM method allows for the detection and discrimination of HTLV-1 and HTLV-2, eliminating the need for post-PCR processing steps such as agarose gel electrophoresis and ethidium bromide staining. As a result, a fast, cost-effective assay simplifies the n-PCR process and is more accessible than immunoassays.

In the post-COVID-19 era, real-time PCR technology has become widely adopted, with many laboratories incorporating it into their practices. This advancement has significantly expanded the scope of molecular diagnostics to include various bacterial and viral diseases, thereby justifying its cost. Once an initial assay is established, the technical expertise required is similar to that needed for other tests.

In Argentina and other South American countries with similar epidemiological characteristics, where most laboratories perform

serological confirmations and have not yet implemented molecular diagnostics, this technique -using syto 9- could serve as an alternative to other molecular methods, such as those using SYBR Green, due to its greater efficiency (Monis et al., 2005). Therefore, it is a faster, simpler, and less labor-intensive technique (2.5 h) compared to the current internal n-PCR method (7 h) or immunoassays (5 h). The adoption of this technique by other laboratories and its validation against the techniques they currently use could complement the results obtained in this approach potentially revealing limitations and improving the efficiency of the reaction.

It is therefore a simpler and more cost-effective technique than the current in-house n-PCR and may serve as a rapid and cost-effective alternative compared to probe-based qPCRs for low budget laboratories equipped with simple molecular diagnostic equipment.

Availability of data and material

The datasets generated and analyzed during the current study are publicly available in the supplemental online files.

Ethics approval

All individuals provided informed consent, which was reviewed and approved by an Ethics Committee (Nexo Asociación Civil) under protocol number 3008/08/2012 at the Institute of Biomedical Research on Retroviruses and AIDS (INBIRS) in Buenos Aires, Argentina.

Consent to participate

All authors have given their consent to participate

Consent for publication

All authors have given their consent for publication.

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Supplementary Figure 1. HTLV-2 sequence used in alignment

Box indicated POF, PIF, POA; arrows in red indicated mismatch annealing with primer (sequence full match with HTLV-1). The sequence bellow showed additional mismatch with primer used at the reaction: GU212854.1 -Pygmy origin- J. Infect. Dis. 203 (9), 1316–1323 (2011); U32873.1 -Brazil origin- J. Virol. 70 (3), 1481–1492 (1996); M63881.1- Mexico origin- J. Med. Virol. 36 (2), 136–141 (1992); Y13051.1- African origin- J. Gen. Virol. 79 (PT 2), 269–277 (1998); Y14365.1-Pygmy origin- J. Virol. 72 (5), 4327–4340 (1998); AF401496.1-Brazil origin-AIDS Res. Hum. Retroviruses 19 (6), 519–523 (2003); GU591299.1 -Pygm origin- Spain AIDS Res. Hum. Retroviruses 27 (5), 579–583 (2011)

CRediT authorship contribution statement

Nicolás Ducasa: Writing – original draft, Methodology, Data curation. **Diego Domínguez:** Methodology. **Paula Benencio:** Methodology. **Laura Alfie:** Methodology. **Patricia Etcheves:** Methodology. **Giampaolo Scarton:** Methodology. **Mirna Biglione:** Writing – review & editing, Visualization, Validation, Investigation, Funding acquisition. **Mariela Caputo:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

No conflict of interest declared.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2024.107395.

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