



Short communication

High-throughput identification and quantification of *Haemonchus contortus* in fecal samplesNoélie Douanne^{a,b}, Victoria Wagner^{a,b}, Denise Bélanger^a, Christopher Fernandez-Prada^{a,b,*}^a Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Canada^b Research Group on Infectious Diseases in Animal Production (GREMIP), Faculty of Veterinary Medicine, University of Montreal, Canada

ARTICLE INFO

Keywords:

Haemonchus contortus

Fluorescence microscopy

Automated fluorescence detection/
quantification

Diagnostic methods

HTS

Sheep

ABSTRACT

Haemonchus contortus are gastrointestinal nematodes of the family Trichostrongylidae that naturally infect small ruminants while grazing, posing a risk to both animal health and farm profitability. Current diagnostics depend on exacting lab techniques, including manual egg counts and larval differentiation, all of which require time, effort, and specialized technicians. The goal of this study was to facilitate and accelerate the identification and quantification of *H. contortus* in fecal samples through the use of fluorescein-isothiocyanate peanut-agglutinin staining in order to allow automated detection using a 96-well microplate reader. Next, the model was to be validated using samples containing unknown quantities of eggs. Automated analysis of fluorescence emission of known quantities of *H. contortus* eggs confirmed an almost perfect linear correlation ($r = 0.9984$, $p < 0.0001$), indicating that this new approach can satisfactorily be used to quantify *H. contortus* eggs on a comparative fluorescence scale. As validation, clinical samples containing an unknown quantity of *H. contortus* eggs were then analyzed by comparing two methods: either Wisconsin Sugar Flotation (WSF) and McMaster counting followed by manual fluorescence microscopy, or WSF coupled with automated microplate reading. Pearson analysis revealed highly significant correlation between manual and automated methods ($r = 0.9999$, $p < 0.0001$), while Bland-Altman plots demonstrated excellent agreement between the two (bias = -0.817 ± 9.94 with 95% limits of agreement from -20.31 to 18.67). Overall, these results demonstrate that high-throughput screening fluorescence detection and quantification of *H. contortus* eggs is both accurate and rapid.

1. Introduction

Globally, livestock are victim to important parasitic diseases. The current economic losses derived from endoparasitic infections greatly impact profitability, particularly for farms whose flocks graze on pastures. Naturally infected with gastrointestinal nematodes (GIN) of the family Trichostrongylidae while grazing, small ruminants are those most affected. In sheep, *Haemonchus contortus* is the GIN species with the greatest pathologic and economic impact due to its many distinctive characteristics. *H. contortus* is able to adapt to very unfavorable conditions and can survive inside its host through hypobiosis. This parasite also has a high fertility rate and is capable of rapid reinfestation, especially in hot and humid conditions (Jacquet et al., 1998; White et al., 2001). Moreover, *H. contortus* is prone to developing resistance against anthelmintics, which dramatically increases the number of therapeutic failures in many countries (Mederos et al., 2010; Barrere et al., 2013). Evidently, the ability to confirm the presence of *H.*

contortus within flocks is essential.

While Wisconsin Sugar Flotation method (WSF), followed by brightfield microscopy using a McMaster slide to manually determine egg count is the process of choice for detection of GIN infection, this approach cannot reliably differentiate *H. contortus* eggs from others belonging to the family Trichostrongylidae. Currently, the primary method used to specifically detect the presence of *H. contortus* is larval coproculture (LC), which is highly demanding, time-consuming, requires specially trained technicians, and can easily fail due to varying laboratory conditions (Falzon et al., 2013). In order to bypass larval identification drawbacks, various experiments have been conducted to determine a more efficient identification method. Several studies reported on the use of lectin binding characteristics to identify eggs of different nematode parasites. In this manner, it was demonstrated that peanut agglutinin (PNA) binds to the eggshell of *H. contortus*, but not to that of other trichostrongyles, which led to the development and further refinement of several PNA-fluorescein-isothiocyanate (FITC) staining

* Corresponding author at: Faculté de Médecine Vétérinaire, Université de Montréal - Département de Pathologie et Microbiologie, Bureau 3119-4, 3200, rue Sicotte, C.P. 5000, Saint-Hyacinthe, Quebec, J2S 2M2, Canada.

E-mail address: christopher.fernandez.prada@umontreal.ca (C. Fernandez-Prada).

<https://doi.org/10.1016/j.vetpar.2018.11.017>

Received 27 July 2018; Received in revised form 16 November 2018; Accepted 20 November 2018

0304-4017/ © 2018 Elsevier B.V. All rights reserved.

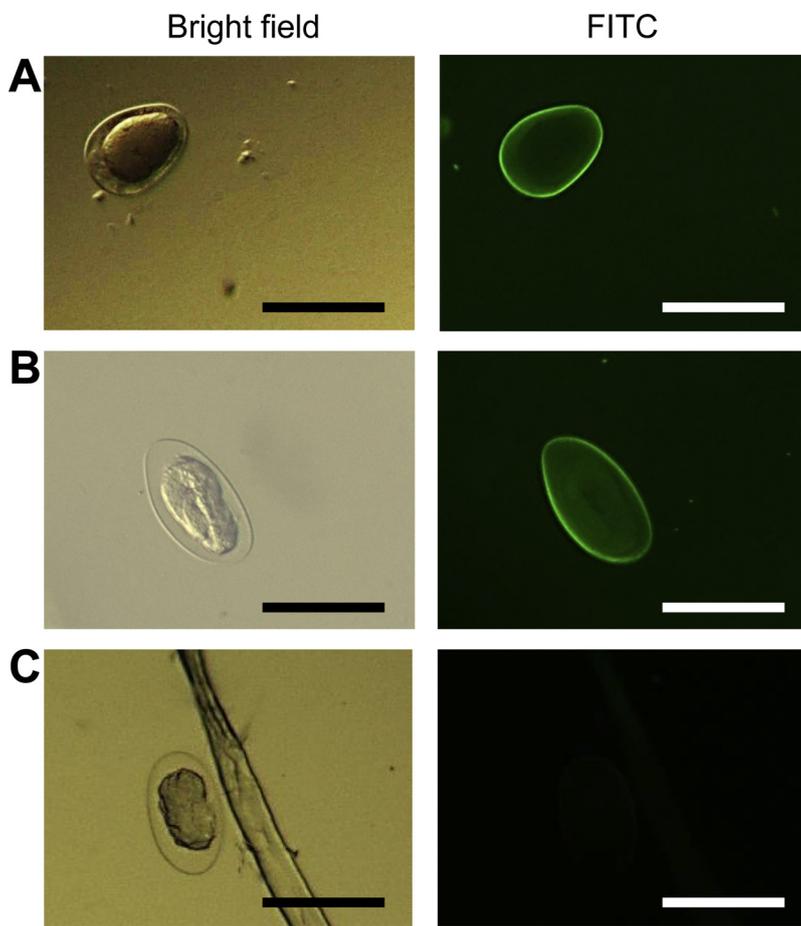


Fig. 1. Manual fluorescence microscopy of GIN eggs for pool creation. Detection of *H. contortus* eggs using PNA-FITC staining. Left-hand photos: brightfield microscopy allows visualization of *H. contortus* (top two rows), but it is almost impossible to differentiate eggs from those of other *Trichostrongylidae* (bottom row). Right-hand photos: PNA-FITC staining and fluorescence microscopy permits identification of *H. contortus* eggs by their green fluorescent outline (A and B), while eggs of other *Trichostrongylidae* remain unilluminated (C). Bar = 100 μ m (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

methods (Palmer and McCombe, 1996; Colditz et al., 2002; Jurasek et al., 2010). Importantly, using these methods, *H. contortus* eggs can be visualized under ultraviolet light for accurate identification by fluorescence microscopy after staining with a PNA-FITC conjugate, which eliminates the need to proceed with cumbersome LC (Jurasek et al., 2010).

Given that rapid and accurate diagnostic techniques are necessary for the prevention and control of parasites, the goal of this study was to evaluate whether identification and quantification of *H. contortus* PNA-FITC-labelled eggs could be expedited using an automated multimode reader for more rapid, accurate diagnostics, all while avoiding bias derived from the subjective visual interpretation of manual microscopy and counting. Correlation between known numbers of PNA-FITC-stained eggs and automated fluorescence was evaluated, and the accuracy of this new approach was then validated by testing clinical samples containing unknown quantities of GIN eggs.

2. Materials and methods

2.1. Samples

Fecal samples were collected between May and August 2016 as part of routine sheep flock monitoring at Haut-Vallon Farm (Frelighsburg, Québec; Canada), and subsequently sent to the Parasitology Diagnostic Service (DS) of the Faculté de médecine vétérinaire of the Université de Montréal (FMV) for analysis. Individual fresh fecal samples were analyzed at the DS within 48 h of sampling by WSF method in order to establish the presence or absence of GIN eggs based on the difference in density between parasite eggs and fecal debris. Samples positive for strongylid eggs were kept at 4 °C to prevent eggs from hatching until further analyses were performed.

2.2. Egg isolation and *H. contortus*-egg pool creation

In order to have enough material for all experiments, six highly concentrated egg stocks were created, each one containing 6000 strongylid-like eggs. To this end, parasite eggs from 15 randomly chosen positive samples were processed using a WSF method, as follows: samples were centrifuged at 1650 \times g for 5 min, resuspended in a saturated sucrose solution, centrifuged at 650 \times g for 2 min, and submitted to flotation for 1 h at room temperature. The coverslip was then removed and rinsed with 1 mL of 1 \times PBS to collect the eggs. The number of eggs corresponding to 3 g of feces was determined by McMaster counting, which allowed the creation of six stock solutions containing 6000 eggs/stock in 1 \times PBS.

The next step was to confirm the proportion of *H. contortus* eggs in each pool by PNA-FITC staining, as previously described in (Jurasek et al., 2010). To this end, 250-egg subsamples derived from each stock solution were centrifuged at 800 \times g for 5 min, resuspended in 1 mL of PNA-FITC lectin from *Arachis hypogaea* (Sigma-Aldrich, Oakville, Ontario, Canada), reconstituted at 5 μ g/mL in 10 mM HEPES, 0.15 M NaCl, pH 7.5, 0.08% sodium azide, 0.1 mM Ca²⁺, 0.01 mM Mn²⁺ (to guarantee preservation during long-term storage), and incubated for 1 h at room temperature with constant agitation. Samples were then centrifuged at 800 \times g for 5 min, the supernatant removed, and egg sediment resuspended in 500 μ L of 1 \times PBS. This step was repeated once more, this time with a final resuspension volume of 50 μ L of 1 \times PBS. Finally, 20 μ L of each sample were deposited in an Ibidi 18-well μ -Slide (IBIDI GmbH, Wisconsin, Germany) with 10 μ L of Ibidi Mounting Medium (IBIDI GmbH, Martinsried, Germany). This procedure was repeated in duplicate (n = 2 for each pool). Slides were examined with a Leica DMI 4000B automated inverted microscope (Leica; Leitz, Wetzlar, Germany) (λ_{ex} = 490/20 nm; λ_{em} = 525/20 nm, at the Virology

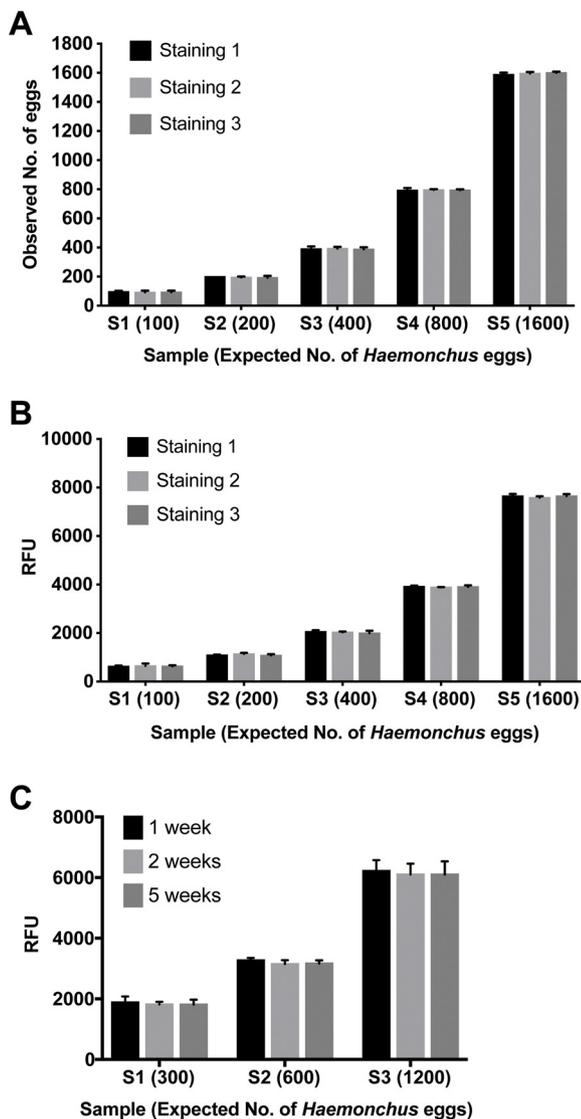


Fig. 2. Staining solution variations and stability. A) Comparison of 3 different, independently-prepared batches of PNA-FITC. Five solutions (S1-5) containing previously known numbers of *H. contortus* eggs (100; 200; 400; 800 and 1600) were stained by means of 3 PNA-FITC solutions, and eggs were counted by manual microscopy. No significant difference between batches was observed. B) Comparison of 3 different, independently-prepared batches of PNA-FITC. Five solutions (S1-5) containing previously known numbers of *H. contortus* eggs were stained by means of 3 PNA-FITC solutions, and raw fluorescence emission (RFU) was measured using a Cytation 5 multimode reader (BioTek, USA) ($\lambda_{\text{ex}} = 480 \text{ nm}$; $\lambda_{\text{em}} = 530 \text{ nm}$). No significant difference between batches was observed. C) Comparison of RFU emission of three different samples (S1-3) containing previously known numbers of *H. contortus* eggs (300; 600 and 1200), stained at 3 different time points (1, 2 and 5 weeks) using the same batch of PNA-FITC. No significant difference between stainings was observed. All three experiments (A, B and C) depict the mean of 3 independent biological replicates ($n = 3$).

laboratory of the FMV). Within each sample, 100 eggs were analyzed by two independent observers, and the evaluation was performed in triplicate. The average percentage of *H. contortus* present in each subsample was used to determine the number of *H. contortus* eggs present in the original pools.

2.3. Correlation between *H. contortus* eggs and fluorescence emission

Next, the correlation between the number of PNA-FITC-stained *H.*

contortus eggs and fluorescence emission was evaluated by means of an automated microplate reader. To this end, *H. contortus* eggs were stained (as described above), and 18 egg solutions (25; 50; 75; 100; 125; 150; 200; 250; 300; 350; 400; 450; 500; 600; 750; 1250 and 2500 *H. contortus* eggs, respectively) were prepared and subsequently dispensed (200 μL) in black 96-well plates with a clear, flat bottom (Corning Inc, USA). Plates were read in a Cytation 5 multimode reader (BioTek, USA) ($\lambda_{\text{ex}} = 480 \text{ nm}$; $\lambda_{\text{em}} = 530 \text{ nm}$). The results of three independent experiments were analyzed using GraphPad Prism version 8.00 (GraphPad Software, La Jolla California, USA). First, the linear correlation between the number of *H. contortus* eggs and fluorescence emission was calculated using Pearson correlation coefficient analysis. The relationship between these two variables was then modeled by fitting a linear equation to observed data.

2.4. Comparison of results between manual and automated fluorescence detection

The final step in this study was to evaluate the accuracy and predicting ability of this novel microplate-based approach. To do so, twenty samples containing an unknown amount of eggs were analyzed in triplicate by means of two methods: A) WSF, followed by McMaster counting and manual fluorescence microscopy; and B) WSF coupled with automated fluorescence detection. Method A was conducted as described above. For Method B, eggs were recovered by flotation and the coverslip rinsed with 1 mL of PNA-FITC (5 $\mu\text{g}/\text{mL}$ in $1 \times \text{PBS}$) in order to collect and stain eggs in a single step. After a 1 h incubation, stained eggs were washed and resuspended in $1 \times \text{PBS}$. At this point, two 2-fold dilutions were prepared for each sample (1, 1/2 and 1/4). 200 μL were then placed in a black 96-well plate and read in a Cytation 5 multimode reader as described above. Three negative controls and three positive ones, with known numbers of *H. contortus* eggs, were included in each run. The fluorescence value corresponding to each well was normalized according to the dilution factor (1, 1/2 and 1/4) and the background of negative controls. The number of *H. contortus* eggs was determined by substitution of the mean fluorescence value of each sample in the linear model previously obtained. The agreement between methods A and B was confirmed by Bland–Altman analysis using GraphPad Prism version 8.00 (GraphPad Software, La Jolla California, USA).

3. Results

3.1. Egg isolation, pool creation, and determination of *H. contortus* quantity by manual microscopy

Strongylid-like eggs were isolated, enriched by WSF, and pooled in six highly concentrated egg stocks (ca. 6000 strongylid-like eggs/stock). *H. contortus* eggs contained in 250-egg subsamples from each stock were visualized based on their bright green fluorescence emission (Fig. 1A and B). Non-*Haemonchus* eggs did not demonstrate any background fluorescence noise under the employed experimental conditions (Fig. 1C), showing the high specificity of PNA-FITC staining. The percentage of *H. contortus* eggs for the different stocks was calculated to be 81 (4860 eggs), 93 (5580 eggs), 85 (5100 eggs), 62 (3720 eggs), 75 (4500 eggs) and 98% (5880 eggs), respectively, for an approximate total of 29,640 *H. contortus* eggs. At this point, potential variation between batches of PNA-FITC in terms of stained eggs and total fluorescence emission was evaluated. Five solutions containing known numbers of *H. contortus* eggs (100; 200; 400; 800 and 1600; respectively), were stained using three independently prepared PNA-FITC solutions. As depicted in Fig. 2, all three solutions stained similar numbers of eggs (Fig. 2A) as determined by manual fluorescence microscopy. Likewise, no differences in terms of total fluorescence emission were observed between samples when analyzed by means of the Cytation 5 multimode reader (Fig. 2B). Finally, the stability of the in-house PNA-FITC solution

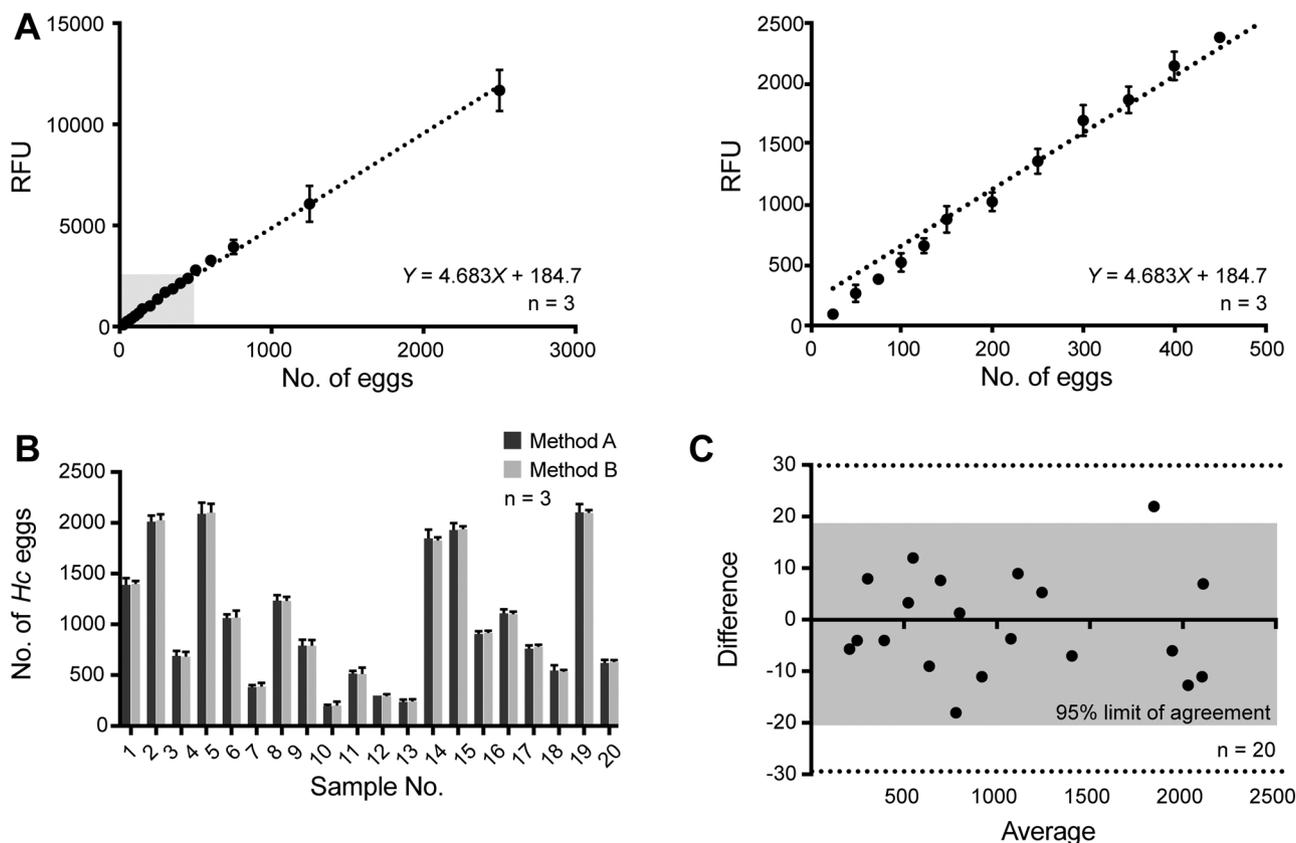


Fig. 3. Number of *H. contortus* eggs vs fluorescence emission and comparison between manual and automated fluorescence microscopy. **A)** *Left panel.* Correlation between number of *H. contortus* eggs and fluorescence emission (RFU = Relative Fluorescence Units) with experiment performed in triplicate (n = 3). Results demonstrate nearly perfect positive correlation ($r = 0.9984$, $p < 0.0001$). The squared grey area is depicted in large scale in *Right panel.* *Right panel.* Magnified section of *Left panel* depicting the correlation between number of *H. contortus* eggs and fluorescence emission (25 to 500 eggs). **B)** Comparison of Method A (WSF and McMaster egg counting, followed by manual fluorescence microscopy) and Method B (WSF, followed by automated fluorescence detection using a multimode microplate reader). The number of *H. contortus* eggs determined by each method was nearly identical. **C)** Bland-Altman Plot for comparison of manual and automated microscopy. Results for both methods are in accordance (bias = -0.817 ± 9.94 , with 95% limits of agreement from -20.31 to 18.67), indicating that HTS fluorescence detection and quantification of *H. contortus* eggs is comparable to the manual technique (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

was evaluated to assess a possible impact of fluorescence decay on the diagnostic test. To this end, three different solutions were prepared and stained containing 300, 600 and 1200 *H. contortus* eggs, respectively, at three different times (1, 2 and 5 weeks) after initial resuspension of the PNA-FITC stock solution. As shown in Fig. 2C, no significant differences between experiments were observed when comparing, using one-way ANOVA, the RFU of three different *H. contortus* samples: S1 (300 eggs), S2 (600 eggs) and S3 (1200 eggs) stained with 1-week ($p = 0.9967$), 2-week ($p = 0.9917$) and 5-week old ($p = 0.9733$) PNA-FITC solution. Each experiment was performed in triplicate.

3.2. Number of *H. contortus* eggs vs. fluorescence emission

A linear regression analysis was performed to model the relationship between the number of *H. contortus* eggs and the fluorescence emission (Fig. 3A right and left panels). The regression equation obtained, under our experimental conditions, was $Y = 4.683X + 184.7$ ($p < 0.0001$). Pearson correlation coefficient analysis confirmed an almost perfect linear correlation between the two aforementioned variables ($r = 0.9984$, $p < 0.0001$). Under these experimental conditions, 25 eggs was the minimum amount of *H. contortus* eggs detectable while maintaining a linear proportion (Fig. 3A right panel).

3.3. Diagnostic of clinical samples and model validation

Analysis of the clinical samples provided to our laboratory showed a

wide range in the content of *H. contortus* eggs (from 209 to 2109 eggs), as depicted in Fig. 3B. Pearson analysis revealed a strong correlation between methods A and B ($r = 0.9999$, $p < 0.0001$), while Bland-Altman plots (Fig. 3C) demonstrated an excellent agreement between the two quantitative approaches (bias = -0.817 ± 9.944 with 95% limits of agreement from -20.31 to 18.67), except for one sample in the high range. D'Agostino-Pearson test ($P = 0.691$) confirmed that the differences between the bias of samples were normally distributed (Bland-Altman validation).

4. Discussion

In this study, we developed a novel method based on the automated detection of PNA-FITC fluorescence, which can rapidly and accurately quantify the number of *H. contortus* eggs present in both controlled experiments (Fig. 3A) and clinical samples (Fig. 3B). While (Jurasek et al., 2010) showed a very good correlation (adjusted $R^2 = 0.72132$) between LC and PNA-FITC staining, our linear model revealed an almost perfect correlation ($r = 0.9984$) between the number of *H. contortus*-eggs and fluorescence emission detected by automated reading. Furthermore, it was demonstrated that the discrepancy between this new high-throughput screening (HTS) method and PNA-FITC staining coupled with manual fluorescence microscopy (Colditz et al., 2002; Jurasek et al., 2010) is extremely low (-0.817 ± 9.94) and is not affected by the number of eggs analyzed. Consequently, both the manual and automated methods can be considered equivalent in terms

of diagnostic accuracy.

In a recent work, (Ljungstrom et al., 2017) explored the benefits and limitations of lectin staining with PNA for the identification of *H. contortus* eggs by comparing this technique with three other diagnostic approaches: McMaster counting, qPCR, and loop-mediated isothermal amplification (LAMP). They clearly identified two major flaws of PNA-FITC staining coupled with manual microscopy: i) tediousness and length of process, making it almost impossible to analyze large sample sets within a reasonable delay; and ii) difficulty accurately quantifying the number of *H. contortus* eggs in a sample. Promisingly, the technique described here overcomes these limitations by eliminating the need for manual counting (McMaster counting usually takes ~ 10 min/sample and manual fluorescence counting ~ 20 min/sample) and by allowing for highly sensitive analysis of up to 30 samples at a time in a 96-well plate with spiked positive controls. This eliminates any possible operator-dependent bias caused by visual interpretation of the eggs in brightfield and/or fluorescence microscopy, which are intrinsically subjective. Additionally, in the current experiments, no significant variation was found between batches of PNA-FITC or after 5 weeks of PNA-FITC storage (Fig. 2).

Since *H. contortus* continues to be highly prevalent and can lead to important losses for farms with grazing sheep, either through a decrease in production or through mortality, a cheap and accurate HTS method becomes essential to rapidly detect the presence of *H. contortus* in order to correctly treat animal disease and monitor the efficacy of anthelmintic treatments (fecal *H. contortus*-egg count reduction tests; *H. contortus*-FECRT).

In conclusion, automated readings proved to be accurate for identification and quantification of PNA-FITC-stained *H. contortus* eggs, as well as inexpensive and straightforward in terms of handling and training. This fluorescence-based HTS approach could therefore be used as a tool to improve *H. contortus* diagnostics for better implementation of control and elimination strategies.

Conflict of interests

The authors have declared that no conflict of interests exists.

Acknowledgments

The authors want to thank the Parasitology Diagnostic Service of the Faculté de médecine vétérinaire of the Université de Montréal (DS-FMV), as well as Dr. Carl Gagnon and Dr. Aida Minguez-Menendez for their kind help in this project. This project was funded by the CDEVQ-MAPAQ 2016-2017 project and supported by the Canada foundation for Innovation (www.innovation.ca), grant number 37324; both awarded to CFP.

References

- Barrere, V., Falzon, L.C., Shakya, K.P., Menzies, P.I., Peregrine, A.S., Prichard, R.K., 2013. Assessment of benzimidazole resistance in *Haemonchus contortus* in sheep flocks in Ontario, Canada: comparison of detection methods for drug resistance. *Vet. Parasitol.* 198, 159–165.
- Colditz, I.G., Le Jambre, L.F., Hosse, R., 2002. Use of lectin binding characteristics to identify gastrointestinal parasite eggs in faeces. *Vet. Parasitol.* 105, 219–227.
- Falzon, L.C., Menzies, P.I., Shakya, K.P., Jones-Bitton, A., Vanleeuwen, J., Avula, J., Stewart, H., Jansen, J.T., Taylor, M.A., Learmount, J., Peregrine, A.S., 2013. Anthelmintic resistance in sheep flocks in Ontario, Canada. *Vet. Parasitol.* 193, 150–162.
- Jacquiet, P., Cabaret, J., Thiam, E., Cheikh, D., 1998. Host range and the maintenance of *Haemonchus* spp. in an adverse arid climate. *Int. J. Parasitol.* 28, 253–261.
- Jurasek, M.E., Bishop-Stewart, J.K., Storey, B.E., Kaplan, R.M., Kent, M.L., 2010. Modification and further evaluation of a fluorescein-labeled peanut agglutinin test for identification of *Haemonchus contortus* eggs. *Vet. Parasitol.* 169, 209–213.
- Ljungstrom, S., Melville, L., Skuce, P.J., Høglund, J., 2017. Comparison of four diagnostic methods for detection and relative quantification of *Haemonchus contortus* eggs in feces samples. *Front. Vet. Sci.* 4, 239.
- Mederos, A., Fernandez, S., VanLeeuwen, J., Peregrine, A.S., Kelton, D., Menzies, P., LeBoeuf, A., Martin, R., 2010. Prevalence and distribution of gastrointestinal nematodes on 32 organic and conventional commercial sheep farms in Ontario and Quebec, Canada (2006–2008). *Vet. Parasitol.* 170, 244–252.
- 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, 447–450.
- White, G.P., Meeusen, E.N., Newton, S.E., 2001. A single-chain variable region immunoglobulin library from the abomasal lymph node of sheep infected with the gastrointestinal nematode parasite *Haemonchus contortus*. *Vet. Immunol. Immunopathol.* 78, 117–129.