An overview of sequencing technology platforms applied to HTLV-1 studies: a systematic review

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Abstract

Human T-lymphotropic virus type 1 (HTLV-1) was the first human retrovirus described. The viral factors involved in the different clinical manifestations of infected individuals are still unknown, and in this sense, sequencing technologies can support viral genome studies, contributing to a better understanding of infection outcome. Currently, several sequencing technologies are available with different approaches. To understand the methodological advances in the HTLV-1 field, it is necessary to organize a synthesis by a rigorous review. This systematic literature review describes different technologies used to generate HTLV-1 sequences. The review follows the PRISMA guidelines, and the search for articles was performed in PubMed, Lilacs, Embase, and SciELO databases. From the 574 articles found in search, 62 were selected. The articles showed that, even with the emergence of new sequencing technologies, the traditional Sanger method continues to be the most commonly used methodology for generating HTLV-1 genome sequences. There are many questions that remain unanswered in the field of HTLV-1 research, and this reflects on the small number of studies using next-generation sequencing technologies, which could help address these gaps. The data compiled and analyzed here can help research on HTLV-1, assisting in the choice of sequencing technologies.

Introduction

It is estimated that 5-10 million people worldwide are infected with human T-lymphotropic virus type 1 (HTLV-1) [1–4]. Infected individuals can develop HTLV-1-associated pathologies such as adult T-cell leukemia/lymphoma (ATLL in 2-5% of patients), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP in 0.25-3.8% of patients), HTLV-1-associated infectious dermatitis (IDH), and other inflammatory diseases such as uveitis and pneumonitis, or they can be classified as asymptomatic carriers [5–7]. The factors involved in the development of a particular clinical manifestation have not yet been elucidated, and HTLV-1-infected individuals remain without specific treatment [8–10].

HTLV-1 genome structure is composed of two flanking regions, known as long terminal repeats (5’ and 3’ LTR), and the structural genes gag, pol, and env. There is also a non-structural region, pX, adjacent to the 3’ LTR that encodes the regulatory and accessory proteins Tax, Rex, and HBZ [11]. Molecular characterization of the viral genome, based on sequencing combined with bioinformatics analysis, provides information on genomic regions such as viral integration sites and allows identification of mutations and epigenetic changes [12]. This information is important for the development of HTLV-1 specific vaccines and therapies.

Although HTLV-1 was the first human retrovirus described, the number of HTLV-1 sequences that have been...
determined is considerably smaller than for other important retroviruses, such as human immunodeficiency virus 1 (HIV-1). In March of 2021, there were 1,048,465 published HIV-1 sequences, while for HTLV-1 there were only 9,980 sequences available in the GenBank database. To perform some specific studies of virus modifications that could be associated to different manifestations in human hosts, it would be necessary to have clinical and epidemiological information about the patients. However, most studies do not give all the information necessary to connect viral mutations with the clinical status of the patient. Even with some sequences already published, an investment in the generation of more HTLV-1 sequences would allow the identification of new mutations that affect infection, which might be helpful for developing new diagnostic strategies.

In 1975, Sanger presented the first DNA sequencing technique, which was widely adopted and is still being used today. This technique is based on the use of modified chain terminators, which are dideoxynucleotides (ddNTPs) [13]. Sequencing techniques later evolved further, resulting in the emergence of next-generation sequencing (NGS), starting with second-generation technology. This technology brought new methodologies for determining nucleotide sequences with greater efficiency and speed, using systems such as 454 from Roche Applied Science, Solexa from Illumina, and Ion Torrent, which expanded the ways of sequencing genetic material [14, 15]. The main examples of second-generation technology are pyrosequencing and sequencing by synthesis (SBS). In this generation, the DNA polymerase acts in conjunction with a chemiluminescent enzyme, which, when complementing a template of a DNA strand, emits chemiluminescent signals, allowing the determination of the sequence [16].

Recently, a third generation has emerged, represented by nanopore sequencing (Oxford Nanopore Technologies) and Pacific Biosciences (PacBio) methodologies [15]. Unlike other sequencing technologies, these methods can be used to sequence unique DNA molecules and to produce longer read lengths in a shorter time than was possible in the previous generations [17]. The nanopore method stands out not only for generating long nucleotide chains through larger devices such as GridION and PromethION but also through small portable devices such as MinION and Flongle. This technique is based on the passage of genetic material through a nanopore membrane, which detects the electrical signals emitted during the passage of each nucleotide [18].

It should be noted that, in recent years, there has been significant technological diversification in genome sequencing, with more efficient, cheaper, and faster devices. Investigating which sequencing technology is most used to determine HTLV-1 genome sequences allows us to understand the limitations and possibilities of research carried out on the viral genome. This may help to fill the gaps in our knowledge about this virus, such as the factors involved in the development of HTLV-1-associated diseases. Considering the importance of the technological choice for sequencing, in this article, we review the different technologies used to generate HTLV-1 sequences and the contributions of these techniques to new investigations of this retrovirus.

**Materials and methods**

This study consists of a systematic literature review carried out in accordance with the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). A systematic search was conducted for studies in which performed partial or total sequencing of the HTLV-1 genome was performed. The articles were searched in the PubMed, Lilacs, SciELO, and EMBASE databases in May 2021. The search algorithm used was composed of subjects from the DeCS/MeSH database and additional keywords: ("Human T lymphotropic virus 1" OR "HTLV-1") AND "sequence*" AND ("molecular sequence data" OR "sequencing"). Through the search algorithm, all titles were cross-checked to identify possible duplicate studies.

For the selection of articles, the following inclusion criteria were applied to select studies: (i) the articles were in Portuguese, English or Spanish; (ii) they were original studies, and (iii) the articles presented complete or partial genome sequences of HTLV-1. Articles published since 2000 were included. The exclusion criteria were as follows: (i) studies not specifying the sequencing method, (ii) studies that did not generate HTLV-1 sequences or did not specify the number of sequences generated, (iii) animal studies, and (iv) studies in which genome sequencing was performed using a cell line. The HTLV-1 subtypes were not considered as a criterion for selection of articles.

The articles found on the platforms were initially filtered and selected by reading the title and abstract. Subsequently, a new selection was made by reading the full text. After reading and analyzing the selected articles, the data were collected and included in this review. The search for published studies was performed independently by two authors (F.O.A. and M.S.C.), and disagreements about all outcomes were resolved by consensus among all authors.

After reading the selected articles, the following content was extracted from each one: (1) basic information (title, authors, year, objectives), (2) study design, (3) sequencing technology and method, (4) subjects (sample origin and region of the HTLV-1 genome sequenced), and (5) number of sequences generated. The data collected from the articles were tabulated using Microsoft Excel. The figures generated in this work were produced using the programs Adobe Photoshop and Microsoft PowerPoint (2019 versions).
This study was registered with the International Prospective Register of Systematic Reviews (PROSPERO) under the number CRD42020218387.

Results

The search for studies identified a total of 574 articles, of which 350 were available in PubMed, 213 in EMBASE, one in SciELO, and 10 in Lilacs. Of these, 65 were excluded due to duplication, 404 were excluded after selection by title and abstract, and 43 after reading the full text. Ultimately, 62 articles were included in the systematic review (Fig. 1).

The articles indicated the use of three sequencing methodologies: Sanger, Illumina, and Ion Torrent. Sanger sequencing, which is the first generation of sequencing, was the most frequently used technique. Even after the emergence of NGS methodologies in 2004, it was observed that most published HTLV-1 studies continued to use the Sanger method preferentially. Among the 62 articles used in this review, 59 used the Sanger method, and, of these, 40 were carried out after 2004 (Table 1).

In most of the studies, a partial HTLV-1 genome sequence was determined. Of the 59 articles that used the Sanger method [19–77], 56 reported partial genome sequences, and three reported complete sequencing of the HTLV-1 genome. Of the four articles that used NGS [12, 75, 78, 79], two reported partial genome sequences and two reported complete genome sequences.

Another important aspect of these articles was the difference in the number of sequences generated for each region of the HTLV-1 genome: 1258 sequences of LTR, 89 sequences of gag, 124 of pol, 777 of env, and 1420 of the pX region (Fig. 2). It is important to highlight that there are four different overlapping open reading frames (ORFs) in the pX region that encode regulatory proteins and the HTLV-1 bZIP domain gene (hbz), which is transcribed in the antisense direction from a promoter present in the 3'LTR. The number of sequences generated for each ORF and hbz are as follows: ORF I, 311; ORF-II, 54; ORF-III, 54; ORF-IV, 1153; hbz, 10. In addition, 14 partial genome sequences with the precise regions not described were found, and 228 complete HTLV-1 genome sequences were reported.

Brazil is the country with the largest number of sequences generated, distributed through 26 sequencing studies, followed by Japan, with seven. Colombia and France had four studies each, and Argentina and Chile each had three. Two studies each were performed in Gabon and Spain, and in Cuba, India, Israel, Italy, Mozambique, the UK, Portugal, and Russia, only one study was performed. Finally, there were also six articles that did not provide information about the origins of the sequences (Fig. 3).

Discussion

In the 41 years since the discovery of HTLV-1, no effective therapeutic treatments or vaccines have been developed, and it is still not clear what determines different infection outcomes. During this period, diverse sequencing technologies have become available. The central aim of this systematic review was to summarize the different technologies used in the HTLV-1 field in order to guide the decision-making processes on the generation of new HTLV-1 genome sequences.

The Sanger method was the most commonly used for generating HTLV-1 sequences, followed by Illumina and Ion Torrent. All of these techniques have advantages and disadvantages. The characteristics of HTLV-1, as well as the specific aspects of each method must be taken into consideration.

One important aspect in HTLV-1 infection is that, after infection, the virus integrates into the host cell DNA as a provirus. Unlike HIV, in HTLV-1 infection, the circulating viral RNA is not easily detected in the plasma or serum, and additional techniques are usually needed prior to sequencing, such as PBMC separation and nested PCR [81, 82]. In this sense, the HTLV-1 sample extraction and preparation steps are an important point to consider during the choice of the sequencing platform to use.

Among the sequencing technology platforms, considering its low error rate, Sanger sequencing is considered the gold standard, despite being first-generation and having a high cost. Furthermore, it is possible to assess the sequencing quality based on other parameters, such as sequence length, sequencing depth, and GC content. One study reported that sequencing quality is more stable and GC depth distribution is better with Ion Torrent than with HiSeq 2000 [83]. Importantly, even when the goal is to sequence larger regions and/or the complete proviral genome, technologies such as Illumina and Ion Torrent produce small sequence reads. This read size, as well as the polymerase chain reaction (PCR) step, can impair the understanding of an essential aspect of HTLV-1 infection: clonality. While in patients with ATLL...
Fig. 1 Flow diagram for the systematic selection of studies to review
Sequencing technology platforms applied to HTLV-1 studies

**Table 1** Summary of information collected from the 62 selected studies, including sample origin, sequencing methodology, equipment, and number of sequences generated

| Reference | Sample origin | Sequencing methodology | Equipment | No. of sequences generated |
|-----------|---------------|-------------------------|-----------|---------------------------|
| [19]      | NA⁵           | Sanger                  | ABI³ 377A DNA sequencer | 218           |
| [20]      | NA            | Sanger                  | Hitachi Fluorescent DNA sequencer SQ-5500 | 39            |
| [21]      | NA            | Sanger                  | Hitachi Fluorescent DNA sequencer SQ-5500 | 64            |
| [22]      | France and UK | Sanger                  | ABI 373 automatic DNA sequencer | 17            |
| [23]      | Chile         | Sanger                  | Automated DNA sequencer | 37            |
| [24]      | Brazil        | Sanger                  | FmOl DNA sequencing system (Promega) | 2             |
| [25]      | France        | Sanger                  | ABI 377A DNA sequencer | 208           |
| [26]      | Japan         | Sanger                  | ABI 373 automatic DNA sequencer | 178           |
| [27]      | NA            | Sanger                  | SQ5500 automated sequencer | 138           |
| [28]      | Colombia      | Sanger                  | NA | 12            |
| [29]      | Italy         | Sanger                  | ABI PRISM automatic sequencer | 6             |
| [30]      | Chile         | Sanger                  | NA | 50            |
| [31]      | Chile         | Sanger                  | NA | 128           |
| [32]      | Spain         | Sanger                  | ABI 310 genetic analyzer | 4             |
| [33]      | Colombia      | Sanger                  | NA | 12            |
| [34]      | France        | Sanger                  | Applied Bioshstems 377 DNA sequencer | 1             |
| [35]      | Japan         | Sanger                  | ABI 377 DNA sequencer | 231           |
| [36]      | Colombia      | Sanger                  | ABI Prism serie 3700 | 11            |
| [37]      | Brazil        | Sanger                  | ABI 373 DNA Sequencer | 3             |
| [38]      | Russia        | Sanger                  | ABI 377 automatic DNA sequencer | 8             |
| [39]      | India         | Sanger                  | ABI automated DNA sequencer | 7             |
| [40]      | Brazil        | Sanger                  | ABI 377 Automated DNA Sequencer | 26            |
| [41]      | France, Gabon and Iran | Sanger | ABI Prism 377 and Ceq2000 sequencer | 65            |
| [42]      | Argentina     | Sanger                  | ABI model 377 automated DNA sequencer | 12            |
| [43]      | Brazil        | Sanger                  | ABI 377 Sequencer | 134           |
| [44]      | Israel        | Sanger                  | ABI automated sequencer | 1             |
| [45]      | Brazil        | Sanger                  | ABI Prism 377 DNA Sequencer | 2             |
| [46]      | Brazil        | Sanger                  | ABI 373 DNA Sequencer | 5             |
| [47]      | NA            | Sanger                  | ABI 310 sequencer | 334           |
| [48]      | NA            | Sanger                  | ABI 310 autossequencer | 316           |
| [49]      | Japan         | Sanger                  | ABI 377 DNA Sequence | 445           |
| [50]      | Brazil        | Sanger                  | ABI 3100 genetic analyzer | 46            |
| [51]      | Argentina     | Sanger                  | ABI Prism 3100 Genetic Analyzer | 44            |
| [52]      | Gabon         | Sanger                  | Automatic sequencing system (Euro Sequence Gene Services) | 34            |
| [53]      | Brazil        | Sanger                  | ABI 3100 genetic analyzer | 8             |
| [54]      | Brazil        | Sanger                  | ABI 3100 genetic analyzer | 5             |
| [55]      | Argentina     | Sanger                  | ABI PRISM 377 Automated DNA sequencer | 114           |
| [56]      | Japan         | Sanger                  | ABI 3730 Sequencer | 19            |
| [57]      | Brazil        | Sanger                  | ABI PRISM 310 Genetic Analyzer | 25            |
| [58]      | Brazil        | Sanger                  | ABI 3100 genetic analyzer | 8             |
| [59]      | Mozambique    | Sanger                  | ABI 3730 Automated DNA Sequencer | 25            |
| [60]      | Colombia      | Sanger                  | ABI PRISM 310 sequencer | 30            |
| [61]      | Portugal and Spain | Sanger | Automated DNA sequencing | 47            |
| [62]      | Brazil        | Sanger                  | NA | 1             |
| [63]      | Brazil        | Sanger                  | ABI 3130 genetic analyzer | 13            |
| [64]      | Brazil        | Sanger                  | ABI 3100 genetic analyzer | 32            |
| [65]      | Brazil        | Sanger                  | ABI 3100 genetic analyzer | 146           |
Table 1 (continued)

| Reference | Sample origin       | Sequencing methodology | Equipment                                      | No. of sequences generated |
|-----------|--------------------|------------------------|------------------------------------------------|---------------------------|
| [66]      | Brazil             | Sanger                 | ABI 3100 genetic analyzer                     | 18                        |
| [67]      | Cuba               | Sanger                 | Genome Lab Dye Terminator Cycle Sequence      | 12                        |
| [78]      | Brazil             | Illumina               | Illumina MiSeq System                         | 90                        |
| [68]      | Brazil             | Sanger                 | ABI 1373 Automated DNA Sequencer              | 14                        |
| [75]      | Brazil             | Sanger and Ion Torrent | ABI 3130xl Genetic Analyzer / Ion 314TM Chip 8-pack | 22                        |
| [69]      | Iran               | Sanger                 | ABI 3730 Sequencer                            | 2                         |
| [70]      | Brazil             | Sanger                 | ABI PRISM 310 Genetic Analyzer               | 2                         |
| [71]      | Japan and Brazil   | Sanger                 | ABI PRISM 3740 Genetic Analyzer              | 14                        |
| [72]      | Brazil             | Sanger                 | ABI PRISM 3100 Genetic Analyzer              | 24                        |
| [12]      | Japan              | Illumina               | Illumina MiSeq or NextSeq                     | 98                        |
| [73]      | Iran               | Sanger                 | NA                                             | 5                         |
| [79]      | Brazil             | Ion Torrent            | Ion 318™ Chip PGM                             | 31                        |
| [74]      | Brazil             | Sanger                 | ABI 3130 Genetic Analyzer                     | 132                       |
| [76]      | Brazil             | Sanger                 | ABI 3100 Genetic Analyzer                     | 21                        |
| [77]      | Brazil and Japan   | Sanger                 | ABI 1373 Sequencer                            | 90                        |

*ABI, Applied Biosystems; NA, not available; *, sequence region not described. Total for each instrument used: ABI 377/377A DNA Sequencer, 12; ABI 3100 Genetic Analyzer, 10; ABI PRISM 310 Genetic Analyzer, 6; ABI 373 DNA sequencer, 4; Hitachi Fluorescent DNA sequencer SQ-5500, 3; ABI 3730 Sequencer, 3; ABI 3130/3130xl genetic analyzer, 3; ABI PRISM automatic sequencer, 3; Automated DNA sequencer, 2; ABI 1373 Automated DNA Sequencer, 2; Illumina MiSeq, 2; ABI Prism 3700, 1; Fmol DNA sequencing system (Promega), 1; Ion 318™ Chip PGM, 1; ABI PRISM 3740 Genetic Analyzer, 1; Illumina NextSeq, 1; Ceq2000 sequencer, 1; Automatic sequencing system (Euro Sequence Gene Services), 1; Genome Lab Dye Terminator Cycle Sequence 1

Fig. 2 Numbers of sequences generated for each region of the HTLV-1 genome
Fig. 3  Geographic origin of HTLV-1 sequences and methodology used for sequence generation

Fig. 4  Number of HTLV-1 sequences generated between 2000 and 2020
there is a monoclonal pattern, in patients with IDH or HAM/TSP, and in asymptomatic carriers, a polyclonal pattern is found [84, 85]. Therefore, the small size of the genome sequencing readout may make it difficult to identify viral quasispecies and may give an unrealistic biological picture.

It is important to highlight that sequencing of viral genomes is important for understanding the infection process [86]. Therefore, the use of few and old sequencing methods, despite the emergence of more innovative, faster, and often less expensive technologies, makes the goal of developing better alternatives for infection control and the understanding of viral pathogenesis increasingly distant. In addition, animal models are important in HTLV-1 research and have allowed significant advances in the understanding of viral infection and pathogenesis. Each animal model has its advantages. Rats are used in studies involving HAM/TSP, and non-human primates are used in studies analyzing the immune response and viral persistence [87].

The emergence of new sequencing protocols has led to a reduction in the time required and production costs [88]. Despite that, no article included in this study used more recent technologies, such as the third-generation sequencing. MinION and PacBio could be an interesting alternative, due to their shorter processing time, despite providing sequences with regular quality, when compared to older methods. These methods can be useful in HTLV-1 research, increasing the number of partial and/or complete sequences available on the platforms and contributing to a better understanding of the virus-host relationship.

In addition to the predominance of the older techniques, most of the studies focused on sequencing specific regions of the genome, with few studies generating complete genome sequences. The LTR and pX regions were the most frequently sequenced. This could be because of the importance of the LTR for the subtyping and the fact that pX encodes the HTLV-1 regulatory proteins. In this context, it is relevant to point out that complete genome sequencing is essential for the identification of gene functions and their involvement in disease as well as for vaccine development.

This systematic review demonstrated a deficit in the number of HTLV-1 sequences. However, this study has an important limitation, since sequences can be deposited in databases such as GenBank without being necessarily associated with a published article. However, our data corroborate an ongoing study carried out by our group that highlights the deficit of complete HTLV-1 genome sequences available in the GenBank database. In this study, we verified that only 242 complete HTLV-1 genome sequences were available in the GenBank database, and most of these sequences did not include clinical and epidemiological information about the patient.

On the other hand, the majority of studies provided geographical information about the samples sequenced. Most of them were from endemic regions such as Japan and Brazil. Another country that deserves attention is Colombia. The Colombian island of Tumaco has a high population density and a very high prevalence of HAM/TSP, which is why this region is a focus of study of HTLV-1 [33]. Moreover, few articles from Africa were found, despite being the continent with the highest endemicity of HTLV-1 [1]. The European continent also contributes to the generation of HTLV-1 sequences, although relatively few articles describe the sequencing. Some studies did not report the origin of the sequence, which limits their epidemiological value. The sum of studies from each country does not correspond to the number of articles included, because some studies include samples from different countries, such as Bandeira et al., 2018 [71].

Interestingly, only 21 articles included in this review were published in the last 10 years, which is equivalent to almost 30% of the total number of studies, revealing that there is still low investment in research in the HTLV-1 field. The encouragement of more investments in HTLV-1 studies may contribute to an increased number of HTLV-1 sequences generated in different geographic regions, and this can assist in the understanding of the global and regional distribution of this virus [1].

There are gaps to be filled in relation to information on HTLV-1 infection. Although it was the first human retrovirus described and has been proven to be associated with the development of diseases, studies on the pathogenesis and treatment of this virus are not encouraged, and worse, investment in research is decreasing [89], demonstrating that HTLV-1 is still a neglected virus [90, 91]. Thus, more investment in HTLV-1 research and the implementation of worldwide prevention strategies will be the main motor for the eradication of these infections.

Conclusion

The analysis of the articles selected for this systematic review showed that the number of studies sequencing the HTLV-1 genome is much lower than for other retroviruses, and most of these studies still opt for Sanger sequencing despite the emergence of new methodologies. This demonstrates a lack of investment in this field. It is important to note that Sanger sequencing has advantages over other methods. However, NGS techniques also have characteristics that may be important for answering questions that remain about HTLV-1 infection. Investments in HTLV-1 research are needed, mainly in the use of more current methodologies, since they are methodologies that have been developed through lessons learned and improved by the previous generation.
Sequencing technology platforms applied to HTLV-1 studies

Author contributions FOA: methodology, formal analysis, investigation, writing. MSC: methodology, formal analysis, investigation, writing. MMNB: methodology, investigation and writing. RCN: methodology. LLG: conceptualization and writing—review and editing. FFAR: conceptualization and writing—review and editing. LAS: conceptualization and writing—review and editing. FKB: conceptualization, formal analysis, writing—review and editing and supervision. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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Declarations

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