Veterinary Parasitology 296 (2021) 109499

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

A novel duplex ddPCR assay for detection and differential diagnosis of *Ascaridia galli* and *Heterakis gallinarum* eggs from chickens feces

B. Tarbiat[a,*], N. Enweji[a], P. Baltrusis[a], P. Halvarsson[a], E. Osterman-Lind[b], D.S. Jansson[c,d], J. Höglund[a]

[a] Department of Biomedical Sciences and Veterinary Public Health, Section for Parasitology, Swedish University of Agricultural Sciences (SLU), P.O. Box 7036, SE-750 07, Uppsala, Sweden

[b] Department of Microbiology, Section for Parasitological Diagnostics, National Veterinary Institute (SVA), SE-751 89, Uppsala, Sweden

[c] Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Box 7054, SE-750 07, Uppsala, Sweden

[d] Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute (SVA), SE751 89, Uppsala, Sweden

ARTICLE INFO

Keywords:
Roundworm
Laying hens
Molecular assay
Poultry
Parasites
Diagnostic
Prevalence

ABSTRACT

Since the EU ban on battery cages, many studies have listed *Ascaridia galli* and *Heterakis gallinarum* as the most common roundworms in the European laying hen population. A complicating factor is that the eggs of these parasites are almost identical. Thus, lack of molecular diagnostic approaches has driven epidemiological studies to take on necropsy for species discrimination, which is labor and cost intensive. Here, we describe a novel diagnostic tool based on droplet digital PCR for simultaneous identification and absolute quantification of the eggs of both of these ascarids in chickens' droppings using two different genus-specific primer-probe sets targeting the second internal transcribed spacer region (ITS-2) in the nuclear ribosomal (rRNA) gene array. No cross-reaction was observed when different combinations of DNA and species-specific primers and probes were tested. The lowest obtained frequency threshold for the detection of *H. gallinarum* in the presence of a constant *A. galli* DNA concentration was determined to be 0.8 %. After validation, we used the assay to analyze field samples collected from several Swedish laying hen farms. Out of 134 samples, 86 (64 %) were positive for *A. galli* while 11 (8.3 %) samples were positive for *H. gallinarum*. These samples were initially analyzed with flotation technique for detection of ascarid eggs. The results of the Cohen's kappa indicated substantial agreement (85.8 %) between the two tests. In conclusion, we have validated a novel molecular-based diagnostic tool for quantification and differentiation between intestinal parasites of major importance in chickens with high precision. Although this study focuses on identification of parasites of laying hens, the findings may well have a bearing on all types of chicken production systems. The present study lays the groundwork for future research into epidemiology of these two important chicken parasite species.

1. Introduction

Due to the implementation of the EU ban on conventional battery cages in 2012, egg producers have since adopted new husbandry systems such as furnished cages, barn production and free-range (outdoor and organic). Non-caged housing has been implemented by almost 92 % of the current Swedish laying hen farms (Öberg, 2020). In barns and on free-range farms, unlike the cage systems, hens are in contact with their droppings. This facilitates transmission of intestinal endoparasites, which are transmitted via stages excreted with the birds' feces and disseminated in the animals' environment. As a result, prevalence of fecal-orally transmitted parasitic roundworms such as *Ascaridia galli* and *Heterakis gallinarum* has greatly increased in laying hens (Permin et al., 1999; Jansson et al., 2010; Kaufmann et al., 2011; Sherwin et al., 2013). Roundworms are also occasionally a problem among other types of chickens (Permin et al., 1999). While most endoparasite-related research in laying hens in Sweden has been dedicated to study *A. galli*, no detailed investigation into the occurrence of *H. gallinarum* has been undertaken. A major reason for this is that validated diagnostic tools that can distinguish between roundworm eggs in poultry droppings are essentially missing.

Most often roundworm infections in poultry are subclinical and go

* Corresponding author.
E-mail address: behdad.tarbiat@slu.se (B. Tarbiat).

https://doi.org/10.1016/j.vetpar.2021.109499
Received 18 March 2021; Received in revised form 6 June 2021; Accepted 7 June 2021
Available online 11 June 2021
0304-4017/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

unnoticed. At the same time manifestation of severe infections especially with *A. galli* in laying has been associated with reduced welfare and health leading to economically important egg production losses (Gauly et al., 2007; Sharma et al., 2019; Stehr et al., 2019; Tarbiat et al., 2020). To prevent the adverse effects of the parasitic diseases on host performance and wellbeing, anthelmintics are today essential for worm control in laying hens. Since their approval for use in poultry in Sweden in 2005 sales of benzimidazoles for this sector increased by 2500 % in the year 2019 (Fag, 2006; Jordbruksverket, 2019).

It is important to distinguish between the two species for several reasons. Firstly, it provides a clearer picture over the infection dynamics and epidemiology of the two parasites (better parasitological screening). *H. gallinarum* is substantially smaller than *A. galli* and produces fewer eggs (Daş et al., 2014; Wongrak et al., 2015), therefore, in dual-infection scenarios *A. galli* eggs encompass majority of the excreted parasite eggs. Identifying few *H. gallinarum* eggs in a crowed of *A. galli* eggs would be of a high value for screening purposes. Secondly, identification of the species present at the right time not only helps taking proper actions (e.g. optimal use of anthelmintics) in the event of infestation but also helps anticipate complications with a protozoan parasite *Histomonas meleagridis* (causative agent of histomonosis, resulting in up to 20 % mortality with high morbidity) (McDougald, 2005). Due to the absence of approved drugs against histomonosis in EU, the control of the aforementioned species primarily relies on managing the infection with *H. gallinarum* levels in the flock, using anthelminthics to reduce the exposure of hens to the cecal worms acting as a vector for the disease-causing protozoan (McDougald, 1998).

Differentiation between the parasite species is often based on the size and morphology of the species in question (including adults and free-living stages of the parasites e.g., larvae or eggs) and their predilection sites in the host. Distinction between these two nematodes through necropsy (an invasive method) is easy as *A. galli* worms are substantially larger and unlike *H. gallinarum* (which inhabits the cecal lumen), they are mainly located in the lumen of the small intestine (Luna-Olivares et al., 2012). However, there are ethical boundaries of sacrificing healthy animals for diagnostic purposes. On the other hand using copromicroscopic detection methods such as the flotation technique to differentiate *A. galli* from *H. gallinarum*, due to morphological similarities of the eggs of the two species, requires professional skills and trained personnel. Since parasite eggs differentiation through traditional methods is subject to error, and performing necropsy on a large scale (e.g., in epidemiological studies) is both cost and labor intensive, there is a need for novel molecular assays as more precise non-invasive alternatives.

Droplet Digital™ PCR (ddPCR) is a technology for absolute quantification of amplicon DNA without the use of standard curves. The ddPCR approach is based on the generation of thousands of nanoliter sized droplets from a single genomic DNA sample and the subsequent primer and probe pair mediated, simultaneous PCR amplification and quantification of short amplicon regions (Hindson et al., 2011). Once, the presence or absence of the target amplicon is measured, the concentration (copy numbers of target amplicon per μL), as well as 95 % confidence intervals are calculated using the integrated Poisson statistics module (www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf). Because no comparative standards are required for DNA quantification of unknown samples, ddPCR has a significant potential to be used in the diagnostic settings (Huggett et al., 2013).

Low intraspecific variation in the second internal transcribed spacer (ITS-2) of the ribosomal DNA gene array (Hoste et al., 1993; Campbell et al., 1995; Gasser and Monti, 1997) makes this region a good candidate to be used in the development of robust and sensitive molecular diagnostic tools for a variety of parasites of veterinary importance. So far, this approach has been developed mainly for gastrointestinal nematodes of ruminants (Elmahalawy et al., 2018; Baltrušis et al., 2019).

The aim of this study was to investigate the use of the ITS-2 region in developing a novel methodology to facilitate the detection and identification of *A. galli* and *H. gallinarum*, the two most common gastrointestinal parasites found in chickens, using species specific primer-probe sets in a single ddPCR assay.

2. Materials and methods

2.1. Parasite worm collection for DNA extraction

DNA used in this study was obtained from adult worms collected for a previous project. Approval was obtained from The Swedish Ethical Committee for Scientific Experiments (5.2.18−01658/2020).

Five naturally infected chickens were obtained from two commercial farms where mixed infections with *A. galli* and *H. gallinarum* were confirmed by onsite veterinarians. Chickens were sacrificed and transferred with cooling pads to our laboratory at the Swedish university of agricultural sciences. Upon arrival, small intestines and cecae were removed, cut open longitudinally and their contents were washed through 1.4 mm and 150 μL sieve with tap water. Adult worms were collected and stored individually at -20 °C in 4.5 mL cryotubes (ThermoFisher) before DNA extraction.

2.2. Fecal sample collection

Anonymized frozen fecal samples from commercial laying hens flocks in Sweden were obtained from the National Veterinary Institute (SVA). These samples originated from the national monitoring program for roundworm infections in layers initiated by the Association of Egg Producers in Sweden (https://www.svenskaagg.se/?p = 19,790&m=). The samples which initially analyzed with the flotation technique at SVA were collected from 79 laying hen farms across the country between August 2019 and March 2020. Permission to use anonymized samples were obtained from SVA.

2.3. DNA extraction

2.3.1. Adult worms

Parasite genomic DNA was extracted from the anterior part of the collected adult worms (to avoid materials from parasite eggs in females) using a DNA purification kit (NucleoSpin® Tissue, Macherey-Nagel) according to the manufacturer's protocol and stored until further use at −20 °C.

2.3.2. Parasite eggs in feces

To obtain a good fecal matter consistency for the best pipetting experience during DNA extraction, a small pilot study was conducted, in which 20 g feces was mixed with tap water at 1:14, 1:4 and 1:2 ratios in 50 mL Falcon tubes. The best consistency of the fecal slurry was obtained by mixing feces and water with 1:2 ratio (data not presented). DNA extraction was then performed in accordance with the protocol of the NucleoSpin® DNA Stool kit using 220 μL of the slurries. This includes a single bead beating step using a universal tissue homogenizer (Precellys Evolution, Bertin Technologies) to facilitate egg disruption.

2.4. Primer/probe development for droplet digital PCR

Primers and probes for *A. galli* and *H. gallinarum* were designed using Primer3 [www.ncbi.nlm.nih.gov/pmc/articles/PMC3424584/] using sequences retrieved from NCBI Genbank. To ensure probe species specificity, each of the probes were placed in less variable regions of the ITS-2 for each nematode but where there were differences between the two genera. Primers and probes were synthesized and purified using HPLC by Integrated DNA Technologies (Leuven, Belgium). To each probe, a fluorescent dye was added to the 5' end and an Iowa Black® Dark Quencher was added to the 3' end (Table 1).

Table 1
Droplet digital PCR primer and probe sequences with respective modifications for *Ascaridia galli* and *Heterakis gallinarum*.

Primer name	5' Modification	Primer sequence	3' Modification	Fragment length (bp)
*A. galli*_Forward		ACTGCTTGATTGCTATTGCC		253
*A. galli*_Reverse		ATACAGCCACTTTTATGCTCC		
*A. galli*_Probe	/5HEX/	TCGCTTGTTTAGTGGCACAT	/3IABkFQ/	
*H. gallinarum*_Forward		ACTGCTGCTCATTCATTGC		310
*H. gallinarum*_Reverse		CGCGTATGGTATTGAATCGTC		
*H. gallinarum*_Probe	/56-FAM/	TGAGTTTGCATAACAGCCCA	/3IABkFQ/	

2.5. ddPCR conditions

The ddPCR experiments were performed (Applied Biosystems 2720 Thermal Cycler) under the following conditions: a single reaction (22 μL reaction volume) containing 11 μL of 2x ddPCR Supermix for Probes (no dUTP, Bio-Rad), 1.1 μL of each 20x stock solution for *H. gallinarum* primers/probe (FAM™) and *A. galli* primers/probe (HEX™), 1 μL DNA and varying amounts of Nuclease-Free Water depending on experimental setups. Approximately 20,000 uniform nanoliter-sized droplets were generated using the automatic droplet generator (AutoDG Instrument) prior to the amplification. The parameters for the amplification steps consisted of 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 62 °C for 1 min (annealing step), followed by an additional 10 min at 98 °C. The temperature for the annealing step was chosen, according to the results of the gradient PCR (data not shown). The detailed process of running the samples using ddPCR technology was described previously (Baltrušis et al. 2018). QuantaSoft™ software (version 1.7.4.0917) was used to assign positive/negative droplets and the fractional abundance (FA) value. Thresholds were manually adjusted in order to separate droplet clusters (Channel 1 (FAM™ dye) – 4000 AU and channel 2 (HEX™ dye) - 1500 AU).

2.6. Evaluation of the ddPCR assay

2.6.1. DNA samples

To evaluate the specificity and the sensitivity of our ddPCR protocol, we set up three experiments (Exp1−3). In Exp1 we aimed to investigate whether or not any cross-reactivity between the two species occurs when different combination of species-specific primer/probe sets (pps), and DNA is used, as shown in Table 2. In Exp2 we aimed to test the limits to which diluted DNA of *H. gallinarum* can be detected in the presence of a constant background concentration of *A. galli* (determine the limit of detection). Sample reactions consisted of a constant *A. galli*-DNA concentration and a dilution series (1:50, 1:100, 1:400, 1:800, 1:1000, 1:5000, 1:10000, 1:15000) of *Heterakis*-DNA. In Exp3, *A. galli* and *Heterakis*-DNA and their specific primers/probe sets were mixed at different ratios, as follows: 1:0, 1:3, 1:1, 3:1 and 0:1.

Table 2
Different combinations of *Ascaridia galli* and *Heterakis gallinarum* species-specific primers/probes (pp) with the target DNA in separate reactions to determine the pp specificity and lack of cross-reactivities in experiment 1. Thresholds were manually adjusted in order to separate droplet clusters (*H. gallinarum*: Channel 1 (FAM™ dye) – 4000 AU and *A. galli*: channel 2 (HEX™ dye) - 1500 AU).

Reaction	*A. galli* DNA	*H. gallinarum* DNA	*A. galli* pp	*H. gallinarum* pp
1	*	–	*	–
2	*	–	–	*
3	*	–	*	*
4	–	*	–	*
5	–	*	*	–
6	–	*	*	*
7	*	*	*	–
8	*	*	–	*
9	*	*	*	*

2.6.2. Fecal samples

In total 134 fecal samples from 79 farms were analyzed. Participating farms had submitted four sub-samples (collected from different places in each flock) which were coded upon arrival at SVA and then mixed and analyzed as one pool with a flotation technique based on 3 g of feces and saturated sugar-salt solution (density: 1.28 g/mL). The results of the SVA analysis were presented in a semiquantitative manner according to a subjective scale. The remaining of the pooled samples were then sent (frozen) to SLU. Upon arrival, the samples were first thawed, then mixed with tap water at 1:2 ratio and analyzed with the validated ddPCR protocol as outlined above.

2.6.3. Statistical analysis

To measure the agreement between flotation technique performed at SVA and our ddPCR assay we used The Cohen's kappa: $k = \frac{p_o - p_e}{1 - p_e}$ where p_o is the relative observed agreement among raters, and p_e is the hypothetical probability of chance agreement (https://idostatistics.com/cohen-kappa-free-calculator/#risultati). To interpret the results we referred to the guidelines provided by Landis and Koch (1977). In Exp. 3, the Pearson correlation coefficient was calculated using an online tool (https://www.socscistatistics.com/tests/pearson/).

3. Results

3.1. Exp.1

The specificities of the pps for the two parasites were evaluated in this experiment. No cross-reaction was observed when different combinations of DNA and parasite-specific pps were evaluated in duplex PCR reactions containing both primer-probe sets (Fig.1).

3.2. Exp. 2

The capacity of our ddPCR assay to detect small amount of *H. gallinarum* amplicon DNA was validated by running mixed DNA samples containing constant *A. galli* concentrations generating on average 242 DNA-copies, and a gradient of diluted *H. gallinarum* genomic DNA. The lowest obtained frequency threshold for the detection of *H. gallinarum* in this experiment was determined to be 0.8 % (Fig. 2).

3.3. Exp. 3

In Exp3, when the DNA belonging to the two species was mixed at various, pre-determined ratios, the obtained fractional abundance measurements were perfectly correlated with the initial ratios ($R^2 = 0.9$) (Fig. 3).

3.4. Exp. 4

Both primer-probe sets were first validated in Exp 1−3. Out of 134 samples, 76 (56 %) were only positive for *A. galli*, 1 (0.7 %) sample was only positive for *H. gallinarum* and 10 samples (7.4 %) were positive for both parasites. An overview of the results of the flotation method performed at SVA and the ddPCR is presented in Table 3. The results of the

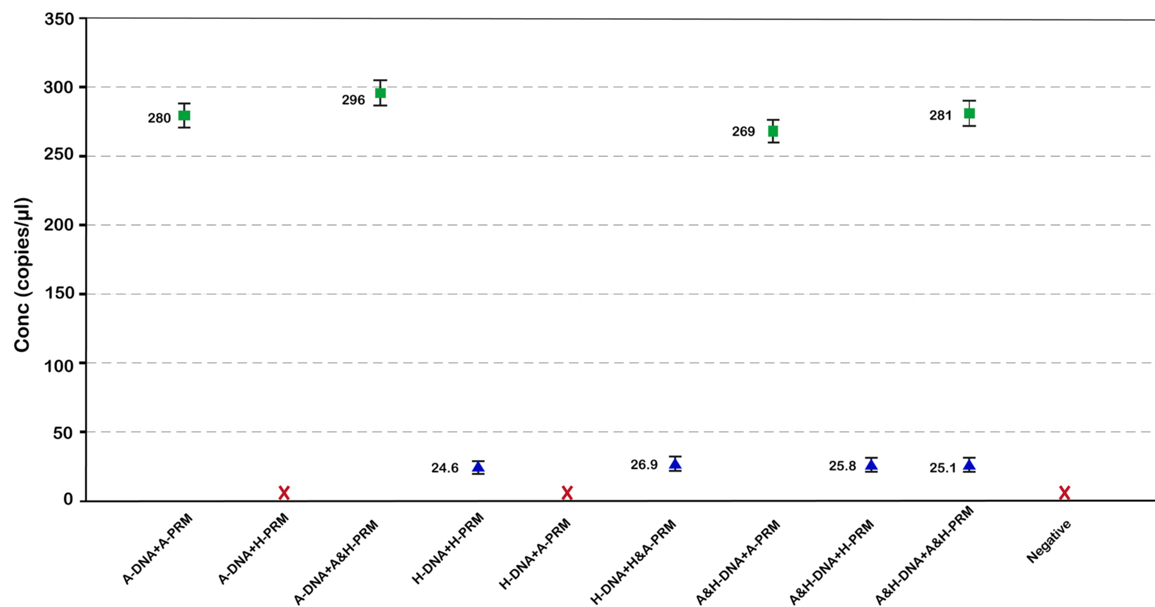

Fig. 1. Cross reactivities between the two species were tested by mixing *Ascaridia galli* (A) and *Heterakis gallinarum* (H) DNA and species-specific primer/probe sets (PRM) in nine reactions. Green squares indicate HEX (A-PRIM) and blue triangles indicate FAM (H-PRIM). Lack of observation is indicated by red X.

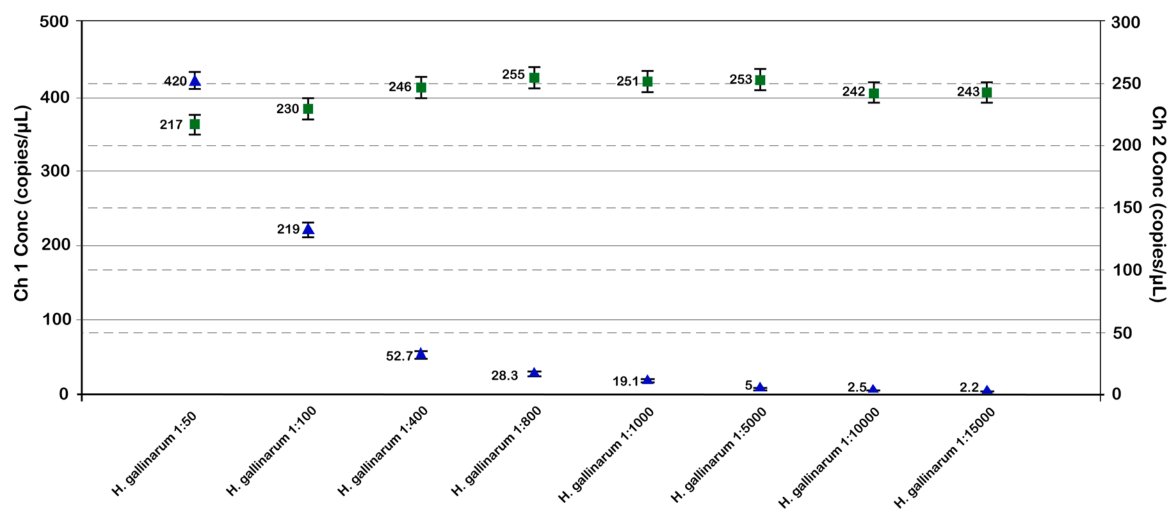

Fig. 2. Limit of detection assay for the determination of the lowest detectable fractional abundances of *Heterakis gallinarum* DNA in mixed samples. The *H. gallinarum* DNA were diluted (to 2%, 1%, 0.2 %, 0.12 %, 0.1 %, 0.02 %, 0.01 and 0.006 % of their initial volume) and mixed with a constant concentration of the *A. galli* DNA. Blue filled triangles correspond to the copy number of *H. gallinarum* DNA molecules and green filled squares to the copy number values for *A. galli* DNA.

Cohen's kappa indicated substantial agreement of 85.8 % (Cohen's k: 0.696).

4. Discussion

Recent advancement in molecular techniques has opened up opportunities to develop novel parasitological diagnostic tools, which are more sensitive and specific compared with conventional diagnostic approaches (e.g., the flotation method). An example of such techniques is droplet digital PCR - a quantitative PCR-method based on water-oil emulsion droplet technology. A major advantage of ddPCR over other PCR based tests is the possibility to analyze the samples on a semi-automatized platform, without the need for standard curves (Hindson et al., 2011). Furthermore, ddPCR provides highly precise measurements of target DNA, improved accuracy in the presence of inhibitors, and overall simplified procedures with reduced variability. Combined, this makes ddPCR stand out from other similar technologies, such as qPCR (Yang et al., 2014; Kuypers and Jerome, 2017).

Like other domestic livestock, chickens can simultaneously be infected with multiple parasite species (Kaufmann et al., 2011). Among the nematodes with the highest prevalence in chickens are *A. galli* and *H. gallinarum* (Sherwin et al., 2013). Morphologically similar free-living stages of these two parasites makes it challenging to differentiate between the two when necropsy is not the preferred option. Polymerase chain reaction-based methods have advantages in overcoming misdiagnosis, in that they allow for the specific detection of parasitic DNA rather than microscopical examination, provided the appropriate DNA target sequence is employed. The ITS-2 is known to be more conserved than ITS-1, yet it has a reasonably high degree of variation even between closely related species (Schultz et al., 2005). Like multiplex qPCR, ddPCR has the capacity to run multiple probes per single reaction, which makes it a good candidate to be used in differential diagnosis. This is not the first time that the ddPCR technology has been applied in a diagnostic context concerning parasites of veterinary and medical interest. The

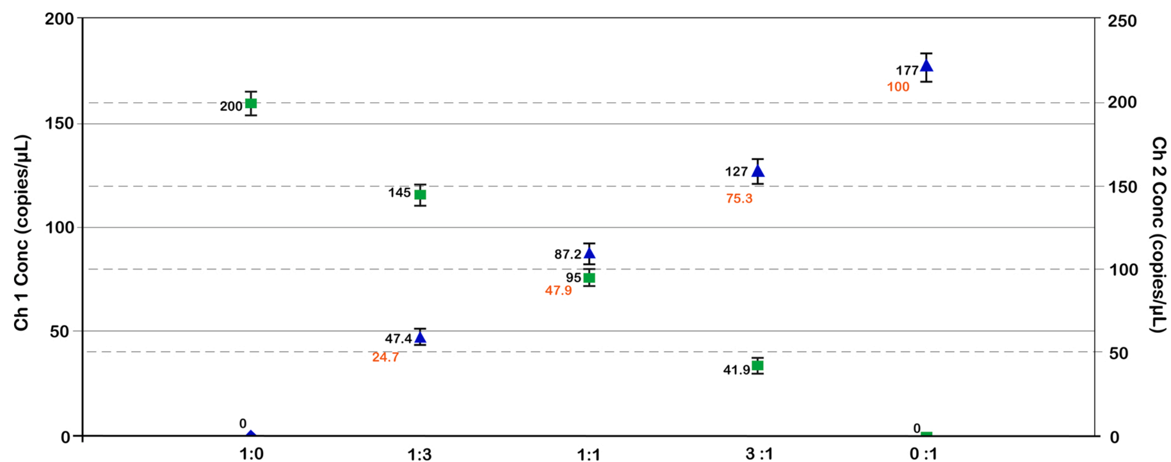

Fig. 3. Fractional abundance (FA) precision test. *Ascaridia galli* and *Heterakis gallinarum* DNA samples were mixed in equal volumes at different ratios (1:0, 3:1, 1:1, 1:3, and 0:1) to evaluate the capacity of the technique to determine the FA of each parasite genus DNA at every dilution ratio and produce an anticipated linear dilution pattern. Blue filled triangles correspond to the copy number of *H. gallinarum* DNA in the initial sample, while green filled squares correspond to the copy number of *A. galli* DNA. Values displayed in orange represent the FA index for *H. gallinarum* DNA copy number.

Table 3
An overview of the results of the ddPCR and the flotation technique. The ddPCR results are based on the detection of *Ascaridia galli* and *Heterakis gallinarum* DNA and presented as +/- samples for respective species. Results of the flotation technique is presented only as roundworm +/- due to the lack of specificity of the test to differentiate between the two species. Lack of observation for respective test is indicated with an asterisk.

Test	Results					
	Roundworm +	Roundworm -	*A. galli* +	*H. gallinarum* +	*A. galli* -	*H. gallinarum* -
Flotation	82	52	*	*	*	*
ddPCR	87	47	86	11	48	123

technique has been validated and used for detection of parasitic species in a wide range of animals such as cattle (Baltrušis et al., 2019) sheep (Elmahalawy et al., 2018), hamsters (Wilson et al., 2015), and human (Koepfli et al., 2016; Weerakoon et al., 2017). Herein we have adopted the ddPCR technique, and for the first time, developed a validated assay that with high precision can detect and simultaneously differentiate between *A. galli* and *H. gallinarum,* which today are key intestinal nematodes of chickens due to the European-wide ban of conventional battery cages (https://eur-lex.europa.eu/eli/dir/1999/74/oj).

Our results show that the ddPCR method allows absolute quantification of both *A. galli* and *H. gallinarum* DNA extracted from adult worms in the same sample. No cross-reactivity was observed when the specific pps were used in the same reaction on DNA extracted from each of the two species. The approach used in this investigation is similar to those used by other researchers to distinguish between common gastrointestinal nematodes of sheep (Elmahalawy et al., 2018) and cattle (Baltrušis et al., 2019) based on the differences in the ITS-2 region of the small-subunit DNA gene array. It is worth mentioning that unlike parasite species of grazing livestock, there are limited numbers of ITS sequences available for *A. galli* and *H. gallinarum* (18 and 33 for *A. galli* and *H. gallinarum* as opposed to 441 for example for *Haemonchus contortus*). Nevertheless, by using the BLAST™ function on NCBI website, we verified that our specific pps were species-specific, in terms of the most important nematodes found in chickens.

There was a gradual decrease in copy numbers for *H. gallinarum* corresponding to the serial dilutions of the worm DNA. The lowest detection level in our experiment was estimated as 0.8 % provided robust FA value for the detection of *H. gallinarum* in the presence of *A. galli*, suggesting that detection of *H. gallinarum* which due to its nature produces a smaller number of eggs, is possible not only in samples from a single host but also in pooled samples from either a single flock or multiple flocks. Although it is not customary to keep turkeys and chickens in a close vicinity, producers of free-range and game birds and owner of hobby flocks often violate this rule. In a farm with a history of histomonosis, early detection and treatment of *H. gallinarum* on a flock can potentially reduce the risk that infection spreads to other flocks or compartments of the same farm.

Data from Exp. 2 was reasonably predictive and ranged between 0.3–2.1 % deviation from predicted values. This finding broadly supports the work of other studies in this area demonstrating accurate identification of FA values using ITS-2 in *Teladorsagia circumcincta* and *H. contortus* (Elmahalawy et al., 2018; Baltrušis et al., 2019).

Annual routine diagnostic necropsy results from commercial laying hens at SVA (Desiree Jansson, personal communication) indicates that the prevalence of *H. gallinarum* is not as high in Sweden as it seems to be in other EU member states (Sherwin et al., 2013; Thapa et al., 2015; Wuthijaree et al., 2017). However, no systematic nationwide investigation has been conducted to estimate the occurrence of *H. gallinarum* in Sweden. As we have demonstrated here, even though there was a substantial agreement between ddPCR and the flotation technique which indeed seems to be a sensitive method, the number of flocks with mono- or dual infection with *H. gallinarum* was underestimated by copromicroscopy as they could only be categorized as ascarid positive. Another example of the limitation pertaining to the interpretation of the results of the copromicroscopy can be found in the work of Sherwin et al. (2013), where the author presented the prevalence (%) of ascarid species determined by egg per gram feces (EPG) and stated that the ascarid species was presumably primarily *A. galli*. Previous research has found that low levels of *A. galli* infection are not economically important (Sharma et al., 2018). If finding parasite eggs in coprological investigations is the criterion to justify anthelmintic use, accurately identifying the species present and the degree of infection is critical under those circumstances. Further research is needed to investigate the impact of high and low level of *H. gallinarum* infection on performance of laying hens.

Another advantage of choosing molecular techniques over the

conventional methods is that ddPCR can be theoretically run on any sample irrespective of its quality or storage condition provided that the target DNA is intact. By contrast, accuracy of the outcome of the fecal egg count (FEC) (flotation or McMaster methods) relies primarily on freshness of the fecal material and requires intact (viable) parasite eggs. Storage of the samples in suboptimal conditions has been shown to alter the viability of *A. galli* eggs (Tarbiat et al., 2015, 2018) and therefore negatively affect the FEC results. Fungal growth can as well damage parasite egg-shell structures, reduce their vitality and therefore, decrease the FEC (Thapa et al., 2017).

By using the ddPCR assay, we achieved a 6% higher detection rate compared with the flotation method. This finding is consistent with that of Höglund et al. (2019) who reported higher detection of *H. contortus* using ddPCR (75 %) compared with microscopy approach (56 %) in Swedish sheep herds. Similar observation has been reported by Sweeny et al. (2011) when the authors identified strongylid eggs using a PCR assay in comparison with McMaster. Readers should bear in mind that the tissue-dwelling stages of *A. galli* and *H. gallinarum* do not produce eggs and are not easily accessible by necropsy, therefore, they are not detectable by either conventional diagnostic tools or molecular methods. The lack of total agreement between the coprological methods and the ddPCR can be partly explained by the fact that the two methods require different preparation procedures to isolate parasite eggs. An important stage in the preparation of the fecal samples prior to the quantitative measurement of parasite eggs is the homogenization of the fecal material. As mentioned earlier, inferior detection level of flotation method could also be due to the presence of damaged or non-viable parasite eggs in the samples. On the other hand, ddPCR is cable of detecting minute amount of the target DNA in the sample. This increases the possibility of detecting non-viable eggs or possibly worm DNA in feces.

Since the results of the flotation method was provided as categorical data, it was not possible to assess the correlation between the number of parasite eggs or worm burden with the DNA copy numbers detected in our ddPCR assay. However, Elmahalawy et al. (2018) reported a positive highly significant correlation between fecal egg count reduction test data and reductions based on universal DNA concentration measured by ddPCR in samples collected from sheep herds infected with *H. contortus.* Further research could usefully explore whether ddPCR can be used to estimate the worm burden or infection pressure based on DNA copy numbers in fecal materials of chickens since both necropsy and copromicroscopy are readily available for use in chickens.

While molecular diagnostic assays are routinely applied in research settings, to our knowledge, they are seldom used in mass screening programs. As no standard curve is needed, ddPCR allows for direct comparison of parasite infection measured in different laboratories. Moreover, variation in quantification between technical replicates has been shown to be low (Koepfli et al., 2016; Elmahalawy et al., 2018). The findings of this investigation complement those of earlier studies and suggest that more accurate diagnostics can be achieved through application of ddPCR technology in chickens and at farm level.

5. Conclusion

The present study establishes a quantitative framework for detecting and differentiating intestinal nematodes of major importance in chickens. This approach will prove useful in expanding our understanding of the prevalence and the distribution of *A. galli* and *H. gallinarum* on commercial laying hen farms. Future research needs to examine more closely the links between parasite-DNA copy numbers and worm burden. This technique has the potential to be used in the monitoring of the infection level in commercial chicken flocks which in turn can regulate anthelmintic treatment and reduce unnecessary anthelmintic use.

CRediT authorship contribution statement

Behdad Tarbiat: Formal analysis, Investigation, Data curation, Writing-Original draft, Writing-Reviewing & Editing, Visualization. **Nizar Enweji:** Review & Editing, Investigation. **Paulius Baltrusis**: Review & Editing. **Peter Halvarsson:** Review & Editing, Investigation. **Eva Osterman-Lind:** Review & Editing. **Desiree Jansson**: Review & Editing. **Johan Höglund**: Conceptualization, Supervision, Project administration, Funding acquisition, Resources, Review & Editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

We would like to show our appreciation to farm managers for providing us the opportunity to harvest adult worms for DNA extraction. This work was supported by the Swedish Farmer's Foundation for Agricultural Research (SLF) (grant number O-19-20-198).

References

Baltrušis, P., Halvarsson, P., Höglund, J., 2019. Molecular detection of two major gastrointestinal parasite genera in cattle using a novel droplet digital PCR approach. Parasitol. Res. 118, 2901–2907.

Campbell, A.J.D., Gasser, R.B., Chilton, N.B., 1995. Differences in a ribosomal DNA sequence of Strongylus species allows identification of single eggs. Int. J. Parasitol. 25, 359–365.

Daş, G., Abel, H., Savaş, T., Sohnrey, B., Gauly, M., 2014. Egg production dynamics and fecundity of Heterakis *gallinarum* residing in different caecal environments of chickens induced by fibre-rich diets. Vet. Parasitol. 205, 606–618.

Elmahalawy, S.T., Halvarsson, P., Skarin, M., Höglund, J., 2018. Droplet digital polymerase chain reaction (ddPCR) as a novel method for absolute quantification of major gastrointestinal nematodes in sheep. Vet. Parasitol. 261, 1–8.

Fag, N.-Å., 2006. Djurläkemedelsanvändning 2005. Jordbruksverket.

Gasser, R.B., Monti, J.R., 1997. Identification of parasitic nematodes by PCR-SSCP of ITS-2 rDNA. Mol. Cell. Probes 11, 201–209.

Gauly, M., Duss, C., Erhardt, G., 2007. Influence of *Ascaridia galli* infections and anthelmintic treatments on the behaviour and social ranks of laying hens (Gallus gallus domesticus). Vet. Parasitol. 146, 271–280.

Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A. J., Bright, I.J., Lucero, M.Y., Hiddessen, A.L., Legler, T.C., Kitano, T.K., Hodel, M.R., Petersen, J.F., Wyatt, P.W., Steenblock, E.R., Shah, P.H., Bousse, L.J., Troup, C.B., Mellen, J.C., Wittmann, D.K., Erndt, N.G., Cauley, T.H., Koehler, R.T., So, A.P., Dube, S., Rose, K.A., Montesclaros, L., Wang, S., Stumbo, D.P., Hodges, S.P., Romine, S., Milanovich, F.P., White, H.E., Regan, J.F., Karlin-Neumann, G.A., Hindson, C.M., Saxonov, S., Colston, B.W., 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal. Chem. 83, 8604–8610.

Höglund, J., Elmahalawy, S.T., Halvarsson, P., Gustafsson, K., 2019. Detection of Haemonchus contortus on sheep farms increases using an enhanced sampling protocol combined with PCR based diagnostics. Vet. Parasitol.: X 2, 100018.

Hoste, H., Gasser, R.B., Chilton, N.B., Mallet, S., Beveridge, I., 1993. Lack of intraspecific variation in the second internal transcribed spacer (ITS-2) of Trichostrongylus colubriformis ribosomal DNA. Int. J. Parasitol. 23, 1069–1071.

Huggett, J.F., Foy, C.A., Benes, V., Emslie, K., Garson, J.A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R.D., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., Bustin, S.A., 2013. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. Clin. Chem. 59, 892–902.

Jansson, D.S., Nyman, A., Vågsholm, I., Christensson, D., Göransson, M., Fossum, O., Höglund, J., 2010. Ascarid infections in laying hens kept in different housing systems. Avian Pathol. 39, 525–532.

Jordbruksverket, 2019. Försäljning, av, djurläkemedel, p. 2019.

Kaufmann, F., Daş, G., Sohnrey, B., Gauly, M., 2011. Helminth infections in laying hens kept in organic free range systems in Germany. Livest. Sci. 141, 182–187.

Koepfli, C., Nguitragool, W., Hofmann, N.E., Robinson, L.J., Ome-Kaius, M., Sattabongkot, J., Felger, I., Mueller, I., 2016. Sensitive and accurate quantification of human malaria parasites using droplet digital PCR (ddPCR). Sci. Rep. 6, 39183.

Kuypers, J., Jerome, K.R., 2017. Applications of digital PCR for clinical microbiology. J. Clin. Microbiol. 55, 1621–1628.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159–174.

Luna-Olivares, L.A., Ferdushy, T., Kyvsgaard, N.C., Nejsum, P., Thamsborg, S.M., Roepstorff, A., Iburg, T.M., 2012. Localization of *Ascaridia galli* larvae in the jejunum of chickens 3 days post infection. Vet. Parasitol. 185, 186–193.

McDougald, L.R., 1998. Intestinal protozoa important to poultry. Poult. Sci. 77, 1156–1158.

McDougald, L.R., 2005. Blackhead disease (histomoniasis) in poultry: a critical review. Avian Dis. 49, 462–476.

Öberg, Å.L., 2020. Marknadsrapport ägg april 2020 (Jönköping, Enheten för handel och marknad).

Permin, A., Bisgaard, M., Frandsen, F., Pearman, M., Kold, J., Nansen, P., 1999. Prevalence of gastrointestinal helminths in different poultry production systems. Br. Poult. Sci. 40, 439–443.

Schultz, J., Maisel, S., Gerlach, D., Müller, T., Wolf, M., 2005. A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. RNA 11, 361–364.

Sharma, N., Hunt, P.W., Hine, B.C., Sharma, N.K., Chung, A., Swick, R.A., Ruhnke, I., 2018. Performance, egg quality, and liver lipid reserves of free-range laying hens naturally infected with *Ascaridia galli*. Poult. Sci. 97, 1914–1921.

Sharma, N., Hunt, P.W., Hine, B.C., Ruhnke, I., 2019. The impacts of *Ascaridia galli* on performance, health, and immune responses of laying hens: new insights into an old problem. Poult. Sci. 98, 6517–6526.

Sherwin, C.M., Nasr, M.A., Gale, E., Petek, M., Stafford, K., Turp, M., Coles, G.C., 2013. Prevalence of nematode infection and faecal egg counts in free-range laying hens: relations to housing and husbandry. Br. Poult. Sci. 54, 12–23.

Stehr, M., Grashorn, M., Dannenberger, D., Tuchscherer, A., Gauly, M., Metges, C.C., Daş, G., 2019. Resistance and tolerance to mixed nematode infections in relation to performance level in laying hens. Vet. Parasitol. 275, 108925.

Sweeny, J.P.A., Robertson, I.D., Ryan, U.M., Jacobson, C., Woodgate, R.G., 2011. Comparison of molecular and McMaster microscopy techniques to confirm the presence of naturally acquired strongylid nematode infections in sheep. Mol. Biochem. Parasitol. 180, 62–67.

Tarbiat, B., Jansson, D.S., Höglund, J., 2015. Environmental tolerance of free-living stages of the poultry roundworm *Ascaridia galli*. Vet. Parasitol. 209, 101–107.

Tarbiat, B., Rahimian, S., Jansson, D.S., Halvarsson, P., Höglund, J., 2018. Developmental capacity of *Ascaridia galli* eggs is preserved after anaerobic storage in faeces. Vet. Parasitol. 255, 38–42.

Tarbiat, B., Jansson, D.S., Wall, H., Tydén, E., Höglund, J., 2020. Effect of a targeted treatment strategy against *Ascaridia galli* on egg production, egg quality and bird health in a laying hen farm. Vet. Parasitol. 286, 109238.

Thapa, S., Hinrichsen, L.K., Brenninkmeyer, C., Gunnarsson, S., Heerkens, J.L.T., Verwer, C., Niebuhr, K., Willett, A., Grilli, G., Thamsborg, S.M., Sørensen, J.T., Mejer, H., 2015. Prevalence and magnitude of helminth infections in organic laying hens (Gallus gallus domesticus) across Europe. Vet. Parasitol. 214, 118–124.

Thapa, S., Mejer, H., Thamsborg, S.M., Lekfeldt, J.D.S., Wang, R., Jensen, B., Magid, J., Meyling, N.V., 2017. Survival of chicken ascarid eggs exposed to different soil types and fungi. Appl. Soil Ecol. 121, 143–151.

Weerakoon, K.G., Gordon, C.A., Williams, G.M., Cai, P., Gobert, G.N., Olveda, R.M., Ross, A.G., Olveda, D.U., McManus, D.P., 2017. Droplet digital PCR diagnosis of human schistosomiasis: parasite cell-free DNA detection in diverse clinical samples. J. Infect. Dis. 216, 1611–1622.

Wilson, M., Glaser, K.C., Adams-Fish, D., Boley, M., Mayda, M., Molestina, R.E., 2015. Development of droplet digital PCR for the detection of Babesia microti and Babesia duncani. Exp. Parasitol. 149, 24–31.

Wongrak, K., Gauly, M., Daş, G., 2015. Diurnal fluctuations in nematode egg excretion in naturally and in experimentally infected chickens. Vet. Parasitol. 208, 195–203.

Wuthijaree, K., Lambertz, C., Gauly, M., 2017. Prevalence of gastrointestinal helminth infections in free-range laying hens under mountain farming production conditions. Br. Poult. Sci. 58, 649–655.

Yang, R., Paparini, A., Monis, P., Ryan, U., 2014. Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of Cryptosporidium oocysts in faecal samples. Int. J. Parasitol. 44, 1105–1113.