Veterinary Parasitology 320 (2023) 109974

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Preliminary results of the recombinase polymerase amplification technique for the detection of *Haemonchus contortus* from Hungarian field samples

Rojesh Khangembam [a,b,*], Nóra Vass [b], Alison Morrison [c], Lynsey A. Melville [c], Alistair Antonopoulos [d,e,**], Levente Czeglédi [b]

[a] *Doctoral School of Animal Science, Faculty of Agricultural and Environmental Management, University of Debrecen, Böszörményi ut. 138, Debrecen 4032, Hungary*
[b] *Department of Animal Science, Institute of Animal Science, Biotechnology and Nature Conservation, Faculty of Agricultural and Environmental Management, University of Debrecen, Böszörményi ut. 138, Debrecen 4032, Hungary*
[c] *Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Scotland, UK*
[d] *School of Biodiversity, One Health, and Veterinary Medicine, University of Glasgow, Glasgow, Scotland, UK*
[e] *Kreavet, Kruibeke, Belgium*

ARTICLE INFO

Keywords:
Haemonchus
RPA
Isothermal amplification
Point of care
Diagnostic

ABSTRACT

Haemonchus contortus is a parasitic nematode of small ruminants responsible for significant economic losses and animal health concerns globally. Detection of gastrointestinal nematode (GIN) infection in veterinary practice typically relies on microscopy-based methods such as the faecal egg count and morphological identification of larval culture. However, mixed co-infections are common and species-specific identification is typically time-consuming and expertise-intensive. Compounded by increasing anthelmintic resistance, there is an urgent need to implement the molecular diagnosis of GIN in the livestock industry, preferably in field settings. Advances in isothermal amplification techniques including recombinase polymerase amplification (RPA) assays could improve this. Yet, constraints in RPA kit availability and amplicon detection systems limit the use of this technology in point of care settings. In this study, we present an early-stage, proof-of-concept demonstration of RPA targeting the internal transcribed spacer (ITS2) region of *H. contortus*. Having tested against eight closely related nematodes and also against five farm isolates in Eastern Hungary, preliminary results derived from a comparative analysis of 3 primer sets showed the assay detects *H. contortus* DNA and has a limit of detection of 10^{-5} ng/μl. We also tested an end-result naked eye detection system using various DNA binding dyes, of which EvaGreen® dye was successful for a qualitative RPA detection that could be adaptable at farm sites.

1. Introduction

Haemonchus contortus is a gastrointestinal nematode (GIN) primarily infecting small ruminants. It is one of the most pathogenic and economically important GINs with a high potential for production loss and animal welfare issues (Miller et al., 2012; Qamar and Maqbool, 2012; Kotze and Prichard, 2016; Besier et al., 2016; Kotze et al., 2020; Rose Vineer et al., 2020). Haemonchosis can lead to severe anaemia, with individual worms capable of consuming up to 50 μl of blood per day (Clark et al., 1962), which can cause a cumulative daily blood loss of approximately 30 ml in cases of severe infection (Albers and Le Jambre, 1983). Although it originated in warmer regions of the world, haemonchosis has been increasingly reported in temperate climates, including in Europe (Rinaldi et al., 2015; Arsenopoulos et al., 2021). To date, reports of haemonchosis in Hungary are still very limited although there are anecdotal claims by sheep farmers in addition to occasional post-mortem diagnoses of the parasite in the country (Khangembam et al., 2021).

Species-specific identification of causative GIN infection is of critical importance when managing livestock (Knoll et al., 2021). Mixed infections with multiple species of GIN are common across sheep farming regions around the world (Roeber et al., 2013; Redman et al., 2019; Zajac and Garza, 2020) and different species of GIN vary in their consequent pathology (Mavrot et al., 2015; Jacobson et al., 2020).

* Corresponding author at: Doctoral School of Animal Science, Faculty of Agricultural and Environmental Management, University of Debrecen, Böszörményi ut. 138, Debrecen 4032, Hungary.
** Corresponding author at: Kreavet, Kruibeke, Belgium.
E-mail addresses: rojesh.kh@agr.unideb.hu (R. Khangembam), Alistair@kreavet.com (A. Antonopoulos).

https://doi.org/10.1016/j.vetpar.2023.109974
Received 1 April 2023; Received in revised form 7 June 2023; Accepted 14 June 2023
Available online 16 June 2023
0304-4017/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Species-specific identification is also important within the overall context of ecological and epidemiological surveillance of GIN infection (Roeber et al., 2017; Borkowski et al., 2020). Morphological differentiation of closely related species is challenging and requires experienced staff (Waghorn et al., 2014). The subjective nature of morphological identification can lead to incorrect diagnoses and consequently inappropriate treatment decisions adopted (Bisset et al., 2014). Recent advances in the application of next-generation sequencing (NGS), such as "Nemabiome" sequencing have been key in expanding species-specific surveillance of mixed co-infections on a larger scale (Avramenko et al., 2015; Avramenko et al., 2018; Santa et al., 2021; De Seram et al., 2022; Francis and Šlapeta, 2022). However, this technology remains cost-prohibitive on a single farm level and for use in lower and middle-income countries where sequencing facilities are not easily accessible.

Isothermal amplification technologies have the capacity to bridge the gap between current point-of-care amenable microscopy-based tests, such as the faecal egg counting (FEC) methodologies recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles et al., 1992; Coles et al., 2006), and the more labour-intensive and complex laboratory-based molecular assays such as PCR (Gasser et al., 2006; Redman et al., 2008; Bisset et al., 2014; Roeber et al., 2017; Antonopoulos et al., 2022). The most well-established isothermal amplification technology to date is loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000). Recent advances in adapting LAMP to the detection of *H. contortus* DNA (Melville et al., 2014; Khangembam et al., 2021) have shown the amenability of isothermal amplification technologies to veterinary parasitology diagnosis. Another contender in the isothermal amplification technology is the recombinase polymerase assay (RPA). RPA was developed as proprietary technology of TwistDx™ and makes use of bacterial recombinase enzymes in conjunction with a single-stranded DNA (ssDNA) binding protein that facilitates the annealing of primers to the target sequence (Piepenburg et al., 2006).

RPA has several key advantages over other isothermal amplification technologies. At the time of writing this study, RPA is reportedly one of the fastest techniques and runs at the relatively low temperature range of 37–42 °C during the amplification step (Piepenburg et al., 2006; Lobato and O'Sullivan, 2018). In addition, RPA is reported to be adapted to existing PCR primers without further modification, although the reaction can at times be inhibited by background DNA in some circumstances (Rohrman and Richards-Kortum, 2015). Finally, RPA is amenable to adaptation to several point of care (POC) applications including lateral-flow detection (Wang et al., 2022; Srisrattakarn et al., 2022) as well as in combination with the Crispr-Cas based Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) technology (Gootenberg et al., 2017, 2018; Kellner et al., 2019). Also, there are reports available on various methods for the detection of RPA end-result including colourimetric visualisation (Lobato and O'Sullivan, 2018). The DNA binding dye, SYBR Green I has been reported successful in end-point detection of isothermal amplification products using LAMP for *Leptospira* spp. in urine (Ali et al., 2017). Similarly, successful RPA amplicon detection has also been reported for various target specimens, to name a few: adulterated meat samples (Cao et al., 2018), *Mycobacterium tuberculosis* (Singpanomchai et al., 2019) and *Staphylococcus aureus* (Srisrattakarn et al., 2020).

In this study, we build on the previous work demonstrating an isothermal assay (LAMP) targeting the highly conserved internal transcribed spacer (ITS2) region (Melville et al., 2014; Khangembam et al., 2021). By adapting and modifying a previously published ITS2 PCR primer set (Redman et al., 2008), we attempt to demonstrate species-specific detection of *H. contortus* DNA through RPA using gDNA extracted from both the laboratory and Hungarian field isolates of *H. contortus*. Lastly, we also attempted to replicate and formulate a qualitative detection system for RPA amplicons using DNA binding dyes as a proof-of-concept RPA assay adaptable to the farm site.

2. Materials and methods

2.1. Parasite Isolates and DNA templates

Nine nematode species, namely, *Cooperia curticei, Haemonchus contortus, Nematodirus battus, Oesophagostomum venulosum, Teladorsagia circumcincta, Trichostrongylus axei, Trichostrongylus colubriformis, Trichostrongylus vitrinus* and *Trichuris ovis* adults were kindly provided by the Moredun Research Institute, Scotland for species-specific testing of the *H. contortus* RPA primer sets. Adult *H. contortus* worms were also obtained from the Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest. Nematode DNA was extracted using the E.Z.N.A.® Tissue DNA Kit (Omega Biotek) according to the manufacturer's instructions. Five DNA samples extracted from sheep faecal samples collected from five different farms located in Eastern Hungary (Tóth, unpublished) were preserved at − 20 °C in the Parasitology Laboratory of the Doctoral School of Animal Science, University of Debrecen. These farm isolate templates were utilised for the field isolate component testing of the *H. contortus* RPA assay. Faecal egg count (FEC) and other reports/farm data were obtained from a parallel study (personal communication).

2.2. Primer design

Primer sets were designed in Geneious 11.1.5 (Biomatters Ltd. Auckland, New Zealand. Initially, ITS2 sequences were downloaded from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and pairwise aligned using MUSCLE alignment (8 iterations). *H. contortus* PCR primer sequences (Redman et al., 2008) were then annotated to the aligned consensus sequence and then modified by increasing the length of primer bind loci to better reflect the TwistDx recommended parameters for RPA primers. The expected amplicon size for the *H. contortus* ITS2 RPA reaction is 320 bp. The primer Hco-RPA-II was selected as optimal for this assay. The primer sequence is listed in Supplementary Table 1.

2.3. RPA reaction

The RPA assay was performed using the TwistAmp™ Basic kit (TwistDx, UK) with a single reaction volume of 50 μl as per the manufacturer's instructions. Betaine (Thermo Scientific™) was included in the reaction to reduce non-specific amplification (Luo et al., 2019). Briefly, each reaction tube contained forward and reverse primer (10 μM) at 2.5 μl each, 29.5 μl of primer-free rehydration buffer, 8 μl of 0.8 M betaine, 4 μl molecular grade water, 1 μl of DNA template and 2.5 μl of 280 mM magnesium acetate making a total of 50 μl final volume. The RPA reaction tubes were incubated using a portable heat block (VWR™ Advanced Mini Dry Block Heater). The reaction time and temperature were optimised at 37 °C for 25 min, with the tubes shaken vigorously at the beginning and the fourth minute. To terminate the amplification, tubes were incubated at 85 °C for 5 min. Post amplification, RPA products were cleaned using either EXOCleanUp FAST kit (VWR) or ChargeSwitch™ PCR Clean-Up kit (Thermo Scientific) according to the manufacturer's instructions. Cleaned RPA products were then visualised on ethidium bromide-stained 2.5% agarose gel electrophoresis and ChemiDoc XRS+ System and Image Lab (BioRad) were used for the analysis of the bands. RPA amplicons were also checked without the post-amplification clean-up step for comparison purposes.

2.4. ITS2 H. contortus species identification PCR

H. contortus ITS2 species identification PCR was set up in 12.5 μl reaction volume as follows: 2.5 μl 5 × GoTaq Green Buffer, 1.25 μl $MgCl_2$ 25 mM, 0.25 μl dNTPs 10 mM each (VWR International), 0.25 μl (100 pmol) each of forward and reverse primers, 0.06 μl GoTaq Flexi polymerase 5 U/μl (Promega), 6.94 μl of molecular grade water (AccuGene) and 1 μl of DNA template, and amplified as follows: denaturation at

94 °C for 2 min; 35 cycles each of 94 °C 30 s, the annealing temperature of 50 °C for 30 s and 72 °C extension for 30 s, with a final extension step of 72 °C 10 min. Results were visualised using 2 % agarose gel stained with ethidium bromide and visualised with ChemiDoc XRS+ System and Image Lab (BioRad).

2.5. RPA end-point detection using a UV lamp and DNA intercalating dye

RPA end-point detection using UV light and intercalating dye was carried out by modification of the protocol initially described by Srisrattakarn et al. (2020). Briefly, 5x EvaGreen® Dye (Biotium) was mixed with the RPA amplicons at a 4:1 ratio in triplicates. To check for the result, the RPA tubes were illuminated by UV rays using the ChemiDoc XRS+ System (BioRad). Similarly, various dye:amplicon ratios (data not shown) were tested using the 10000 × GelRed™ (Biotium) at working concentrations of 10 ×, 100 ×, 200 × and 300 × and in triplicates.

3. Results

3.1. HCo-RPA assay: optimisation and cross-reactivity

Our RPA assay screened three primer sets (Supplementary Table 1) based on the highly conserved ITS2 gene of *H. contortus*. The Hco-RPA-I primer set yielded amplification but also showed some non-specific amplification, which was not improved by the addition of betaine (Fig. 2-B). Single-species PCR and LAMP were performed after gel excision of non-specific amplicons resulting from the Hco-RPA-I assay, *H. contortus* DNA was not detected. The Hco-RPA-II primer had the best results (Fig. 1-B & C) and the non-specific amplifications observed previously were not detected following the addition of betaine (optimised at 0.8 M concentration) in the RPA master mix, indicating that betaine addition increased primer specificity. Also with the betaine addition, a significantly better result was observed with the ChargeSwitch™ PCR Clean-Up kit for the post-amplification cleaning (Supplementary Fig. 1). The ChargeSwitch™ PCR Clean-Up kit took longer to perform compared to the enzymatic-based clean-up principle of the VWR ExoCleanUP FAST kit (about 20–25 min and 8–15 min respectively for 8 reaction tubes).

Initially, the *H. contortus* RPA primers were tested against extracted DNA from *H. contortus*, in addition to other common GIN of veterinary importance: *C. curticei, N. battus, O. venulosum, T. circumcincta, T. axei, T. colubriformis, T. vitrinus* and *T. ovis*. Amplification was detected at the expected size for *H. contortus* (Fig. 1-A) of 320 bp. No amplification was seen at the expected size for any of the remaining species assayed. The specificity of the assay was also verified by the single species ITS2 PCR (Fig. 1-B) where the eight related ovine GIN species obtained from the Moredun Research Institute, Scotland were used as control templates to confirm species specificity. Only the *H. contortus* template gave a clear band at the expected amplicon size of 320 bp. Following confirmation of species-specific amplification, the limit of detection of the assay was established by using a 10-fold serial dilution of positive control *H. contortus* DNA. The limit of detection of the RPA assay for *H. contortus* was determined to be 0.1 ng/μl (Fig. 1-C).

3.2. Detection of H. contortus DNA in Hungarian field samples

The *H. contortus* ITS2 RPA assay was then used to detect the presence of *H. contortus* DNA in five Hungarian field samples (Fig. 2). Three out of the five farms, namely F1, F2 and F5, gave positive results for the presence of *H. contortus* DNA with primer set Hco-RPA-I, while the remaining two farms, F3 and F4 gave negative results (Fig. 2-B). The same set of farm DNA templates was utilised for RPA with the best primer set Hco-RPA-II along with betaine optimisation. This affirmed that Hco-RPA-II primer set gave better results showing clearer bands and lesser non-specific amplification (Fig. 2-C). This was also confirmed by the ITS2 species identification PCR (Fig. 2-A).

3.3. Detection of H. contortus in Hungarian field samples using UV illumination with DNA intercalating dyes

The results of the UV illumination using two different intercalating dyes, namely 5x EvaGreen® and various working concentrations of 10,000 × GelRed™ dyes, are shown in Fig. 3 and Supplementary Fig. 2-A–C respectively. For the EvaGreen® dye, the known *H. contortus* positive farm samples (F1, F2 and F5) presented a uniform red glow under UV illumination. Samples from F3 and F4 (known *H. contortus* negative farm samples) showed a visible fluorescent green precipitate (Fig. 3-A). However, no difference is detectable under white light (Fig. 3-B).

For the GelRed™ dye working concentrations of 100 ×, 200 × and 300 ×, none of the concentrations tested gave significant results, with positive and negative samples being indistinguishable under UV light (Supplementary Fig. 2-A). For the 10 × GelRed™ working concentration, two positive control template samples (of the triplicate) showed a red glow under UV light, while the non-template control and the remaining positive control template RPA tube gave no colour under UV illumination (Supplementary Fig. 2-C). When observed under white light, all the 10x concentration tubes gave no colour (Supplementary Fig. 2-B).

4. Discussion

Unlike LAMP (Notomi et al., 2000), RPA uses one primer pair in a manner analogous to PCR (Piepenburg et al., 2006), and it has been reported that PCR primers can be directly used as RPA primers without

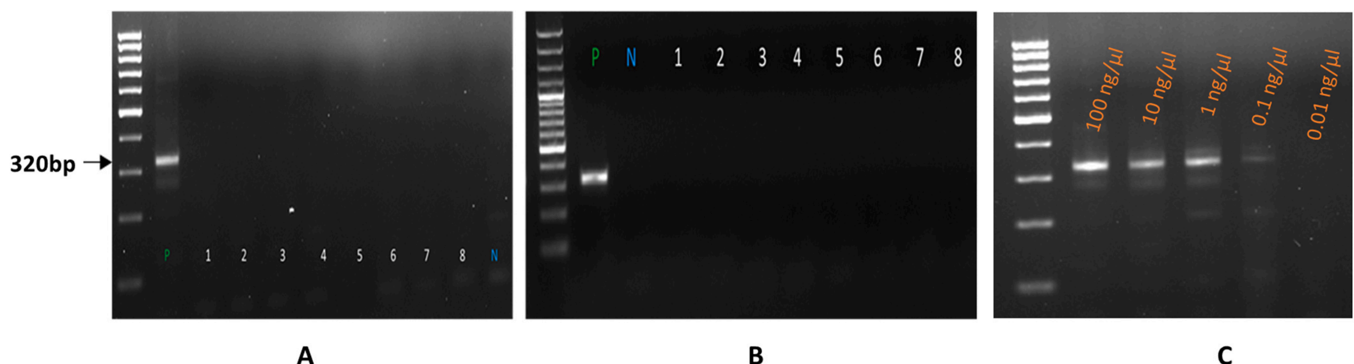

Fig. 1. Summary of species specificity and limit of detection of the *H. contortus* RPA assay. Hco-RPA-II primer set was used for the RPA assay. 100 bp ladder. A: *H. contortus* RPA assay. B: *H. contortus* ITS2 species identification PCR. C: Limit of detection of *H. contortus* RPA assay via serial dilution of *H. contortus* gDNA; RPA reactions included betaine addition. P = Positive control *H. contortus* DNA; N = non-template control; *1 = N. battus, 2 = T. axei; 3 = T. vitrinus; 4 = T. colubriformis; 5 = T. circumcincta; 6 = O. venulosum; 7 = T. ovis; 8 = C. curticei.*

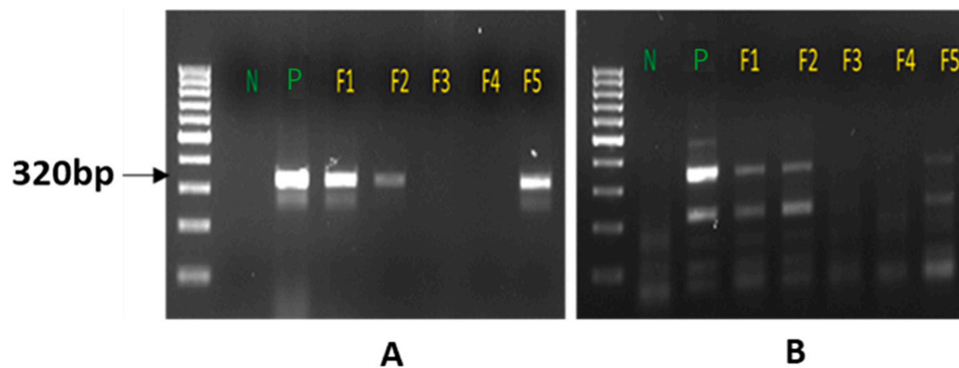

Fig. 2. Analysis of farm-isolate gDNA templates. 100 bp ladder. A: ITS2 PCR; B: RPA with Hco-RPA-I primer set with betaine; C: RPA with the best primer set Hco-RPA-II and optimised with betaine; P = Positive control *H. contortus* DNA; N = non-template control; F1–F5 = faecal sample DNA templates from farms in Eastern Hungary.

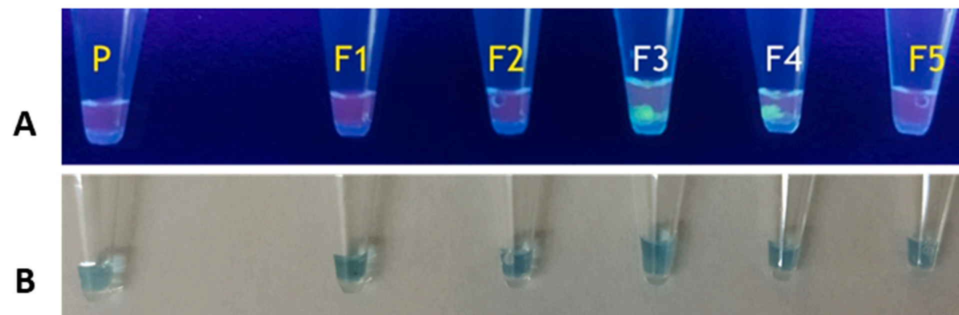

Fig. 3. RPA amplicons from farm-derived gDNA samples, detection using UV illumination using 5 × EvaGreen® dye; A: UV illumination view; B: white light view; P = Positive control *H. contortus* DNA from Budapest; F1–F5 = faecal sample DNA templates from farms in Eastern Hungary.

further modification, although with reduced efficiency (TwistAmp® DNA Amplification Kits Assay Design Manual). Out of the three primer sets tested for this assay (Supplementary Table 1), Hco-RPA/PCR is an original unmodified PCR primer (Redman et al., 2008). This was tested first to determine if *H. contortus* ITS2 primers could be used directly. The remaining RPA primer sets were modified according to the TwistDX Ltd RPA primer design criteria. The unmodified ITS2 PCR primer set (Hco-RPA/PCR) was tested but no significant amplification was observed. This was nonetheless an important aspect of the assay design trial as this would significantly speed up future development for RPA, a research point which the kit manufacturer encourages to report the findings. Had this been proven successful, this could have presented a significant advantage compared to LAMP, which usually requires extensive primer design and optimisation with specialised software (Torres et al., 2011). However, our results indicated that this approach is unlikely to yield optimal results for *H. contortus*. It should also be noted that post-amplification clean-up steps did not result in any significant improvements to reduce the non-specific amplification despite using two different kits. This non specific amplification could be due to primer binding at secondary loci. However, our assay utilised two different commercial post-amplification clean-up kits and yielded varying results where the EXOCleanUp FAST kit (VWR) gave quicker results but with some unwanted bands (Supplementary Fig. 1). Thus, the choice of a post-amplification cleaning kit could also affect the quality of the resulting output and the overall result-output time. The use of a relatively cheap thermal block (VWR™ Advanced Mini Dry Block Heater) with low power requirements and a high degree of portability was chosen to improve the POC amenability of the assay. This simple thermal block was sufficient to provide the optimised time-temperature of 37 °C for 25 min amplification step and a final 85 °C for 5 min for the reaction termination step.

In this study we showed *H. contortus* specific amplification when tested against the DNA templates from eight related helminth species. This was also confirmed by the agreement with the result of the single species ITS2 PCR, with no amplification detected in any species other than *H. contortus*. The present RPA technique reported a lower analytical sensitivity as compared to two other LAMP assays for the detection of *H. contortus*, where the limit of detection was between 1×10^{-5} ng (Melville et al., 2014) to 2.5×10^{-5} ng (Khangembam et al., 2021). A recent RPA study also reported a limit of detection of 100 fg of *H. contortus* DNA (Wu et al., 2021). The differences between these studies and our own are likely attributable to several factors including the divergence of Hungarian *H. contortus* isolates genetically, which is supported further by a lower limit of detection for the LAMP assay developed by Melville et al. (2014), and when applied by Khangembam et al. (2021) to Hungarian field isolates. Secondly, our assay approach prioritised the speed of development of a working proof-of-concept assay by modifying existing PCR primers to RPA. Hence, the principal aim for our assay was to demonstrate a rapid adaptation and adoption pipeline for researchers and clinicians within the field of veterinary parasitology, mainly for GIN parasites, to improve species-specific identification within specific local contexts. This is particularly important as current conventional diagnostics are no longer optimal, but molecular assay development lags far behind other fields, such as human medicine. This requires a concerted effort from the research community to demonstrate effective diagnostic tools (Kaplan, 2020) including proof-of-concept assays.

Our optimised RPA technique was also tested using DNA extracted from faecal samples from various farms in Eastern Hungary. It should be noted that farm F3 reported a low EPG (mean EPG = 5.76) with insignificant trichostrongyle egg counts, while F4 had no detectable trichostrongyle egg counts (Supplementary Table 2). Besides, F1 and F2 farms had already been analysed with peanut agglutinin (PNA) fluorescence microscopy and confirmed positively (Khangembam et al.,

unpublished) the presence of the parasite, as PNA fluorescence microscopy has been shown to selectively detect (Palmer and McCombe, 1996; Abbas and Hildreth, 2019) only *H. contortus* eggs. The results of the RPA were also crossed examined using ITS2 species-specific PCR. This shows that our assay is robust and can accurately and specifically detect *H. contortus* in farm isolates. Although the sensitivity is lower than that described by Wu et al. (2021), this nevertheless demonstrates that the RPA assay developed herein offers a comparable detection accuracy to that of the ITS2 species identification PCR in practice for Hungarian field isolates.

A further aim of this study was to reduce the need for equipment, time to result, and the required expertise of operating staff in using the RPA technique, thereby moving towards the development of an efficient pen-side molecular diagnostic assay. This remains impractical in conventional RPA assays, particularly if the post-amplification clean-up step and gel electrophoresis steps have to be performed. Preliminary findings of the RPA without the post-amplification clean-up and results visualisation using DNA intercalating dyes are shown in Supplementary Fig. 2. Initially, we attempted to eliminate the recommended post-amplification clean-up. We found that RPA amplicons could still be detected albeit with some 'smearing' in the lane of the positive bands, while the negative templates showed no bands with relatively fainter smearing (Supplementary Fig. 2-C). This smearing has been explained by the kit manufacturer (TwistDx Ltd) as possibly due to the interference by the crowding agent and proteins. As a follow-up to this and in line with our objectives, we also tested if the amplicons could be visualised either with UV illumination or by the naked eye in the presence of a DNA intercalating dye.

Of the many DNA intercalating dyes available, SYBR Green I is one of the most widely used dye-detection systems. Despite this, the main disadvantage is that SYBR Green I is expensive and often used for qPCR systems that require expensive equipment unsuitable for use outside of a well supplied laboratory. Hence, we tested if the comparatively cheaper alternative dyes, EvaGreen® and GelRed™, could replace SYBR Green I in our assay. Following the optimisation of the dye: amplicon ratio, we were able to detect a colour change indicating RPA amplification under UV light, however, this was not possible under white light (Fig. 3). The EvaGreen® dye showed promising results in this preliminary trial as the change in colours of the tested RPA tubes was visualised clearly under UV illumination. Nevertheless, it should also be noted that the EvaGreen® dye used here was of a low concentration (5 ×) and this might have also contributed to the failure of any colourimetric change detection by unaided eyes. This low concentration proved practical in this trial as this was more readily accessible in laboratories dealing with thermal amplification systems as well as cheaper in the market. The use of a handheld UV-lamp of decent quality can easily offset the need for UV illumination to detect any colourimetric change. GelRed™ dye, however, showed inconsistent results; at a working concentration of 10x, the GelRed™ dye correctly illuminated two of three positive control templates under UV (Supplementary Fig. 2-B). This could be due to the mismatched dye: amplicon ratio or interference by the RPA reagents. Our attempts to rectify and improve this using various working concentrations (100 ×, 200 × and 300 ×) proved unsuccessful (Supplementary Fig. 2-A), resulting in no change in the colour of the tubes either in white light or UV illumination. Hence, we acknowledge further work is necessary for this detection system to be fully optimised for an effective farm-side detection system.

Despite this setback, these preliminary results constitute an important first step towards developing naked-eye detection of RPA amplicons. The limited source of RPA kits and the detection system significantly complicate the development of new RPA tests hindering the design and optimisation of reaction conditions, custom-designed kit components are available but costly (Lobato and O'Sullivan, 2018). This has slowed the development and adoption of RPA in the context of point-of-care diagnostics, with a majority of the current RPA development restricted to research settings. It is nonetheless necessary to demonstrate the potential of this technology, as it offers significant advantages over conventional PCR-based molecular tests without compromising the sensitivity and specificity, particularly for resource-limited settings such as a farm.

5. Conclusion

The present study demonstrates the preliminary results of a proof-of-concept RPA assay, optimised for the identification of *H. contortus* which could be suitable for farm-side detection following further optimisation. We sought to explore a minimal equipment set-up to ensure applicability to field-based settings wherever possible. We also trialled our assay in some selected sheep farms in and around Eastern Hungary where farmers reported suspicion of the presence of *H. contortus*. Hungary still lags behind neighbouring countries in reporting the prevalence of *H. contortus* infections. There is limited data and information available from the country which is in part due to the lack of deployment of fast, cheap, and reliable diagnostics both for large-scale monitoring, and smaller-scale flock management. Finally, our results indicate that RPA is amenable to naked-eye detection using existing DNA intercalating dyes, although further optimisation of the dye concentration and amount is necessary. This could open the path to the development of a rapid farm-site RPA diagnostic assay with the kit available at the moment. This in turn suggests that RPA could serve as a suitable molecular diagnostic assay both for veterinary clinicians and for adoption within veterinary diagnostic laboratories in Hungary and Central Europe, with significant translational potential.

CRediT authorship contribution statement

Lynsey Melville: Writing – original draft, Resources, Control parasite specimen, Technical advice and Manuscript. **Alison Morrison:** Writing – original draft, Resources, Control parasite specimen, Technical advice and Manuscript. **Nóra Vass:** Writing – review & editing, Project administration, Data curation, Supervision, Sampling, Funding acquisition and Manuscript. **Levente Czeglédi:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis. **Alistair Antonopoulos:** Writing – review & editing, Writing – original draft, Methodology and Manuscript, Formal analysis, Conceptualization. **Rojesh Khangembam:** Writing – review & editing, Writing – original draft, Validation, Methodology and Manuscript, Investigation, Formal analysis, Conceptualization, Laboratory works.

Declaration of Competing Interest

The authors declare that they have no direct conflict of interest with this work.

Acknowledgement

The study was supported by the EFOP-3.6.3-VEKOP-16-2017-00008 project which is co-financed by the European Union and the European Social Fund. We would also like to acknowledge the James Herriot Scholarship Fund in Veterinary Medicine from the University of Glasgow, School of Veterinary Medicine for its contribution by funding the doctoral studies of Alistair Antonopoulos. Much gratitude to the Stipendium Hungaricum Scholarship from the Tempus Public Foundation, Hungary which provides funding to the doctoral studies of Rojesh Khangembam. Also, our appreciation to the Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest for providing adult *H. contortus* samples.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at doi:10.1016/j.vetpar.2023.109974.

References

Abbas, I., Hildreth, M., 2019. Egg autofluorescence and options for detecting peanut agglutinin binding for the identification of Haemonchus contortus eggs in fecal samples. Vet. Parasitol. 267, 69–74.

Albers, G., Le Jambre, L., 1983. Erythrocyte potassium concentration: a simple parameter for erythropoiesis in sheep infected with Haemonchus contortus. Res. Vet. Sci. 35 (3), 273–276.

Ali, S.A., Kaur, G., Boby, N., Sabarinath, T., Solanki, K., Pal, D., Chaudhuri, P., 2017. Rapid and visual detection of Leptospira in urine by LigB-LAMP assay with pre-addition of dye. Mol. Cell. Probes 36, 29–35.

Antonopoulos, A., Doyle, S.R., Bartley, D.J., Morrison, A.A., Kaplan, R., Howell, S., Neveu, C., Busin, V., Devaney, E., Laing, R., 2022. Allele specific PCR for a major marker of levamisole resistance in Haemonchus contortus. Int. J. Parasitol.: Drugs Drug Resist. 20, 17–26.

Arsenopoulos, K.V., Fthenakis, G.C., Katsarou, E.I., Papadopoulos, E., 2021. Haemonchosis: a challenging parasitic infection of sheep and goats. Animals 11 (2), 363.

Avramenko, R.W., Bras, A., Redman, E.M., Woodbury, M.R., Wagner, B., Shury, T., Liccioli, S., Windeyer, M.C., Gilleard, J.S., 2018. High species diversity of trichostrongyle parasite communities within and between Western Canadian commercial and conservation bison herds revealed by nemabiome metabarcoding. Parasites Vectors 11 (1), 1–13.

Avramenko, R.W., Redman, E.M., Lewis, R., Yazwinski, T.A., Wasmuth, J.D., Gilleard, J. S., 2015. Exploring the gastrointestinal "nemabiome": deep amplicon sequencing to quantify the species composition of parasitic nematode communities. PLoS One 10 (12), e0143559. https://doi.org/10.1371/journal.pone.0143559.

Besier, R., Kahn, L., Sargison, N., Van Wyk, J.A., 2016. The pathophysiology, ecology and epidemiology of Haemonchus contortus infection in small ruminants. Adv. Parasitol. 93, 95–143.

Bisset, S.A., Knight, J.S., Bouchet, C.L., 2014. A multiplex PCR-based method to identify strongylid parasite larvae recovered from ovine faecal cultures and/or pasture samples. Vet. Parasitol. 200 (1–2), 117–127. https://doi.org/10.1016/j.vetpar.2013.12.002.

Borkowski, E.A., Redman, E.M., Chant, R., Avula, J., Menzies, P.I., Karrow, N.A., Lillie, B. N., Sears, W., Gilleard, J.S., Peregrine, A.S., 2020. Comparison of ITS-2 rDNA nemabiome sequencing with morphological identification to quantify gastrointestinal nematode community species composition in small ruminant feces. Vet. Parasitol. 282, 109104 https://doi.org/10.1016/j.vetpar.2020.109104.

Cao, Y., Zheng, K., Jiang, J., Wu, J., Shi, F., Song, X., Jiang, Y., 2018. A novel method to detect meat adulteration by recombinase polymerase amplification and SYBR green I. Food Chem. 266, 73–78.

Clark, C.H., Kiesel, G.K., Goby, C.H., 1962. Measurements of blood loss caused by Haemonchus contortus infection in sheep. Am. J. Vet. Res. 23, 977–980.

Coles, G.C., Bauer, C., Borgsteede, F.H., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44 (1–2), 35–44.

Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A., Vercruysse, J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 136 (3–4), 167–185. https://doi.org/10.1016/j.vetpar.2005.11.019.

De Seram, E.L., Redman, E.M., Wills, F.K., de Queiroz, C., Campbell, J.R., Waldner, C.L., Parker, S.E., Avramenko, R.W., Gilleard, J.S., Uehlinger, F.D., 2022. Regional heterogeneity and unexpectedly high abundance of Cooperia punctata in beef cattle at a northern latitude revealed by ITS-2 rDNA nemabiome metabarcoding. Parasites Vectors 15 (1), 1–11.

Francis, E.K., Šlapeta, J., 2022. A new diagnostic approach to fast-track and increase the accessibility of gastrointestinal nematode identification from faeces: FECPAKG2 egg nemabiome metabarcoding. Int. J. Parasitol. 52 (6), 331–342.

Gasser, R.B., Hu, M., Chilton, N.B., Campbell, B.E., Jex, A.J., Otranto, D., Cafarchia, C., Beveridge, I., Zhu, X., 2006. Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. Nat. Protoc. 1 (6), 3121–3128.

Gootenberg, J.S., Abudayyeh, O.O., Lee, J.W., Essletzbichler, P., Dy, A.J., Joung, J., Verdine, V., Donghia, N., Daringer, N.M., Freije, C.A., 2017. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science 356 (6336), 438–442.

Gootenberg, J.S., Abudayyeh, O.O., Kellner, M.J., Joung, J., Collins, J.J., Zhang, F., 2018. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. Science 360 (6387), 439–444.

Jacobson, C., Larsen, J.W., Besier, R.B., Lloyd, J.B., Kahn, L.P., 2020. Diarrhoea associated with gastrointestinal parasites in grazing sheep. Vet. Parasitol. 282, 109139.

Kaplan, R.M., 2020. Biology, epidemiology, diagnosis, and management of anthelmintic resistance in gastrointestinal nematodes of livestock. Vet. Clin.: Food Anim. Pract. 36 (1), 17–30.

Kellner, M.J., Koob, J.G., Gootenberg, J.S., Abudayyeh, O.O., Zhang, F., 2019. SHERLOCK: nucleic acid detection with CRISPR nucleases. Nat. Protoc. 14 (10), 2986–3012. https://doi.org/10.1038/s41596-019-0210-2.

Khangembam, R., Tóth, M., Vass, N., Várady, M., Czeglédi, L., Farkas, R., Antonopoulos, A., 2021. Point of care colourimetric and lateral flow LAMP assay for the detection of Haemonchus contortus in ruminant faecal samples. Parasite *28*.

Knoll, S., Dessì, G., Tamponi, C., Meloni, L., Cavallo, L., Mehmood, N., Jacquiet, P., Scala, A., Cappai, M.G., Varcasia, A., 2021. Practical guide for microscopic identification of infectious gastrointestinal nematode larvae in sheep from Sardinia, Italy, backed by molecular analysis. Parasit. Vectors 14 (1), 505. https://doi.org/10.1186/s13071-021-05013-9.

Kotze, A.C., Prichard, R.K., 2016. Anthelmintic resistance in Haemonchus contortus: history, mechanisms and diagnosis. Adv. Parasitol. 93, 397–428. https://doi.org/10.1016/bs.apar.2016.02.012.

Kotze, A.C., Gilleard, J.S., Doyle, S.R., Prichard, R.K., 2020. Challenges and opportunities for the adoption of molecular diagnostics for anthelmintic resistance. Int. J. Parasitol. Drugs Drug Resist. 14, 264–273. https://doi.org/10.1016/j.ijpddr.2020.11.005.

Lobato, I.M., O'Sullivan, C.K., 2018. Recombinase polymerase amplification: basics, applications and recent advances. Trac Trends Anal. Chem. 98, 19–35.

Luo, G.-C., Yi, T.-T., Jiang, B., Guo, X.-l, Zhang, G.-Y., 2019. Betaine-assisted recombinase polymerase assay with enhanced specificity. Anal. Biochem. 575, 36–39.

Mavrot, F., Hertzberg, H., Torgerson, P., 2015. Effect of gastro-intestinal nematode infection on sheep performance: a systematic review and meta-analysis. Parasites Vectors 8 (1), 1–11.

Melville, L., Kenyon, F., Javed, S., McElarney, I., Demeler, J., Skuce, P., 2014. Development of a loop-mediated isothermal amplification (LAMP) assay for the sensitive detection of Haemonchus contortus eggs in ovine faecal samples. Vet. Parasitol. 206 (3–4), 308–312. https://doi.org/10.1016/j.vetpar.2014.10.022.

Miller, C.M., Waghorn, T.S., Leathwick, D.M., Candy, P.M., Oliver, A.M., Watson, T.G., 2012. The production cost of anthelmintic resistance in lambs. Vet. Parasitol. 186 (3–4), 376–381. https://doi.org/10.1016/j.vetpar.2011.11.063.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28 (12), E63.

Palmer, D.G., McCombe, I.L., 1996. Lectin staining of trichostrongylid nematode eggs of sheep: rapid identification of Haemonchus contortus eggs with peanut agglutinin. Int. J. Parasitol. 26 (4), 447–450.

Piepenburg, O., Williams, C.H., Stemple, D.L., Armes, N.A., 2006. DNA detection using recombination proteins. PLoS Biol. 4 (7), e204.

Qamar, M., Maqbool, A., 2012. Biochemical studies and serodiagnosis of haemonchosis in sheep and goats. J. Anim. Plant Sci. 22 (1), 32–38.

Redman, E., Packard, E., Grillo, V., Smith, J., Jackson, F., Gilleard, J.S., 2008. Microsatellite analysis reveals marked genetic differentiation between Haemonchus contortus laboratory isolates and provides a rapid system of genetic fingerprinting. Int. J. Parasitol. 38 (1), 111–122. https://doi.org/10.1016/j.ijpara.2007.06.008.

Redman, E., Queiroz, C., Bartley, D.J., Levy, M., Avramenko, R.W., Gilleard, J.S., 2019. Validation of ITS-2 rDNA nemabiome sequencing for ovine gastrointestinal nematodes and its application to a large scale survey of UK sheep farms. Vet. Parasitol. 275, 108933 https://doi.org/10.1016/j.vetpar.2019.108933.

Rinaldi, L., Catelan, D., Musella, V., Cecconi, L., Hertzberg, H., Torgerson, P.R., Mavrot, F., De Waal, T., Selemetas, N., Coll, T., Bosco, A., Biggeri, A., Cringoli, G., 2015. Haemonchus contortus: spatial risk distribution for infection in sheep in Europe. Geospat. Health 9 (2), 325–331. https://doi.org/10.4081/gh.2015.355.

Roeber, F., Jex, A.R., Gasser, R.B., 2013. Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance–an Australian perspective. Parasites Vectors 6 (1), 1–13.

Roeber, F., Hassan, E.B., Skuce, P., Morrison, A., Claerebout, E., Casaert, S., Homer, D.R., Firestone, S., Stevenson, M., Smith, L., Larsen, J., 2017. An automated, multiplex-tandem PCR platform for the diagnosis of gastrointestinal nematode infections in cattle: an Australian-European validation study. Vet. Parasitol. 239, 62–75. https://doi.org/10.1016/j.vetpar.2017.04.011.

Rohrman, B., Richards-Kortum, R., 2015. Inhibition of recombinase polymerase amplification by background DNA: a lateral flow-based method for enriching target DNA. Anal. Chem. 87 (3), 1963–1967.

Rose Vineer, H., Morgan, E.R., Hertzberg, H., Bartley, D.J., Bosco, A., Charlier, J., Chartier, C., Claerebout, E., De Waal, T., Hendrickx, G., 2020. Increasing importance of anthelmintic resistance in European livestock: creation and meta-analysis of an open database. Parasite 27, 69.

Santa, M.A., Rezansoff, A.M., Chen, R., Gilleard, J.S., Musiani, M., Ruckstuhl, K.E., Massolo, A., 2021. Deep amplicon sequencing highlights low intra-host genetic variability of Echinococcus multilocularis and high prevalence of the European-type haplotypes in coyotes and red foxes in Alberta, Canada. PLoS Negl. Trop. Dis. 15 (5), e0009428.

Singpanomchai, N., Akeda, Y., Tomono, K., Tamaru, A., Santanirand, P., Ratthawongjirakul, P., 2019. Naked eye detection of the Mycobacterium tuberculosis complex by recombinase polymerase amplification—SYBR green I assays. J. Clin. Lab. Anal. 33 (2), e22655.

Srisrattakarn, A., Tippayawat, P., Chanawong, A., Tavichakorntrakool, R., Daduang, J., Wonglakorn, L., Sooksongsoontorn, P., Lulitanond, A., 2020. Direct detection of methicillin-resistant in Staphylococcus spp. in positive blood culture by isothermal recombinase polymerase amplification combined with lateral flow dipstick assay. World J. Microbiol. Biotechnol. 36 (11), 1–11.

Srisrattakarn, A., Panpru, P., Tippayawat, P., Chanawong, A., Tavichakorntrakool, R., Daduang, J., Wonglakorn, L., Lulitanond, A., 2022. Rapid detection of methicillin-resistant Staphylococcus aureus in positive blood-cultures by recombinase polymerase amplification combined with lateral flow strip. PLoS One 17 (6), e0270686. https://doi.org/10.1371/journal.pone.0270686.

Torres, C., Vitalis, E.A., Baker, B.R., Gardner, S.N., Torres, M.W., Dzenitis, J.M., 2011. LAVA: an open-source approach to designing LAMP (loop-mediated isothermal amplification) DNA signatures. BMC Bioinform. 12 (1), 1–7.

TwistAmp® DNA Amplification Kits Assay Design Manual. ⟨https://www.twistdx.co.uk/wp-content/uploads/2021/04/twistamp-assay-design-manual-v2-5.pdf⟩.

Waghorn, T., Knight, J., Leathwick, D., 2014. The distribution and anthelmintic resistance status of Trichostrongylus colubriformis, T. vitrinus and T. axei in lambs in New Zealand. N. Z. Vet. J. 62 (3), 152–159.

Wang, H., Yang, X., Tuo, D., Liu, Y., Zhou, P., Shen, W., Zhu, G., 2022. Rapid detection of sweepoviruses through lateral flow dipstick-based recombinase polymerase amplification. Acta Virol. 66 (2), 186–191. https://doi.org/10.4149/av_2022_208.

Wu, Y.D., Wang, Q.Q., Wang, M., Elsheikha, H.M., Yang, X., Hu, M., Xu, M.J., 2021. Development of a lateral flow strip-based recombinase polymerase amplification assay for the detection of Haemonchus contortus in goat feces. Korean J. Parasitol. 59 (2), 167.

Zajac, A.M., Garza, J., 2020. Biology, epidemiology, and control of gastrointestinal nematodes of small ruminants. Vet. Clin. North Am. Food Anim. Pract. 36 (1), 73–87. https://doi.org/10.1016/j.cvfa.2019.12.005.