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Review

Current Applications of Digital PCR in Veterinary Parasitology: An Overview

Constantina N. Tsokana [1], Isaia Symeonidou [1,*], Georgios Sioutas [1], Athanasios I. Gelasakis [2] and Elias Papadopoulos [1]

[1] Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; nt.tsokana@live.com (C.N.T.); gsioutas@vet.auth.gr (G.S.); eliaspap@vet.auth.gr (E.P.)

[2] Laboratory of Anatomy and Physiology of Farm Animals, Department of Animal Science, School of Animal Biosciences, Agricultural University of Athens, 11855 Athens, Greece; gelasakis@aua.gr

* Correspondence: isaia@vet.auth.gr; Tel.: +30-69-38008589

Abstract: Digital PCR (dPCR) is an emerging technology that enables the absolute quantification of the targeted nucleic acids. The body of research on the potential applications of this novel tool is growing in human and veterinary medicine. Most of the research on dPCR applications in veterinary parasitology is concentrated on developing and validating new assays to detect and quantify parasites of great financial impact in the food-producing animal industry. Several studies describe the utility of dPCR for individualized medicine in companion animals. Most frequently, dPCR performance is assessed compared to quantitative PCR or Next Generation Sequencing platforms, while others also compare the accuracy of dPCR with traditional parasitological techniques considered gold standard methods. Other researchers describe dPCR assays for surveillance purposes, species identification, and quantification in mixed parasitic infections, the detection of mutations indicative of anthelmintic resistance, and the identification of new targets for drug development. This review provides an overview of the studies that employed dPCR in investigating animal parasites and parasitic diseases from a veterinary perspective and discusses how this novel technology could advance and facilitate diagnosis, surveillance, and the monitoring of response to treatment, or shed light on current gaps in our knowledge of the epidemiology of significant veterinary parasitic diseases.

Keywords: digital PCR; *Dirofilaria immitis*; *Echinococcus multilocularis*; gastrointestinal nematodes; poultry; protozoa; ruminants; *Schistosoma japonicum*; veterinary parasitology

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1. Introduction

The advent of molecular methods has been a game changer and deepened our understanding of veterinary parasitology. The invention and application of DNA-based molecular tools, such as Polymerase Chain Reaction (PCR) and quantitative PCR (qPCR), followed by high-throughput sequencing technologies, gave new insights into molecular diagnostics and research in this field [1].

Digital PCR (dPCR) is an emerging technology that enables the absolute quantification of the targeted nucleic acids. It is regarded as a vastly improved version of conventional PCR and qPCR [2]. For the time being, most studies on parasitic diseases of veterinary importance involve developing and validating dPCR assays and evaluating their performance compared to qPCR or Next Generation Sequencing (NGS) platforms. Several studies also compare the sensitivity of dPCR with traditional parasitological techniques that are considered gold standard methods [3–6]. Other researchers described dPCR assays for surveillance purposes, species identification, and quantification in mixed infections, the detection of mutations indicative of anthelmintic resistance, as well as the identification of new targets for drug development [5,7–9].

This novel technology is based on endpoint quantification after sample division. It also provides information on the precise analyte concentrations in the original sample, expressed in DNA copies per microliter, along with confidence interval estimations [2]. The dPCR assays usually combine probe-based PCR (typically TaqMan probes) with a microfluidics analysis platform. One of the major advantages of dPCR compared to qPCR is that it does not require reference samples and a standard curve for the quantification of the parasitic burden [10]. Moreover, the assay's performance is not affected by PCR inhibitors, as the sample partitioning has a dilution effect for the inhibitors [4]. However, the cost per sample is still significantly higher than conventional and qPCR, and it lacks the potential to perform DNA sequencing to confirm a positive result due to the extremely low number of amplicons per droplet (Table 1) [10,11].

Table 1. Advantages and disadvantages of the dPCR technology.

Advantages	Disadvantages
Absolute quantification	High cost
No reference samples required	Inability to sequence the PCR amplicons
No standard curve required	
Better performance in the presence of inhibitors	
High sensitivity	

Among the different dPCR forms, the droplet dPCR (ddPCR) is the most commonly used in veterinary parasitology [4,7,12]. The general principles and technical advantages have been described in detail elsewhere and are beyond the scope of this review article [2,13,14]. Concisely, the PCR reaction is subdivided into thousands of small partitions (droplets), uniform in volume, by various available methods. These droplets optimally contain only a single or no template DNA molecule. After PCR amplification, a specialized reader measures the number of fluorescent droplets that corresponds to the number of template molecules with a particular fluorescence (Figure 1) [10].

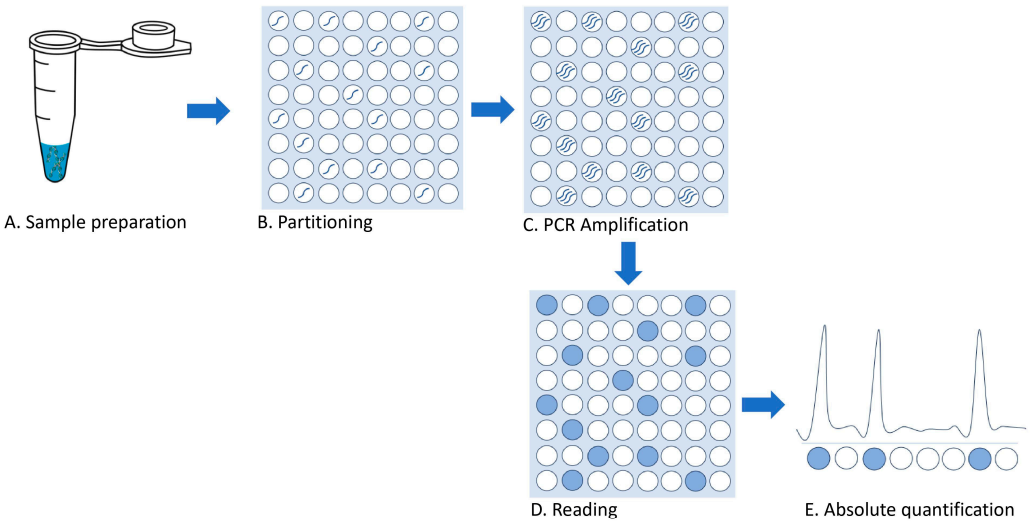

Figure 1. Visualized workflow of the ddPCR assay illustrating the subdivision of a single sample containing the target sequence (**A**) into thousands of droplets, (**B**) the PCR amplification in each droplet, and (**C**) the measurement of the number of fluorescent droplets (**D**) that corresponds to the number of template molecules with a particular fluorescence leading to absolute quantification (**E**).

This review provides an overview of the studies that employed dPCR in investigating animal parasites and parasitic diseases from a veterinary perspective. We also discuss how this novel technology could provide added value, aid and benefit diagnosis, surveillance,

and the monitoring of response to treatment, or could shed light on current gaps in our knowledge of the epidemiology of important veterinary parasitic diseases.

2. Protozoa

2.1. Eimeria spp.

Traditionally, the description and differentiation of *Eimeria* spp. are based on the oocysts' shape, size, and features [15]. Precise species identification is essential for coccidiosis control since it reveals the degree of drug or vaccine resistance [15]. However, species identification based on oocyst morphology is challenging due to the variations within and between oocyst morphometrics [16]. Consequently, several molecular tools have been employed for discriminating at the species level and others, such as qPCR, for quantifying the parasitic burden [17–19].

Snyder et al. developed a highly specific ddPCR to discriminate seven *Eimeria* species-*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. tenella*, and *E. praecox* [4]. The researchers used a single PCR amplicon labeled with genus-specific and species-specific probes targeting conserved and specific regions. The targeted cytochrome c oxidase subunit III gene in the mitochondrial genome (mtCOIII) allowed accurate species differentiation due to its interspecific variability. The specificity was high even in samples containing DNA from more than one species [4]. The relative species quantification obtained from the ddPCR was in agreement with the NGS assay used. The ddPCR assay presented high sensitivity for all the seven described *Eimeria* spp.; it detected low concentrations of DNA: 0.00001 ng/µL for *E. brunetti*, *E. praecox*, and *E. tenella*; 0.0001 ng/µL for *E. maxima* and *E. mitis*; and 0.001 ng/µL for *E. acervulina* and *E. necatrix* [4].

One limitation of this assay is that it does not provide quantification data for oocysts. Instead, it provides information on the relative abundance of different *Eimeria* species in a sample based on the number of copies of DNA for each species. Therefore, the results should be interpreted in combination with the oocysts per gram data. Moreover, non-specific binding was observed between *E. necatrix* and *E. tenella* [4]. Nonetheless, the authors suggested that the newly developed ddPCR assay would provide valuable information on coccidiosis, a disease of major financial impact in the poultry sector, and they proposed many potential applications in commercial chicken production systems [4].

2.2. Haemosporidian Parasites

Haemosporidian parasites, transmitted by dipteran blood-sucking insects, are cosmopolitan in birds [20,21]. Clinical manifestations appear during the acute phase of infection when erythrocytic parasitemia is high, while low-intensity infections do not usually become evident [22]. Some species cause severe disease in avian hosts [22], and coinfections with different species or genetic lineages, which are often particularly virulent, have been reported worldwide [23,24]. Currently, the identification of avian haemosporidian is mainly based on the analysis of blood samples by traditional light microscopy and qPCR [25]. However, both methods present limitations. Microscopy has low sensitivity; it requires high-quality blood smears that are difficult to achieve in field conditions and high infection levels. As for qPCR, although it is more sensitive compared to microscopy, it is difficult to standardize and requires reference samples of absolute known parasite DNA concentrations that limit the inter-laboratory comparisons [23,26].

Huang et al. developed a ddPCR protocol for the detection and absolute quantification of avian haemosporidians belonging to *Plasmodium* spp., *Haemoproteus* spp., and *Leucocytozoon* spp. [26]. The method's performance was assessed compared to qPCR, nested PCR, and light microscopy in blood samples from raptors. The newly developed ddPCR assay enabled reliable quantification even in samples with minimum parasitic load, i.e., one parasite copy in 10^5 host genomes. The ddPCR assay showed equal sensitivity and high agreement with qPCR. Notably, the quantity assessment was more accurate than qPCR, and the assay demonstrated higher consistency among technical duplicates and reactions, especially in low parasitic loads. Unlike nested PCR and light microscopy, ddPCR identified

more than 60% and 74% of the negative samples, respectively, as positive. The researchers suggested that despite the higher cost of ddPCR compared to qPCR, it could be a better choice in several cases, such as the comparison of the absolute quantifications reported from different laboratories, when reference samples are not available, as well as in long-term studies where sample storage could negatively affect the DNA integrity. Moreover, they highlighted the potential of ddPCR for investigating abortive infections, which are difficult to identify but may be detected by quantifying very low-intensity infections [26].

In the same context, in a previous study in Australia, ddPCR targeting the mitochondrial 18S rRNA gene was used to quantify the parasitic load of *Leucocytozoon podargii* in tissues collected during necropsy from a tawny frogmouth, which exhibited signs consistent with a *Leucocytozoon* infection. Histopathological analysis of the respective tissues revealed significant changes in the lung tissue. This finding was in agreement with the absolute quantification data provided by ddPCR; the infected lung tissue displayed the highest *Leucocytozoon* load [27].

2.3. *Cytauxzoon felis*

Recently, a ddPCR assay was designed to detect and quantify *Cytauxzoon felis* (*C. felis*) in feline blood samples during early infection and throughout treatment [28]. The researchers evaluated the performance of the test in clinical samples from cats suspected of *C. felis* infection correlated to the traditionally used blood smear microscopy; the two methods showed 100% agreement. They also compared ddPCR to nested PCR and qPCR for *C. felis* in blood samples from experimentally infected cats pre- and post- antiprotozoal treatment. Although no significant differences in the detection rate were found between the molecular methods in cases of severe cytauxzoonosis—probably due to the high circulating parasite load before treatment—the ddPCR and qPCR assays performed better in samples collected ten days post-treatment. Regarding the quantification of the parasite load, the ddPCR assay detected as low as 0.0000231 ng DNA/reaction in the reference samples from acute cases and 0.00232 ng DNA/reaction in the reference samples from chronic cases. It is also worth mentioning that ddPCR identified infection up to one day prior to the development of clinical signs in experimentally infected cats. Thus, this assay successfully detected the early stages of the disease, even when employed in small sample volumes with low DNA concentrations [28]. This is extremely important in the case of *C. felis*, as accurately detecting the earliest stages of infection is critical for initiating treatment. In addition, the clinical progression of the disease is rapid, and most cats die or are euthanized within 24 h of presentation in the veterinary clinic [29]. The authors suggested that ddPCR is also useful for monitoring the parasite load throughout treatment by means of the absolute quantification of target DNA copies in samples acquired pre- and post-treatment [28].

2.4. *Cryptosporidium spp.*

Quantitative molecular methods are increasingly gaining ground in *Cryptosporidium* spp. diagnostics due to their invaluable advantage of estimating the parasitic load in human, animal, and environmental samples [30]. The low infectious dose (10–100 oocysts) of *Cryptosporidium* spp., the ability to survive in the environment for long periods, and the inherent resistance to drinking water disinfectants [31] make the quantification of the parasitic load necessary for further diagnostic and management steps. In addition, quantitative molecular methods have a significantly lower limit of detection (LOD) than microscopy, which requires 1×10^4–5×10^4 oocysts per mL of feces [32].

In a 2014 study, the researchers developed and validated a ddPCR assay for detecting and quantifying *Cryptosporidium* spp. DNA in samples from sheep, cattle, and humans. They compared the performance of the newly developed assay with qPCR and showed a high degree of linearity and positive correlation between the two tests for the reference and clinical samples examined [33]. The ddPCR was superior in precision and was not affected by the presence of inhibitors. This is especially important when the presence of inhibitors is anticipated in the biological samples examined, like feces. The authors also suggested that

ddPCR could be helpful for calibrating qPCR standards to deliver more precise standard curves [33]. Later, when Zahedi et al. investigated the occurrence of *Cryptosporidium* spp. and *Giardia* spp. in dam water in sheep farms, they followed this suggestion and quantified the reference samples that they used in *Cryptosporidium* qPCR with ddPCR [34].

Recently, a study showed how ddPCR coupled with the phenol-chloroform DNA extraction method is a valuable tool for monitoring and detecting *Cryptosporidium* spp. in wastewater samples [35]. Environmental samples like water are challenging due to the low parasitic load, the complexity of their matrix that involves a higher concentration of PCR inhibitors, and the additional concentration step that is required to obtain sufficient biomass containing oocysts [36,37]. In that study, the researchers developed and evaluated a ddPCR assay for *Cryptosporidium* spp., which presented high sensitivity and a very low LOD of 5.93 copies, equivalent to less than one oocyst per reaction [35]. The ability to detect such small starting quantities of protozoan parasites is essential in water samples since the infectious dose can be as low as ten oocysts [38].

2.5. Leishmania infantum

Many qPCR assays have been developed for detecting and quantifying *Leishmania* DNA in clinical samples and provided new insights into the kinetics of the parasitic load [39]. Usually, high burdens are associated with progressive disease, while a marked decrease appears following effective treatment. However, the parasitological cure is not anticipated, and most dogs remain infected for life. On top of the above, even dogs with zero parasitic burdens after treatment may present fluctuations over time [39,40]. Quantitative assays display high sensitivity in detecting *Leishmania* DNA and, thus, are increasingly popular in diagnosing canine leishmaniosis, as well as monitoring the disease progression following treatment and the potential for relapses. They have also been extensively used for species identification in animal, human, and vector studies. Although sensitive, rapid, and with reduced possibility of contamination, qPCR does not provide absolute quantification and, currently, there is no standardized method [41,42].

In the 2023 study of Pereira et al., a ddPCR assay for *Leishmania infantum* (*L. infantum*) DNA detection was developed and validated [43]. The researchers assessed its performance in spleen samples from dogs with confirmed canine leishmaniosis following necropsy compared to qPCR. The two molecular methods presented perfect agreement (100%) and a positive correlation between the number of copies detected by ddPCR and the quantification cycles found in qPCR. No correlation was found between the copy numbers recorded in ddPCR and the morphological changes in the spleen of the dogs included in the study. This finding is in concordance with the study of Vasconcelos et al., which demonstrated that dogs with severe disease, as well as asymptomatic or mildly symptomatic animals with moderate to extensive splenic tissue disorganization, can have high parasitic loads [44]. The authors suggested that ddPCR is advantageous over qPCR as it does not require a standard curve and is easy to standardize; therefore, it should be further used to investigate the relationship between parasitic load and the pathogenesis of the disease in dogs [43].

2.6. Theileria spp.

Currently, the diagnosis of bovine theileriosis is based on clinical signs coupled with detecting the parasites in blood samples with light microscopy. Species identification is pivotal in bovine theileriosis. Unlike the most pathogenic species, *Theileria annulata* (*T. annulata*) and *Theileria parva* (*T. parva*), the other *Theileria* spp. are not life-threatening and do not require veterinary intervention [45]. However, microscopy presents several limitations; the identification of the low blood parasite load is complex and may fail to detect early infection status. Furthermore, the presence of morphologically identical parasites like *Babesia* spp. may be misleading [46]. Thus, employing highly sensitive and specific tools for disease diagnosis at early stages is paramount in bovine theileriosis. The treatment relies on the administration of buparvaquone, and the efficient elimination of the parasite is anticipated when early detection and treatment initiation occur. Additionally,

resistance to buparvaquone has been reported, and delayed diagnosis may lead to deadly lymphoproliferative disease [47].

Recently, a multiplex ddPCR assay was developed for identifying and quantifying *Theileria* spp. and *T. annulata* in clinical samples [45]. The researchers assessed the efficacy of the ddPCR assay compared to a qPCR assay and showed that they were equally efficient. The ddPCR presented high sensitivity for *Theileria* spp. (100%) and *T. annulata* (97.8%) identifying quantities as low as 8.5 copies/µL of *T. annulata* DNA. Noticeably, the ddPCR assay had higher sensitivity than qPCR at lower parasite burdens and exhibited considerably less variability. The method was also performed on DNA samples from treated and untreated *Theileria*-infected cell lines with buparvaquone to assess the treatment's efficacy. The study's findings displayed the ability of this novel tool to track the treatment response in clinical cases of bovine theileriosis, which is considered a critical feature [45].

2.7. Toxoplasma gondii

In a 2022 study, the researchers developed and validated a ddPCR assay for detecting and quantifying *Toxoplasma gondii* (*T. gondii*) DNA in meat samples from intermediate hosts [48]. They determined the accuracy of the new assay compared to qPCR and showed an almost perfect agreement between the two methods. The ddPCR presented high sensitivity (97.5%) and specificity (100%) and resulted in higher positivity rates in the meat samples (7.6% vs. 1.2% in qPCR), with a detection limit of 8 genomic copies/µL [48]. Increased sensitivity is essential for *T. gondii* detection in meat samples because of the non-homogeneous distribution of tissue cysts and the small size of the sample that is usually examined [49]. This is why many molecular tools that have been developed up to now present unexpectedly low sensitivity. The authors suggested that ddPCR is a promising tool and should be further tested in food matrices like milk and dairy products, as well as vegetables, to rapidly detect low parasitic loads of *T. gondii* [48].

Earlier, a dPCR platform was used to investigate the hypothesis that edible fishes can harbor and effectively transmit *T. gondii* to marine organisms and human consumers. In this context, Marino et al. examined edible fishes using dPCR and qPCR. They showed by dPCR that fish specimens (skin/muscle, intestine, and gills) belonging to 12 different fish species were contaminated with *T. gondii* DNA to the extent of 1 to 5.7×10^4 copies/mL. The authors suggested that although fishes are not deemed as competent hosts for *T. gondii*, they could possibly act as mechanical carriers and, thus, be accidentally involved in the transmission cycle of the parasite [50].

3. Trematodes

Schistosoma japonicum

Schistosoma japonicum (*S. japonicum*) causes intravascular disease in humans and is endemic in the People's Republic of China, the Philippines, and Indonesia [51]. Among several human schistosome species, *S. japonicum* is zoonotic; it infects domestic and wild animals, thus complicating the national control strategy for the disease in the human population [52]. Although most studies on schistosomiasis focus on humans, as China gets closer to eliminating the disease, it becomes essential to investigate the possible role of other hosts in maintaining low levels of transmission and the contamination of the environment [9].

In a 2017 study, fecal samples from rodents, goats, dogs, cattle, and water buffaloes were collected and examined using traditional parasitological techniques and qPCR to detect and quantify the *S. japonicum* DNA levels in different hosts [9]. A previously described ddPCR assay was also performed on the collected goat fecal samples. Goats, cattle, and water buffaloes were more frequently infected. The molecular methods presented increased sensitivity more than the traditional parasitological techniques, which significantly underestimated the actual prevalence of infection in all animal species examined. Notably, using ddPCR, a considerably higher prevalence of infection was recorded in goats (46.4%) compared to qPCR (6.9%). This discrepancy is probably due to inhibitors in the fecal sam-

ples that negatively affect the performance of qPCR but exhibit no effect on ddPCR. Based on the findings of ddPCR, the authors highlighted the importance of obtaining precise estimates of the prevalence of infection in animal species that could be involved in the transmission cycle as reservoirs or as maintenance hosts. They also suggested the inclusion of goats as targets of control to achieve the aim of eliminating schistosomiasis in China [9].

4. Cestodes

Echinococcus multilocularis

In the case of *E. multilocularis*, infection in intermediate hosts is regularly detected through the macroscopic examination of the target organs, like the liver, for lesions [53]. However, the macroscopic examination may fail to detect exposure to the parasite, thus leading to an underestimation of the true infection prevalence [12]. Consequently, macroscopic examination is typically followed by histopathology, immunohistochemistry, or molecular tests [53].

Massolo et al. applied a ddPCR assay and assessed its suitability for detecting *E. multilocularis* DNA in liver samples from intermediate hosts independently of macroscopic lesions. The ddPCR assay provided the highest positivity rates for *E. multilocularis* DNA (15.09%) compared to qPCR (4.72%) and the presence of macroscopic lesions (1.88%) [12]. Applying such high-sensitivity molecular methods in prevalence studies can significantly increase the accuracy of the prevalence estimations.

As depicted in the study of Massolo et al., great differences in the prevalence estimates may appear even between highly sensitive tools, as in the case of ddPCR and qPCR [12]. According to the authors, this discrepancy could be related to the presence of bile acids in the liver, which act as inhibitors and negatively affect the performance of qPCR. They also suggested that results from high-sensitivity molecular methods, like ddPCR, should always be interpreted cautiously. Evidence of *E. multilocularis* DNA does not necessarily correspond to early, successfully developing lesions. It could represent cases of exposure to the parasite, which was eliminated by the immune system, and early or abortive infections, which are not expected to develop into successful infections. Nonetheless, the above study highlighted that sensitive molecular tools like ddPCR could better estimate the exposure rates of intermediate hosts, identify the transmission foci, define the competence of less common intermediate hosts, and refine epidemiological models [12].

5. Nematodes

5.1. Gastrointestinal Nematodes

5.1.1. *Haemonchus contortus*

Haemonchus contortus (*H. contortus*) is one of the most pathogenic and abundant gastrointestinal nematodes in small ruminants. Infection may result in anemia, production losses, and mortality, mainly in lambs and pregnant ewes [54]. Effective treatment aims to reduce the worm burden and pasture contamination, thus preventing the establishment of infective-stage larvae (L3). Treatment is based on broad-spectrum anthelmintics of three major drug classes, i.e., benzimidazoles, imidazothiazoles/tetrahydropyrimidines, and macrocyclic lactones. However, anthelmintic resistance has increased globally and has become a significant concern for the sheep industry [55].

The occurrence of *H. contortus*-resistant field strains in all three most commonly used anthelmintic drug classes is well documented [56–58] and has led to the need for reliable screening and diagnostic techniques in routine settings. The Fecal Egg Count Reduction Test (FECRT) is the gold standard method for identifying clinical anthelmintic resistance in flocks [59]. However, this method provides a rough estimation and lacks sensitivity. In addition, it is negatively affected by several parameters, such as the level of excretion, the aggregation of fecal egg counts (FECs), the sample size, and dilution factors [7,60]. Toward this end, several molecular tools have been developed and validated for screening, detecting, and evaluating resistance to anthelmintic drugs [61]. Until now, the following

studies employed ddPCR assays in *H. contortus* investigation, focusing on identifying molecular markers that could be used to detect resistant field strains.

In 2018, Baltrusis et al. validated and optimized a ddPCR assay using single adult *H. contortus* worms collected from 13 countries to detect and quantify the F200Y mutant allele that is linked to anthelmintic resistance to benzimidazole drugs [7]. They targeted the β-tubulin isotype 1 gene, which is well known to present increased variability within populations of *H. contortus* and to result in resistance to benzimidazoles. Moreover, to obtain a reference read, they used ovine fecal larval culture samples containing mixed *H. contortus* strains (wild type and resistant strains) from Swedish sheep farms pre- and post-treatment with albendazole that had been molecularly characterized by an NGS platform (PacBio RSII Sequencing). When comparing the fractional abundance values (the percentage of the variant allele in the sum of background and variant alleles) of the resistance-determining mutant allele between NGS and the newly developed ddPCR assay, the two methods showed an excellent agreement and yielded highly similar fractional abundance values. Interestingly, although the initial FECRTs performed on post-treatment larval culture samples across various farms showed an efficient egg count reduction, NGS and ddPCR characterized the same strains as resistant. This finding suggests that although the resistant strains decreased after treatment, they persisted regardless of the treatment, and underlines the lack of FECRT sensitivity in detecting resistant strains. The authors suggested that ddPCR could be a powerful tool for mutation detection that needs to be further tested before being implemented in routine fecal examinations [7].

In a 2020 study, Baltrušis et al. used pooled samples of mixed larvae cultures containing *H. contortus* from 67 farms in Sweden in a 6-year period to estimate the presence of the two most frequently observed mutations, F167Y and F200Y, in the isotype 1 β tubulin gene using an in-house ddPCR and pyrosequencing. They also compared the samples collected pre- and post-treatment with ivermectin or albendazole regarding changes in the frequency of the F200Y mutation. Similar to the previous study [7], the two methods had an excellent agreement concerning the frequency of the mutations. The F167Y mutation frequency was low, but the F200Y was dominant in the samples from most of the farms included in the study, irrespective of the year of sampling. Furthermore, the fractional changes of the mutation F200Y on farms before and after treatment with ivermectin or albendazole provided solid evidence only for the selection of the F200Y mutation after treatment with albendazole [62].

Later, Baltrušis et al. used the previously mentioned ddPCR assay for detecting the F200Y mutation [7] to estimate the frequency of the mutated β tubulin allele in L1 of *H. contortus* that had been hatched under gradually increasing thiabendazole concentrations. In this experimental study, the researchers used two laboratory-maintained strains with distinct resistance statuses, and they showed that F200Y can be used as the main genetic marker to estimate and evaluate the degree of benzimidazole resistance in this parasite species [63].

An earlier study examined the suitability of dyf-7 Single Nucleotide Polymorphisms (SNPs) as a molecular marker for ivermectin resistance in *H. contortus* strains [64]. The developed and validated ddPCR assay targeted the dyf-7 SNPs of *H. contortus*, using mixed species larval cultures from sheep feces pre- and post-treatment with ivermectin. The study included a number of Swedish sheep farms where FECRT demonstrated ivermectin failure. The ddPCR assay was robust for detecting mutations of the dyf-7 allele frequency in mixed larval cultures with a low threshold (≈3 copies/μL). However, this study showed that mutations in dyf-7 are not involved in ivermectin resistance because the fractional abundance was not increased in the post-treatment samples. Moreover, the frequency of the mutations was high in the post-treatment samples despite the ivermectin treatment being 100% effective based on the FECRT [64].

Similarly, ddPCR assays have been developed in other studies to detect genetic markers that could be associated with levamisole resistance in small ruminants. To that end, a ddPCR assay targeting the hco-acr-8 L-AchR subunit gene, previously identified as associ-

ated with levamisole resistance, was used [65]. That study showed that hco-acr-8 L-AchR is not a predictive marker for levamisole resistance. Although the "resistant" allele was highly prevalent, the researchers did not observe a decrease in levamisole's efficacy or an increased frequency of surviving animals in the field populations treated with levamisole [65]. On the contrary, in another survey, a non-synonymous mutation in the exon 4 of this gene, resulting in the S168T substitution, was identified as a major determinant of levamisole resistance that could be used as a potential molecular marker in sheep populations. The researchers accomplished the application of ddPCR to define and assess the relative frequency of the S168T mutation. Moreover, following treatment with levamisole, they examined multiple phenotypically distinct *H. contortus* isolates and field larvae populations from Swedish sheep farms [66].

5.1.2. *Trichuris* spp.

Lately, a ddPCR assay targeting the Internal Transcribed Spacer (ITS) gene of *Trichuris* spp. was applied to sheep fecal samples, and its performance was evaluated compared to qPCR [6]. The ddPCR assay demonstrated good reproducibility and consistency even in low parasitic loads. The LOD was lower (less than 3.17 copies per reaction), and the sensitivity was higher than qPCR. Notably, the ddPCR assay detected more positive samples (80.6%) than qPCR (72.4%). At the same time, it presented high specificity without cross-amplification of other relevant gastrointestinal nematodes. This ddPCR assay could be useful for the accurate and early diagnosis of the infection in sheep and a valuable tool for the diagnosis and prevention of the disease [6].

5.1.3. Mixed Infections with Gastrointestinal Nematodes in Ruminants

Although *H. contortus* has been recognized as a parasite of major concern in small ruminants due to its increased pathogenicity and rapidly emerging anthelmintic resistance [55], pasture-grazing sheep are also exposed to several other nematode species, resulting in multi-species infections [67]. Mixed infections are especially important in cases of low levels of *H. contortus* infection that are difficult to detect with conventional parasitological techniques [68]. Up to now, a wide variety of DNA-based approaches exist for detecting, quantifying, and discriminating the different gastrointestinal nematode species [69].

In 2018, a ddPCR assay was developed and validated for the absolute quantification and identification of three of the most abundant genera of strongylids in sheep: *Haemonchus*, *Teladorsagia*, and *Trichostrongylus*. This assay employed one universal target for all strongylid gastrointestinal nematodes and three genus-specific targets, thus allowing the estimation of the relative abundance of each parasite species with high precision even in samples containing multiple genera of strongylids. Noticeably, no cross-amplifications were observed between the genera, verifying that the ddPCR assay is genus-specific. Moreover, the ddPCR assay presented a good agreement with FECRT. Regarding *H. contortus*, the researchers found excellent agreement between the *Haemonchus*-specific ddPCR assay and a well-established qPCR assay [60]. They suggested that ddPCR could be used to evaluate the efficacy of different anthelmintics and could successfully complement FECRT and other routine diagnostic methods [3].

The ddPCR assay developed by Elmahalawy et al. [3] was later used to determine the occurrence and quantify the DNA copies of the three major strongylid genera infecting sheep in the following surveys. Högberg et al. investigated how activity patterns along with standard diagnostic indicators and FEC are influenced in naïve grazing lambs when they are exposed to gastrointestinal nematodes under natural grazing conditions [70]. Höglund et al. provided an overview of the development of anthelmintic resistance in sheep farms in Sweden based on surveillance data [71]. Finally, Högberg et al. investigated the effect of weaning age on animal performance in lambs naturally exposed to nematodes [72].

Later, another ddPCR assay was developed to discriminate gastrointestinal nematodes frequently identified in mixed infections in cattle. In this case, the ddPCR assay targeted *Cooperia* spp. and *Ostertagia* spp. Both genera were amplified using the same primer pair,

and the discrimination was enabled using different probes in duplex reactions. The assay produced promising results in terms of fractional abundance and LOD for each parasite genus in the presence of the other. It detected *Cooperia oncophora* (*C. oncophora*) DNA when it represented only 2.5% of the template and *Ostertagia ostertagi* (*O. ostertagi*) DNA, even when it corresponded to only 0.67% of the template [73].

5.1.4. Dual Infections with *Ascaridia galli* and *Heterakis gallinarum* in Poultry

As regards poultry nematodes, the flotation technique is sensitive, but the differentiation of *Ascaridia galli* (*A. galli*) and *Heterakis gallinarum* (*H. gallinarum*) is difficult. It requires professional skills and trained personnel due to the morphological similarities between the eggs of the two nematodes. On top of that, in dual infections, *A. galli* eggs encompass most of the excreted parasite eggs because *H. gallinarum* produces fewer eggs, thus underestimating the occurrence of mixed infections [74,75]. As for the necropsy, the two species are easily identified based on their substantial differences in size and location. However, it is considered an invasive procedure where healthy animals are sacrificed for diagnostic purposes [5,76].

Heterakis gallinarum may also be involved as an intermediate host in the life cycle of *Histomonas meleagridis*, the causative agent of histomonosis, which, in the absence of approved drugs for chicken in the EU, can reach up to 20% mortality, with high morbidity in chicken. Thus, the early detection and treatment of the *H. gallinarum* infection are crucial in flocks with a history of histomonosis as it comprises one of the primary management tools [77].

In a 2021 study, the researchers developed a duplex ddPCR to identify and quantify *A. galli* and *H. gallinarum* DNA in chicken feces and evaluated its accuracy compared to the flotation technique [5]. The two methods showed substantial agreement. The ddPCR assay displayed a 6% higher detection rate than the flotation technique. It also enabled the simultaneous detection and differentiation, with no cross-amplification, between these two intestinal nematodes of major importance. It is noteworthy that ddPCR had such a low LOD that enabled the identification of small amounts of *H. gallinarum* DNA in samples with dual infection with *A. galli*. In particular, in samples with a constant *A. galli* concentration, the lowest detection level of *H. gallinarum* DNA was as low as 0.8% [5]. This critical feature makes ddPCR useful for diagnostics at individual and flock levels and overcomes the limitations of the flotation technique and necropsy, the traditional diagnostic procedures.

The authors also emphasized that ddPCR does not rely on the quality and storage conditions of the samples. At the same time, this is not the case for the flotation technique, in which the freshness of the fecal material and the integrity of the parasite eggs strongly affect its sensitivity. This study showed that ddPCR could be a useful monitoring tool for *A. galli* and *H. gallinarum* dual infections in commercial chicken flocks [5].

5.2. Dirofilaria immitis

A 2020 study used ddPCR to identify the role of the ecdysone signaling system during the developmental regulation of *Dirofilaria immitis* (*D. immitis*) microfilariae in its arthropod host, using an in vitro culture condition that mimics the mosquito host environment. This investigation aimed to give insights into the in vitro development of *D. immitis* microfilariae and provide data for novel targets for drug development. The ddPCR was employed during the transcript-level study and provided data for the absolute quantification of the target nucleic acids present in the sample. This study displayed that the ecdysone signaling system might play an important role in filarial nematode developmental transitions, and gave another example of the potential applications of ddPCR in veterinary parasitology [8].

Lately, Curry et al. used ddPCR to measure the level of the expression of the *D. immitis* P-glycoprotein 11 (DimPgp-11) SNP in macrocyclic lactone-susceptible and resistant isolates. This study confirmed that genetic changes in the P-glycoprotein 11 gene, encoding an

ATP-binding cassette transporter, are associated with the macrocyclic lactone-resistant phenotypes and could be employed as markers of drug resistance [78].

6. Conclusions

Novel molecular tools have emerged and are widely used even in routine diagnostics. In this context, the body of the literature is growing in human and veterinary medicine for the potential applications of dPCR. Currently, the vast majority of studies on this novel tool in veterinary parasitology focus on developing and validating dPCR assays for detecting and quantifying parasites of great financial impact on the food-producing animal industry. Some steps have been taken to apply dPCR for individualized medicine in companion animals.

The dPCR assays developed so far presented high sensitivity and specificity, as well as low levels of detection, comparable to or even higher than qPCR and NGS platforms. This new molecular method accurately detects low parasitic loads in clinical samples, facilitating early diagnosis and treatment initiation at individual and flock levels, even in mixed infections. Regarding the parasitic diseases that species identification is crucial for diagnosis and treatment, dPCR is a promising tool. As for the absolute quantification of the parasitic burden, it will be an invaluable advantage for monitoring disease progression and treatment outcomes. Moreover, the use of dPCR in surveillance and epidemiological studies will increase our knowledge of the prevalence estimates and the role and competence of different hosts. Last but not least, dPCR has the potential to be used for the discovery of new molecular markers to identify resistance to parasiticides and new targets for drug development.

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Abbreviations

Cytochrome c oxidase subunit III gene in the mitochondrial genome (mtCOIII); deoxyribonucleic acid (DNA); digital PCR (dPCR); droplet digital PCR (ddPCR); fecal egg count (FEC); Fecal Egg Count Reduction Test (FECRT); Internal Transcribed Spacer (ITS); limit of detection (LOD); Next Generation Sequencing (NGS); Polymerase Chain Reaction (PCR); quantitative PCR (qPCR); ribonucleic acid (RNA); ribosomal RNA (rRNA); and Single Nucleotide Polymorphisms (SNPs).

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