Chapter

Metabarcoding and Digital PCR (dPCR): Application in the Study of Neglected Tropical Diseases

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Abstract

neglected tropical diseases such as Chagas disease, dengue, Zika, chikungunya, and malaria cause millions of deaths each year and they are caused by a variety of pathogens whose diagnosis is very limited or subject to conventional testing, making a treatment less accessible, accurate and timely diagnosis for choosing their treatments. Traditional methods for pathogen detection have not been able to meet the growing need for diagnosis and control. The incorporation of new technologies such as next-generation sequencing (NGS) and digital PCR (dPCR) represent a better diagnostic possibility due to their ability to absolutely quantify pathogens with high selectivity and precision. Our planet is currently experiencing environmental changes of an unprecedented magnitude and rate, including climate change, globalized pollution, biodiversity loss, and land use changes, so neglected diseases require a comprehensive understanding of the ecology of vectors in the different eco-epidemiological contexts, as well as of the transmission cycles of pathogens and their transmission dynamics. In this sense, NGS and dPCR open a new panorama for a better understanding of these diseases with the aim of proposing new programs for their care.

Keywords: neglected tropical diseases (NTDs), DNA, metabarcoding, dPCR, next-generation sequencing (NGS), diagnosis, pathogen detection, digital droplet PCR (ddPCR), ecohealth

1. Introduction

neglected tropical diseases (NTDs) can be caused by viruses, bacteria, parasites, fungi, and toxins. They occur mainly in tropical areas of 149 countries on the planet, mainly concentrated in Africa and Latin America, and are called "neglected" because at the political, health, and research levels, what is necessary has not been done to eliminate them, and, furthermore, the money allocated to serving them is practically nil. According to the World Health Organization (WHO), there are 20 NTDs and one-sixth of the world's population suffers from at least one of these diseases [1, 2]. The majority of the affected population live in poor socioeconomic conditions, with low income, low educational level, and precarious housing where, in addition to the

beliefs, attitudes and behaviors of these people cause social exclusion. NTDs predominate in tropical and humid climates, mainly in rural areas, conflict zones, and regions of difficult access. However, urban areas can also be affected. The presence of these diseases has been increasing due to factors such as climate change [3–5]. The absence of timely and accurate diagnoses, quality medical care, medicines, vaccines, and access to drinking water and sanitation services are the factors that increase the presence of NTDs and can only be faced with leadership and political effort economic as a whole since they do not top the list of priorities in public health policies. The development in the research of new and better medicines and diagnostic methods by the industry is very scarce. In addition, reliable statistical data makes it difficult for these diseases to be known by society [6–9].

These diseases are of increasing concern, as the geographic range of tropical diseases is expanding due to climate change, urbanization, changing agricultural practices, deforestation, and biodiversity loss [10]. Infectious diseases can be unpredictable with the potential risk of global outbreaks. However, the way we characterize pathogens has changed dramatically. Although increasingly sophisticated diagnostic tools have improved the ability to detect the presence of the pathogens that cause these diseases, this ability comprises only a small part of the set of tools necessary to generate an accurate etiological diagnosis to manage these health threats. For this, the ecoepidemiology of diseases must be taken into account, with an approach to the interdependence of individuals and their connection with the levels of individual, social, molecular, and environmental organization that participate in the causal processes of diseases [11, 12]. Over the years, molecular methods have evolved and improved, from conventional endpoint polymerase chain reaction (PCR) to real-time quantitative PCR (qPCR), DNA microarrays, digital droplet PCR (ddPCR), loop-mediated isothermal amplification (LAMP), and metagenomic approaches based on high-throughput next-generation DNA sequencing (HT-NGS). Such technological achievements have contributed to expanding the set of analytical tools used to solve various research objectives, including disease diagnosis. The next-generation sequencing (NGS) has generated a trigger in the discovery and characterization of pathogens, leaving behind old culture methods and techniques and making it possible to detect non-culturable pathogens that were previously difficult to diagnose. On the other hand, due to its sensitivity and quantitative characteristics, digital polymerase chain reaction (dPCR) is a potential candidate to become an attractive new method among molecular technologies for parasite detection and quantitative analysis, favoring a better understanding of the transmission dynamics of the pathogens that cause these diseases [13–18].

2. Next generation sequencing (NGS)

2.1 DNA sequencing: history and current status

The description of the molecular structure of DNA in 1953 by Watson and Crick, reported in their article "molecular structure of nucleic acids: A structure for deoxyribose nucleic acid" [19], became the basis of genomic research allowing technological development and research. At the beginning of the 70s, studies on DNA sequencing began, Maxam and Gilbert in February 1977 stated that DNA can be sequenced by a chemical procedure [20]. However, the first enzymatic method for DNA sequencing was proposed by Sanger and Coulson in 1975 [21]. In 1982, Caruthers and Hood

developed the first automated method for sequencing DNA [22], which was capable of sequencing fragments from 5 to 75 base pairs (bp), and in 1986, Hood and Smith designed the first automatic sequencer that used laser beams that recognize fluorescent markers on DNA [23–25]. By 1985, the first automated sequencers using gels (Applied Biosystems PRISM® 373) or polymer-coated capillaries (ABI PRISM® 310) appeared [26]. Later, in 2004, new sequencing methods emerged, based on pyrosequencing and the so-called next generation sequencing (NGS) platforms. Currently, due to the fact that new sequencers have appeared on the market that are capable of applying other sequencing technologies in parallel, it is more appropriate to speak of High-Throughput Sequencing (HTS) or massive sequencing. There are some variants of this technology such as sequencing by ligation (*sequencing by oligonucleotide ligation and detection*), sequencing by synthesis and semiconduction, and sequencing by synthesis in clusters (sequence length up to 600 bp). A third generation of sequencers is those that use single molecule sequencing (*single molecule real-time* [SMRT]) that allows sequencing of much longer molecules, up to 30 kb [27–29]. There are other sequencing technologies in the development phase, those based on nanopores; which are based on the identification of the different bases of the DNA chain, thanks to an optical signal or by the variation that occurs in an electric current when the chain passes through a nanopore anchored to a membrane, in situ nucleic acid sequencing; which is a progression of single-cell RNA sequencing methods, is performed intracellularly within intact tissues, thus preserving the spatial context of gene expression within and between cell types and sequencing based on direct observation with microscopy that uses electron microscopy and allows the DNA sequence to be read directly by optical methods without the need for amplification (**Figure 1**) [30, 31] .

2.2 Inside to the NSG methodology

High-throughput DNA sequencing techniques are capable of sequencing a large number of different DNA sequences in a single reaction, which is why they are also

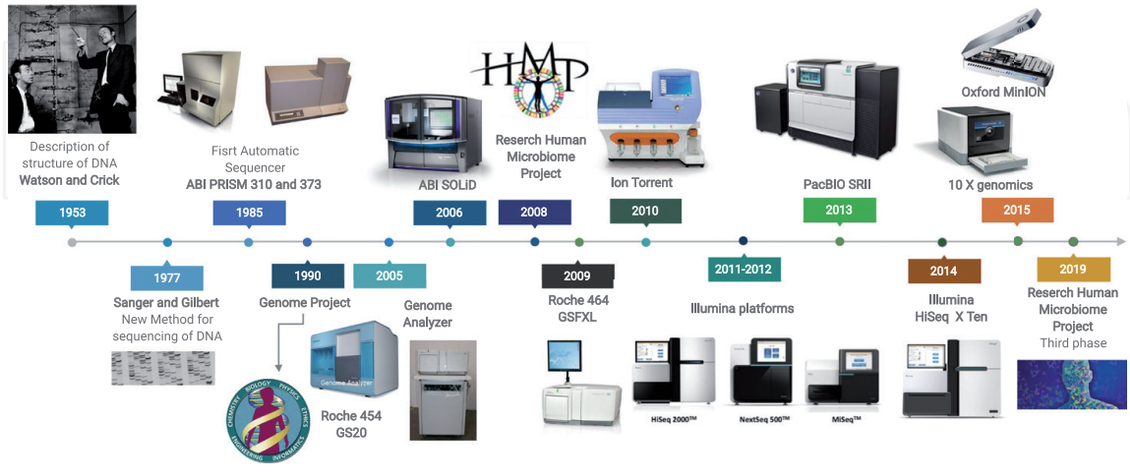

Figure 1.
Sequencing technology timeline. Research in DNA sequencing technologies is in continuous development. In the last decade, NGS tests have established their value as a diagnostic test, given their good performance for the detection of genetic diseases and the discovery of new pathogenic variants. This technology has facilitated molecular diagnosis, being a more efficient and faster tool in gene sequencing, and has facilitated the identification and classification of multiple genetic variants together with their respective pathological association. Additionally, the costs of sequencing and the duration in which the diagnosis is defined have been reduced, which allows timely prevention measures to be established in patients with high risk in the future.

known as "next generation" and "massive parallel" DNA sequencing. In general, the material to be sequenced is double-stranded DNA; however, genomic DNA, reverse transcribed RNA or cDNA, immunoprecipitated DNA can be used [32]. Nucleic acid templates are first prepared for sequencing (library generation), this step differs according to the platform to be used. For a short read, three steps generally apply: (1) fragmentation of DNA to application-specific template lengths, (2) ligation of adapters to facilitate attachment of fragments to solid surfaces (such as microchips, microspheres, or nanowells) or to circularize, and (3) amplification of templates for provide enough copies of each template to allow the sequencer to detect them. Libraries can be read from one end only (single-ended read), or from both ends (double-ended reads). Some technical errors can occur when preparing libraries that are related to the amplification and sequencing of the PCR products, mainly inherent polymerase errors and inefficiencies associated with the guanine-cytosine (GC) content of the template. To solve this, libraries have been prepared without the use of the PCR reaction for short-read platforms, increasing the fidelity of the sequence. Long read platforms do not require an amplification step.

After library preparation, which attaches adapters to approximately 450 bp DNA fragments, the templates are annealed to a glass slide bearing complementary adapters. Once attached to the solid surface, the fragments are amplified by PCR from either one end or both ends, producing billions of sets of DNA fragments from the clonal template that can be sequenced simultaneously. Raw obtained sequence reads are typically set to FASTQ format [33]. Subsequently, the sequences are aligned with a reference genome using an alignment tool [34] in order to obtain a map file of the sequence alignment (read assembly and base sequence) (**Figure 2**). A typical whole genome sequence will produce on the order of 5 million SNVs and 250,000 short

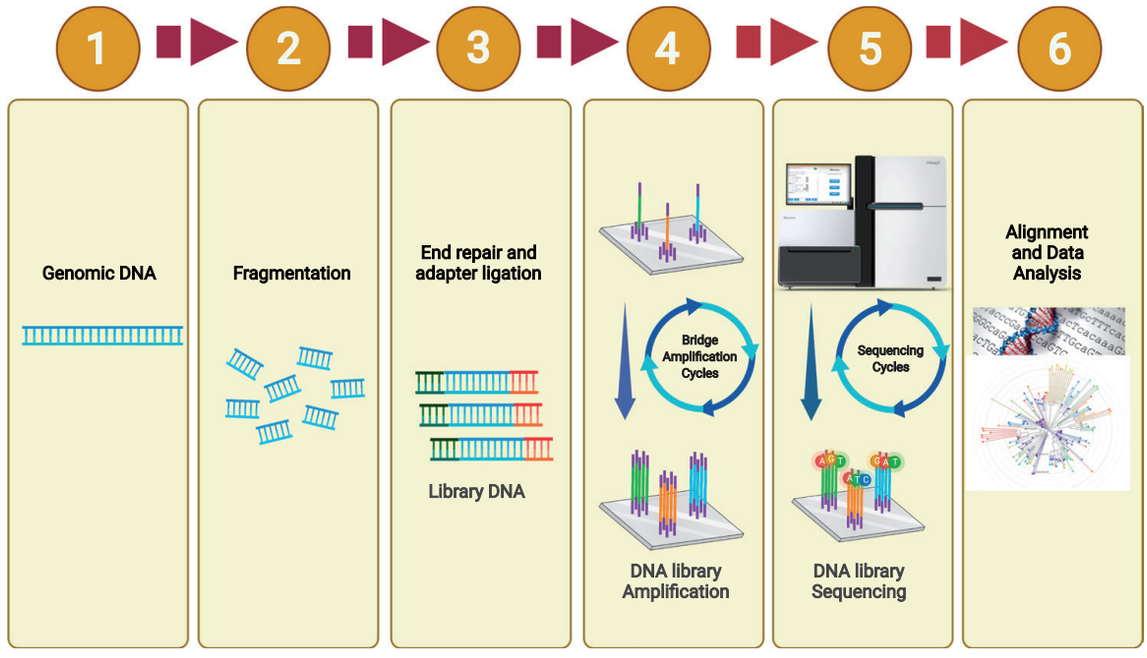

Figure 2.
Next generation sequencing (NGS) is a group of technologies designed to sequence large numbers of DNA segments massively and in parallel, in less time and at a lower cost per base. The methodological approach can be summarized in five steps: 1) segmentation of the DNA into several fragments, 2) labeling of the DNA by means of primers or adapters that indicate the starting point for replication, 3) amplification of the DNA fragments labeled with adapters by methods based on polymerase chain reaction, 4) sequencing or reading of the DNA fragments, and 5) reconstruction of the complete sequence by means of reference sequences and export to data storage files.

indels. Ignoring common single nucleotide polymorphisms and short insertions or deletions (indels), there are typically about 300,000 unique variants left to consider (**Figure 2**) [31, 35, 36].

3. Digital polymerase chain reaction (dPCR)

3.1 The evolution of PCR

Since the development of the polymerase chain technique (PCR) in 1971 when Gobind Khorana described the technique by explaining the replication of a DNA fragment using two primers [37]. But it was in 1983 that Kary Mullis and his colleagues at the Californian company Cetus Corporation first carried it out while working on making oligonucleotides and using primers for DNA sequencing. Used two primers that aligned with each of the DNA strands, added *Escherichia coli* DNA polymerase I and triphosphated nucleotides. As a result, they obtained the exponential replication of the DNA fragment flanked by the primers [38]. This endpoint PCR technique has evolved thanks to two factors, the discovery of the Taq polymerase and the thermal cycler (**Figure 3**). Thus, today we can find variants such as: reverse transcription PCR (RT-PCR) [39, 40], Circularized RT-PCR (cRT-PCR) [41], inverse PCR [42], nested PCR [43], in situ PCR [44], multiplex PCR [45], multiplex-ready PCR [46], asymmetric PCR [47], Ligation-anchored PCR [48], long fragment PCR [49], Fluorescent Quantitative PCR (Real Time PCR; qPCR) [50], Immune PCR (IPCR) [51], and digital PCR (dPCR) (**Figure 3**). dPCR was first conceptualized in the 1990s. It is based on limiting dilutions, PCR and Poisson distribution [52]. dPCR is classified into digital droplet PCR (ddPCR) and digital chip PCR (cdPCR). cdPCR presents a challenge to achieve high throughput, however, ddPCR can overcome this impediment [53]. It is worth mentioning that isothermal amplification methods called LAMP (LOOP-mediated isothermal amplification) have been developed that, unlike PCR technology, amplify the target sequence at a constant temperature of 60–65°C. based on strong strand displacement DNA polymerase and 4–6 specifically designed primers can be used that can recognize and amplify six or eight regions of a given target

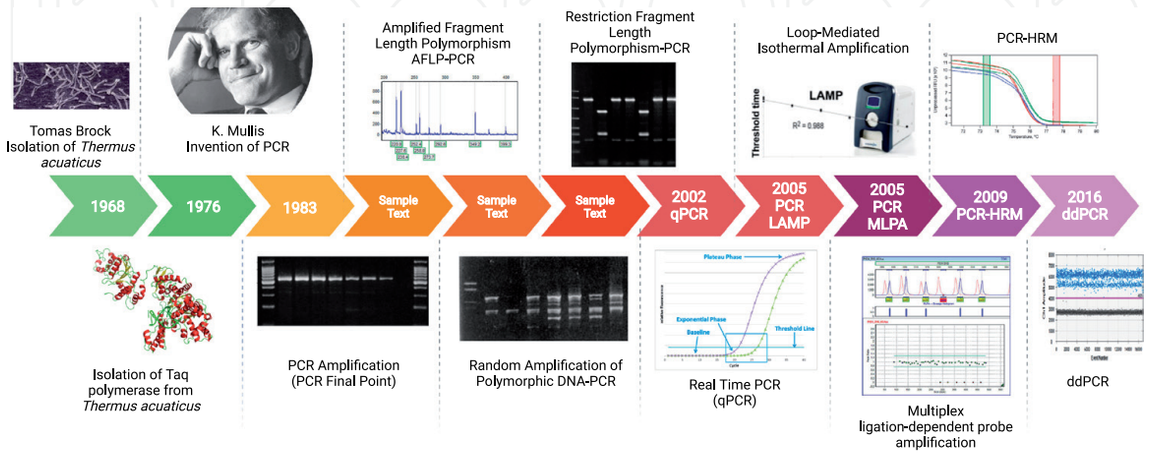

Figure 3.
Evolution of the PCR technique. PCR technology has undergone continual improvements. The applications of PCR are multiple, encompassing from the evolution to the clinic, passing through the genetics, molecular biology, and biotechnology; in addition to applications in agriculture and livestock. The applications of this new technique seem to have no limits.

gene to achieve higher analytical specificity. Amplification signals can be detected by photometry of the turbidity of the reaction or by colorimetry of the fluorescent intensity of the intercalated dyes. This approach offers a fast (1 h), a low-cost, easier-to-use, thermocycler-free alternative method for PCR [54, 55].

3.2 Principles and benefits of dPCR

The basis of ddPCR is to distribute a nucleic acid-containing sample to thousands of independent partitions. There are several ways to create these droplets: manual partitioning, immiscible liquid chemistry, atomization, etc. The generated droplets contain only one target DNA molecule or none at all [56]. To determine target DNA copies without bias, templates with target DNA must be randomly distributed and microdroplets must be produced in large numbers. These partitions can be individually amplified through thermal cycling. Unlike qPCR, which produces an exponential signal and quantifies samples by comparing their CTs (threshold cycle) to a standard curve generated by well-defined samples; by determining the concentration of the sample using an "analog" method, ddPCR technology generates linear digital signals that allow quantitative analysis of the PCR product, being able to detect very rare mutations with high precision and sensitivity, and these amplicons can be quantified without a curve standard [53]. Quantification of DNA molecules is performed by a combination of Poisson distribution and dilution templates at the single-molecule level. The number of templates correlates positively with positive wells, so the exact number of template copies can be calculated. The use of the presence and absence of signals to indicate the target DNA makes a direct "digital" measurement of the samples [57] (**Figure 4**). Furthermore, due to high pipetting handling in sample preparation and PCR conditions, even with a standard curve, the data disparity in qPCR is greater than in ddPCR. ddPCR can be used to detect low concentrations of DNA [58, 59].

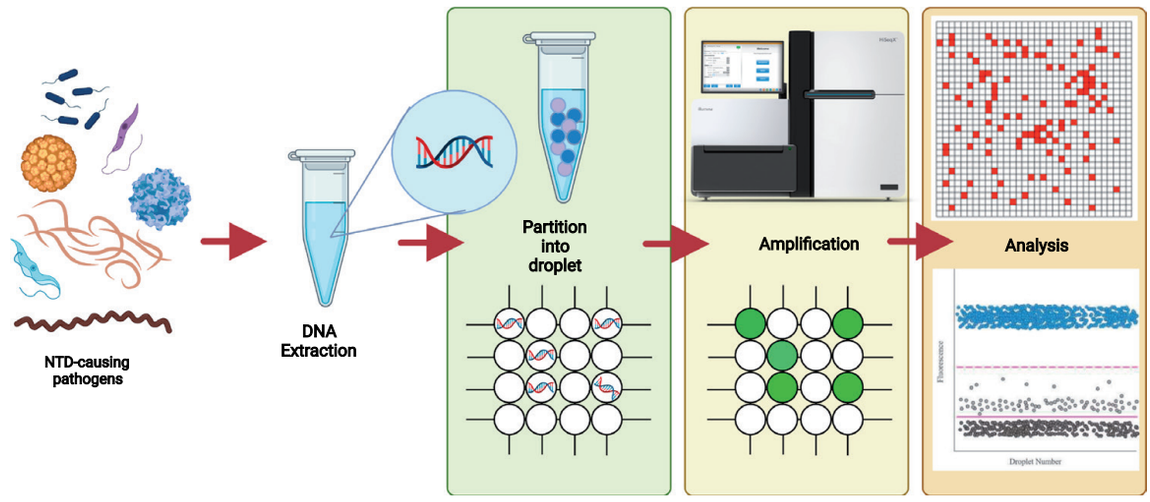

Figure 4.
Digital droplet polymerase chain reaction (ddPCR) is a highly sensitive quantitative polymerase chain reaction (PCR) method based on fractionation of the sample into thousands of individual nano-sized water-in-oil reactions. Recently, ddPCR has become one of the most accurate and sensitive instruments for DNA detection. Consists of simple steps to follow: 1) prepare PCR-ready samples before starting ddPCR, 2) droplet generation, 3) droplet amplification by PCR; 4) Reading of drops, and 5) analysis of results. Positive droplets, which contain at least one copy of the target, exhibit greater fluorescence than negative droplets.

4. Uses of NSG and dPCR in the diagnosis of the causal agents of NTDs

NTDs are caused by several types of common and rare pathogens. A common concern with conventional testing methods is the limitation in the range of pathogens that can be detected and the lack of sensitivity for their diagnosis. Obstacles such as incomplete knowledge of natural history make it difficult to understand the ecology and pathogenesis of rare and neglected diseases. Emerging technologies, including NGS and dPCR, provide opportunities to accelerate the diagnosis and development of treatments for these diseases [60, 61]. The application of NGS in Leishmania isolates has allowed the characterization of populations through the identification and analysis of variations. Information on population structure can reveal important insights into disease dynamics and identify genetic backgrounds associated with parasite virulence and ecology [62]. The metagenomic analysis of the Leishmania vectors revealed the microbiota present in them, these studies will allow us to understand how the microbiota interacts with the parasite vectors and to develop tools for biological control [63]. Also by means of NGS, it has been observed that some HLA class I and class II genes could be involved in the predisposition of cutaneous leishmaniasis [64].

Mycetoma is one of the neglected tropical diseases, characterized by painless subcutaneous inflammation, multiple paranasal sinuses, and discharge containing aggregates of the infectious organism known as pimples. Studies of host genetic variation in mycetoma susceptibility by NGS will allow the identification of new treatments for mycetoma and will also improve the ability to stratify 'at risk' individuals, allowing the possibility of developing preventive and personalized clinical care strategies in the future [65]. The application of NGS in the study of malaria has greatly contributed to a better understanding of Plasmodium biology as well as host–parasite interactions [66].

Trypanosoma cruzi, the etiological agent of Chagas disease, represents a challenge due to its repetitive nature. Only three of the parasite's six recognized discrete typing units (DTUs) have their draft genomes published, and, therefore, analyzes of genome evolution in the taxon are limited, thus the assembly of short NGS reads can be applied for the detection of highly repetitive genomes [67]. Also, single nucleotide polymorphisms (SNPs) have been identified in the protein sequences of *T. cruzi* [68] and studies have been carried out with an EcoHealth approach [69]. These results may lead to a better understanding of Chagas disease and will provide further development of biomarkers for the prognosis, diagnosis, and development of drugs for the treatment of Chagas disease.

Comparisons of *Treponema pallidum* genomic sequences using NGS have revealed a modular structure of several genomic loci. This diversification of *T. pallidum* genomes appears to be facilitated by genome recombination events within the strain [70]. On the other hand, unbiased sequencing of the Zika virus genome obtained by NGS from the cerebrospinal fluid of one patient revealed that no virus mutations associated with anatomical compartments were detected [71] and NGS has also been used in different experimental and epidemiological settings to understand how the adaptive evolution of dengue variants shapes the dengue epidemic and disease severity through its transmission [72].

ddPCR has been shown to be more accurate than qPCR; therefore, it has been finely modified to detect low-abundance nucleic acids, which might be more suitable for clinical diagnosis [59]. Human strongyloidiasis is one of the neglected tropical diseases caused by infection with soil-transmitted helminth *Strongyloides stercoralis*. Conventional stool examination, a method commonly used for diagnosis of *S.*

stercoralis, has low sensitivity, especially in the case of light infections. However, the use of ddPCR showed high sensitivity and specificity for the detection of *S. stercoralis* in stool samples. This technique can help improve diagnosis, especially in cases of mild infection. In addition, the ddPCR technique could be useful for the detection of patients before starting immunosuppressive drug therapy and the follow-up after treatment of strongyloidiasis [73]. The usefulness of the ddPCR platform in the detection of *T. cruzi* infection has also been evaluated. The clinical sensitivity and specificity of the assay were both 100%, with perfect agreement between positive and negative qPCR and ddPCR results in the clinical samples tested. However, the fact of not performing a calibration curve in ddPCR offers an advantage for its use in the diagnosis of *T. cruzi* [74]. Moreover, RT-ddPCR in dengue diagnosis could help harmonize DENV quantification results and improve field findings, such as identifying a DENV titer threshold that correlates with disease severity [75, 76]. The use of ddPCR to absolutely quantify human malaria parasites successfully detects *Plasmodium falciparum* and *Plasmodium vivax*, and the sensitivity of ddPCR to detect *P. falciparum* is significantly higher than qPCR [77].

NGS and ddPCR have recently shown great potential for pathogen detection, however, in a comparative study between these techniques, the results were subject to their respective limitations and strengths, the ddPCR method being more useful for rapid detection of common isolated pathogens, while the mNGS test is more appropriate for diagnosis where classical diagnostic methods (microbiological or molecular) fail to identify the causative pathogens [78]. NGS is a new technology that holds the promise of improving our ability to diagnose, interrogate, and track infectious diseases. For its part, the third generation of the PCR; ddPCR can be used to directly quantify and clonally amplify DNA, the latter has been widely used in the detection

NTD: Pathogen	Study objective*	Method	Reference
Anquilostomiasis *Necator americanus Ancylostoma duodenale*	—	—	—
Ascariosis *Ascaris lumbricoides*	Identification of repeated sequences for diagnostic use	NGS	[79]
Schistosomiasis *Schistosoma duodenale*	—	—	—
Cysticercosis *Taenia solium*	Diagnosis in cerebrospinal fluid	NGS	[80, 81]
	Identification and characterization of microsatellites		[82]
	Analysis and identification in soil samples	ddPCR	[83, 84]
Chagas disease *Trypanosoma cruzi*	Detection of *T. cruzi* infection	ddPCR	[74]
	Identification of trypanosomatid species in mammalian reservoirs, human patients, and sandfly vectors from endemic regions of leishmaniasis	NSG	[85]
	Detection of *T. cruzi*-specific antibodies		[86]
	EcoHealth/One Health approach		[69]

NTD: Pathogen	Study objective*	Method	Reference
Leishmaniasis	Identification of trypanosomatid species in mammalian reservoirs, human patients, and sandfly vectors from endemic regions of leishmaniasis	NSG	[85]
	Assembling nuclear genomes		[87]
	Antimony resistance gene analysis		[88]
	Parasite-host interaction for the search for target proteins used in prognosis and/or infection control		[89]
	Descriptive analysis of genomes		[62]
	Diagnosis from Cutaneous Leishmaniasis in patients	ddPCR	[90]
Leprosy *Mycobacterium leprae*	Detection of Infections	ddPCR	[91, 92]
	Identification of risk genes that affect susceptibility to leprosy	NSG	[93]
Trachoma *Chlamydia trachomatis*	Detecting and quantifying ocular	ddPCR	[94, 95]
Yaws *Treponema pallidum*	Subspecies identification	NSG	[96]
	Reconstruction of genomes		[97]
	Evolutionary origin		[98]
	study syphilis pathogenesis	NSG and ddPCR	[99]
Rabies Lyssavirus	Phylogenetic analysis	NSG	[100]
	Genetic and antigenetic characterization	NSG	[101]
Dracunculiasis (guinea-worm disease)	—	—	—
Buruli ulcer *Mycobacterium ulcerans*	Transcriptome analysis	NSG	[102]
	Analysis of recurrent infections due to continuous exposure		[103]
Trichuriasis *Trichuris trichiura*	Clinical diagnosis	ddPCR	[104]
Malaria *Plasmodium vivax Plasmodium falciparum*	Quantification in samples from asymptomatic patients	ddPCR	[77]
Onchocerciasis *Onchocerca volvulus*	analysis of the presence of Wolbachia	NSG	[105]
Elephantiasis (Lymphatic filariasis) *Wuchereria bancrofti*, *Brugia malayi*	Identification of sequences for diagnostic use	NSG	[106]
	Detection and quantification in blood samples and mosquitoes		[107]
	Differential gene expression	ddPCR	[108]
Fascioliasis *Fasciola hepatica* *Fasciola gigantica*	Identification of virulence genes	NSG	[109]
	Sequencing of the complete mitochondrial genome		[110]

NTD: Pathogen	Study objective*	Method	Reference
Sleeping sickness *Trypanosoma brucei*	Aneuploidy analysis	NSG	[111]
Dengue DENV	Quantification in clinical samples	RT-ddPCR	[75]
	Identification of viral pathogens in clinical samples from travelers	NSG	[112]
Zika ZIKZV	Detection and quantification in swimming pool water	RT-ddPCR	[113]
Chikungunya CHIKV	Identification and confirmation of pathogens in samples of known etiology	NSG	[114]
CHIKV, DENV, *P. vivax*, ZIKV	Epidemiological surveillance	NSG	[115]

**Only the papers deposited in the PubMed database are presented.*
DENV, Dengue virus, CHIKV, Chikungunya virus; ZIKV, Zika virus.

Table 1.
Use of NSG and ddPCR in the study of NTDs.

of low abundance nucleic acids, being useful in the diagnosis of infectious diseases and may be a better option than qPCR for clinical applications in the future. However, it is clear that to date there are few studies on the application of these technologies to the study of NTDs (**Table 1**).

5. NGS and dPCR as tools for understanding NTDs

Understanding health from different areas, based on the circumstances in which people are born and develop their lives, health systems, public policy, and environmental factors, among others, is to understand it from the approach proposed by Ecohealth. This comprehensive vision takes into account the diversity of factors that affect the presence or absence of any disease in humans and not only considers that people's health depends on the effects of pathogenic microorganisms or toxic substances. Ecohealth addresses what are known as social determinants of health [116, 117]. To analyze these determinants, it must be understood that there are singular, particular, and general dimensions that condition the health status of individuals and societies.

The control of NTDs requires novel visions and approaches that effectively address the determinants of transmission of a group of very diverse and complex pathologies that only share in some cases being transmitted by vector insects, but that have very different clinical characteristics. Their diagnoses require techniques of varying complexity and cost, they are transmitted in very specific contexts (urban, rural, and jungle), they affect the general population to varying degrees, their treatment varies in efficacy and cost, and control faces different technical challenges and operations that make the success of the programs very uncertain. On the other hand, frequent changes in environmental and ecological conditions, the dynamics of social and economic forces and the influence of cultural and gender determinants dominate the patterns of presentation and control of vector-borne diseases (endemic, epidemic, emergency, deletion, etc.). That is why the traditional approaches focused exclusively on the vector, based on the intensive and massive use of insecticides, with vertical

programs isolated from health services and operational personnel far away or oblivious to the needs of the communities, are not having an impact on the profile epidemiology of each of the NTDs such as dengue, Chagas disease, and malaria to mention some NTDs [118, 119]. Based on these needs, the Ecohealth model emerges, incorporating a transdisciplinary approach in the study and care of DNTs, emphasizing social participation as a central actor in the solutions to these health problems, and proposes an approach to inequality of gender as an element to insert in prevention and control programs. The model demands a multi- and intersectoral vision to contain them in a sustainable manner and provides evidence to strengthen operational programs [120]. In this sense, NSG and dPCR can easily be very powerful tools for the comprehensive study of DNTs from an Ecohealth point of view. For example, using next-generation sequencing metagenomic analysis, a variety of viral families in mammals and rodents were studied, and the results obtained allowed us to know the viral community in wildlife that at a given moment could become a critical point for the development of an emerging disease [121]. Other work on the presence of enteroviruses (EV) in nonhuman primates through phylogenetic analysis revealed that one virus strain was related to human clinical isolates, suggesting zoonotic behavior [122]. Studies related to Chagas disease vectors have made it possible to analyze the blood-feeding sources of multiple species of triatomines with much greater sensitivity and also to identify multiclonal infections with *T. cruzi*, which must be taken into account to develop transmission networks and characterize the risk of human infection, eventually leading to better control of disease transmission [69, 123–125]. These studies represent a powerful approach to unravel and understand transmission dynamics at microgeographic levels of Chagas disease.

On the other hand, ddPCR, which is considered a biotechnological refinement of conventional polymerase chain reaction methods that can be used to directly quantify and clonally amplify DNA, has been widely used in the detection of low-abundance nucleic acids and is useful in diagnosing infectious diseases including

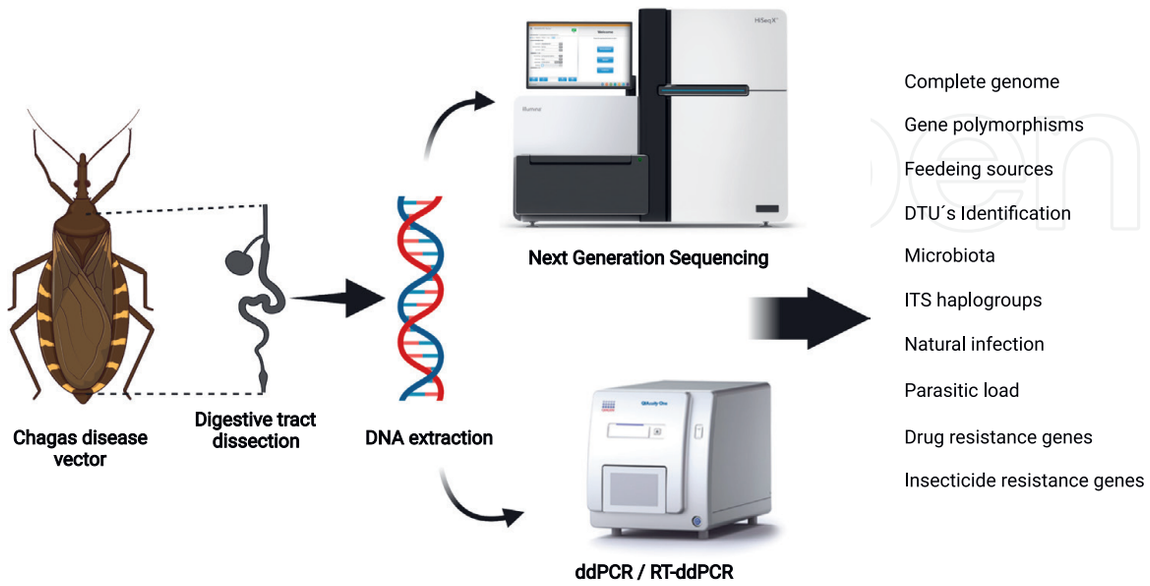

Figure 5.
The promotion of research with ecosystem approaches to human health (EcoHealth) is of great importance to contribute to improving the health of communities in the poorest regions of the world, for example, in Chagas disease, where new technologies, such as NSG and ddPCR, may have a better scope for a better understanding of the disease and thus propose new intervention policies. This strategy should be applicable to the study of all NTDs.

viral, bacterial, and parasitic infections, concluding that ddPCR provides a more sensitive, accurate, and reproducible detection of low-abundance pathogens [126, 127]. For example, Multiplex RT-ddPCR could help characterize defective genomes by simultaneously quantifying multiple regions of the same DENV RNA molecule, and in samples where quantities are limited, the application of RT-ddPCR could identify patients with burdens. Elevated viral levels, especially during the first days of the disease, could not be carried out with other methodologies due to the scarcity of the sample [75]. Therefore, the inclusion of NSG and dPCR techniques should be used jointly for the comprehensive study of NTDs so that in the future they allow the generation of interventions or sustainable strategies for vector control and transmission prevention of the NTDs (**Figure 5**).

6. Drawbacks of NSG and dPCR

One of the most common issues affecting the efficiency of NGS and dPCR is sample quality. Although platforms are often tested and compared using highly selected samples, real-life samples do not behave in the same way. Although "next generation" massively parallel DNA sequencers have been shown to offer many potential benefits in performing genetic analyses, especially for large-scale projects, one of the main drawbacks limiting their use is the relatively low error rate highly compared to standard short-read methods. In general, the main disadvantages observed in the different existing platforms are the presence of a bias in the CG ratio, high cost, and errors in the homopolymers (substitution/deletion errors in the readings) because their length is not correctly deduced from the electrical signal, the introduction of new algorithms, post-sequencing correction tools and the SNV/insertions-deletions (INDEL) tool [128], low output, short reads, and high error rate [129]. Likewise, there are also problems with long-length reads. In general, NSG and other long-read sequencing platforms suffer from a high error rate [130]. On the other hand, accuracy stands out as the most important issue for all recently developed technologies. For example, in metagenomic whole genome sequencing (mWGS), which analyzes all of the DNA or RNA in a given sample, in addition to high cost, an additional consideration for performing mWGS is the abundance of human DNA present in many types of clinical samples, such as blood and respiratory secretions [131]. Another barrier to the implementation of NGS in the diagnosis of infectious diseases has been the time it takes to complete all the steps for NGS, this turnaround time relative to conventional methods has limited the clinical relevance of NGS results for decision-making decisions about patient care [132].

On the other hand, although dPCR has numerous advantages over qPCR, it has not yet been able to fully replace the use of qPCR. It has been reported that high concentrations of nucleic acid could saturate the dPCR reaction, highlighting the importance of adequate dilution [133–136]. The low throughput compared to qPCR and the longer response times of current dPCR systems have not allowed this technique to enter routine analysis. On the other hand, the exclusivity of reagents based on the platform used is another limitation as it does not allow working with a different platform, including the multiplexing approach. Some dPCR protocols show a dynamic range similar to or smaller than that offered by a qPCR assay, which in some cases decreases the sensitivity of the assay, and lastly, the costs of equipment and reagents are still somewhat inaccessible [74, 137].

7. Conclusions

Reflecting on the public health problem that NTDs implies worldwide, improving the efficiency of the diagnosis of these pathologies is of vital importance for the development of more effective treatments. At the same time, an accurate diagnosis will limit the abusive use of drugs, thus reducing the appearance of resistance phenomena in the pathogens that cause these diseases. The traditional clinical diagnosis of infectious diseases consists of a differential analysis supported by a series of tests in order to identify the causal pathogen, which ranges from microscopic observation, the culture of microorganisms, and the detection of antibodies to the amplification of nucleic acids. Although molecular diagnostic assays are a rapid way to diagnose the most common infections, almost all current conventional microbial assays target a limited number of pathogens, these limitations mean that pathogens are not detected in up to 60% of cases and sometimes the use of cultures from clinical samples is required, where many of these pathogens are difficult to culture. Although the application of these molecular methods is a routine tool for the diagnosis of numerous diseases, they have not yet been fully used for the diagnosis of NTDs. In this sense, regardless of the current advantages and disadvantages of NGS and dPCR, these techniques have a promising application to contribute to an improvement in the diagnosis of NTDs, since they provide rapid results, allow the determination of parasitaemia in asymptomatic infections and low density, as well as the detection of pathogens difficult to cultivate. Both techniques have a higher sensitivity than conventional techniques used for diagnosis. However, more evaluations are needed, and especially the development of studies that combine NSG and dPCR technologies for the generation of comprehensive knowledge that allows a better understanding of NTDs in different ecoepidemiological contexts, as well as in the dynamics of the transmission cycles of the pathogens that cause these diseases and thus meet the goals, intermediate measures, and indicators of the roadmap on neglected tropical diseases 2021–2030 to prevent, control, and eliminate NTDs.

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Conflict of interest

The authors declare no conflict of interest.

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