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Research paper

Egg autofluorescence and options for detecting peanut agglutinin binding for the identification of *Haemonchus contortus* eggs in fecal samples

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ABSTRACT

Quantifying eggs from *Haemonchus* and other trichostrongyle genera in sheep and goat fecal samples is important for evaluating control and treatment strategies for this family of nematodes with divergent pathologies, capabilities for anthelmintic resistance and environmental susceptibilities. Unfortunately, egg morphology among most of the genera do not differ enough to support the accurate identification of these genera with standard microscopic techniques. Several studies have identified specific lectins which bind selectively to sugars located on the egg surfaces for individual genera among the trichostrongyles. To detect lectins binding to these eggs, they must be directly or indirectly bound to fluorophores, and observed with an epi-fluorescence microscope. The binding of multiple lectins to isolated eggs from a fecal sample can be simultaneously detected if fluorophores are used whose excitation and emission spectra do not overlap, and this would enable the development of a fluorescence-based diagnostic test that identifies multiple trichostrongyle genera within each sample. The present study compared the usefulness of different, commercially available detection systems for use in detecting lectin binding to trichostrongyle eggs. Comparisons were made using the detection of PNA binding to *H. contortus* eggs with the goal of finding three systems with color spectra that do not overlap. These evaluations included both fluorophores directly conjugated to PNA in a one-step incubation protocol and a two-step incubation protocol involving biotinylated PNA and streptavidin conjugated to different fluorophores. Autofluorescence can affect the efficiency of any fluorescence-based detection system, and significant autofluorescence was observed among the unstained *H. contortus* eggs with the DAPI-type fluorescence filter, but it was significantly lower with the FITC-type filter and was virtually absent with the rhodamine-type filter. This study demonstrated that all the PNA detection methods tested with *H. contortus* eggs generated fluorescence intensities (FIs) that were significantly above the autofluorescence generated by the eggs among the three different fluorescence filters. Fluorescence intensities from PNA directly conjugated to either the FITC or rhodamine fluorophores were not different, but the lower autofluoresence in the rhodamine-type filter will enable this fluorophore to be detected more efficiently. Use of biotinylated PNA combined with streptavidin-conjugated to synthetic fluorophores (Alexa Fluor 405, 488 and 546) significantly increased FIs over that of the directly conjugated PNA, but there were no significant differences in FIs among these three biotin-avidin conjugation fluorophores. This biotin-avidin system required two incubation steps. Doubling the concentration of PNA also provided increased FI, at least for the biotin-avidin system. Adding an additional amplification step to the biotin-avidin system involving biotinylated anti-streptavidin followed by the streptavidin-Alexa Fluor complex also provided additional fluorescence.

1. Introduction

Trichostrongyle nematodes are a very common cause of sheep and goat parasitic gastroenteritis throughout the world, and the several genera that constitute these trichostrongyles (e.g. *Haemonchus*, *Ostertagia*, *Teladorsagia*, *Nematodirus*, and *Trichostrongylus*) vary in their pathogenicity and control strategies enough that there are clinical advantages to diagnosing them to at least the genus level (Preston et al., 2014). This is particularly important for the genus *Haemonchus,* which can cause sudden death in heavily infected sheep and goats (Besier et al., 2016). Trichostrongyle infections can be easily diagnosed to the family level by finding their characteristic eggs in fecal samples. Each

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animal is typically infected with worms from multiple trichostrongyle genera, and with the exception of *Nematodirus* spp., there are only small, overlapping differences in egg morphology among the trichostrongyle genera. This makes it virtually impossible to accurately diagnose the various genera based only on the morphology of the egg stage (Georgi and McCulloch, 1989).

A lectin-staining technique involving peanut agglutinin (PNA) was developed in order to differentiate *Haemonchus contortus* from two other common trichostrongyles of sheep and goats, *Teladorsagia circumcinta* and *Trichostrongylus columniformis* (Palmer and McCombe, 1996). For this study, ten different lectins, including PNA, were evaluated for their ability to bind to the three species. The lectins were either visualized with a one-step binding protocol involving lectins directly conjugated to the green-fluorescing fluorophore fluorescein isothiocyanate (FITC) or in a multiple step protocol involving unconjugated lectins that were detected with a rabbit derived anti-lectin antibody and an anti-rabbit secondary antibody bound to FITC. FITC fluorescence surrounding the appropriate eggs was then visualized with an epi-fluorescence microscope equipped with the appropriate filters. PNA was the only genus-specific lectin identified, and it bound specifically to *H. contortus*. Flow cytometery has also been used to detect four different FITC-conjugated lectins binding to five different species of trichostrongyles, and again PNA was shown to bind strongly and specifically to both *Haemonchus placei* and *H. contortus*, but the other lectins were not specific for any of the species tested (Colditz et al., 2002). The usefulness of PNA to microscopically identify *Haemonchus* spp. eggs in clinical facal samples from sheep was documented by comparing its results to those achieved through identifying cultured larvae from the same samples (Jurasek et al., 2010). This was more recently confirmed in comparisons of PNA binding of *H. contortus* eggs with established polymerase chain reaction and loop mediated isothermal amplification protocols (Ljungstrom et al., 2017). This study found that while the two molecular methods provided increase sensitivity to *Haemonchus* eggs, the PNA method enabled quantification of the *Haemonchus* eggs. In a study involving four different developmental stages (adults, eggs, and sheathed and exsheathed 3rd stage larvae) from *H. contortus* and *T. circumcinta*, 19 lectins were evaluated, four of which had not yet been tested for any trichostrongyle species (Hillrichs et al., 2012). For this evaluation, biotinylated lectins were used and then detected with avidin conjugated to the red-fluorescing fluorophore Alexa Fluor® 546. Once again, PNA bound selectively to *H. contortus*, while the newly tested lectin, *Lens culinaris* agglutinin (LCA) bound selectively to *T. circumcinta* eggs. The same 19 biotinylated lectins detected with the same avidin-Alexa Fluor multi-step protocol were used to evaluate their binding to eggs isolated from the adults of six trichostrongyle taxa harvested from naturally infected sheep (Umair et al., 2016). This study demonstrated that the six taxa could be identified through the binding of six lectins: PNA for *H. contortus*; LCA for *Teladorsagia* sp; *Aleuria aurantia* agglutinin (AAL) for *Trichostrongylus* sp; *Psophocarpus tetragonolobus*-II (PTLII) for *Nematodirus* sp; *Lotus tetragonolobus* lectin (LTL) for *Cooperia* sp and wheat germ agglutinin (WGA) for *Chabertia ovina*.

The ability of at least six different lectins binding to six different genera of trichostrongyles eggs creates the potential possibility of simultaneously determining the genus identities for most of the trichostrongyle eggs excreted from each animal in a herd. This would be particularly useful in a fecal egg count reduction test for measuring the level of anthelmintic resistance for each trichostrongyle genera in a herd. A diagnostic test that simultaneously identifies multiple genera would need to use multiple lectins detected with multiple-colored fluorophores. Many fluorophores with different wavelength are available for detecting these lectins, but it is technically difficult to use more than three of them simultaneously because their excitation and emission spectra cannot overlap. Generally, fluorophores emitting photons in the blue, green and/or red spectra are used if only three fluorophores are needed, but fluorophores in the near-UV, the near-infrared or yellow spectra could be added if more are needed.

The purpose of the present study was to compare the usefulness of different, commercially available detection system for use in detecting lectin binding to trichostrongyle eggs. Comparisons were made using the detection of PNA binding to *H. contortus* eggs with the three most common spectra: blue, green and red. PNA directly conjugated to FITC and rhodamine are commercially available, and the fluorescence intensities (FIs) resulting from the single-step protocol involving these two products were compared to multiple-step protocols involving biotinylated PNA and streptavidin conjugated to three different fluorophores. The effects of adding an additional amplification step involving an anti-streptavidin was also evaluated.

2. Materials and methods

2.1. Source of Haemonchus contortus eggs

H. contortus eggs were harvested from a small group of commercial lambs which were shown to contain *Haemonchus* as their only trichostrongyle species (Grosz et al., 2013). Eggs were isolated from freshly deposited fecal samples using the modified Wisconsin sucrose flotation technique (Cox and Todd, 1962), and floated eggs were collected into distilled water and concentrated with centrifugation (Grosz et al., 2013). To verify that *H. contortus* was the only species in these samples, PCR amplification of the ITS-2 region of ribosomal DNA of different trichostrongyles was performed using variable genus specific (Grosz et al., 2013) and species-specific (Santos et al., 2014) primers, under conditions described by Grosz et al. (2013). Freshly harvested egg suspensions (used less than 72 h after fecal collection) were filtered through a 50 μm nylon mesh filter and collected on a 30 μm filter. The eggs were then washed from the 30 μm filter and concentrated with centrifugation.

2.2. Autofluorescence analysis

The natural autofluoescence of *H. contortus* eggs was evaluated using the 3 fluorescence filter cubes used for the lectin study by adding 10 μl of egg suspension to a small drop of Vectashield (Vector Laboratories, Burlingame, Ca) mounting media on a microscope slide, and photographing them with an Olympus BX53 epifluorescence microscope equipped with 3 Semrock filter cubes (DAPI-5060C filter: EX377/50, EM447/60, DM409; GFP-4050 A filter [i.e. FITC filter]: EX466/40, EM525/50, DM495; TRITC-B filter [i.e. rhodamine filter]: EX543/22, EM593/40, DM562) and CellSens software (Olympus Corp., Tokyo, Japan). These photographs were taken at 50 and 100 ms with the black background defined in an area not containing eggs or debris using the TRITC-B filter. Background values for samples viewed with the other 2 filters were measured in relation to the TRITC-B filter. Autofluorescence intensities (AFIs) associated with egg photographs from each filter were measured using the FIJI version of ImageJ (Rueden et al., 2017; Schindelin et al., 2012). Only the 8-bit color channel associated with each of the appropriate filters (i.e. blue channel for DAPI filter, green channel for GFP-FITC filter and red channel for TRITC-B filter) was used for the analysis; pixel data from each channel consisted of brightness intensities ranging from 0-255. The mean raw pixel intensity was calculated from the region of interest associated with each egg; background values were also calculated from a region of interest just outside of each egg that contained the same area value. The autofluorescence intensity for each egg was calculated by subtracting the background value from the raw mean egg fluorescence intensity value.

2.3. Eggs-lectin binding

The PNA lectin products (Rhodamine-conjugated, FITC-conjugated and biotin-conjugated) were purchased from Vector Laboratories (Burlingame, Ca). Freshly harvested egg suspensions were washed 3

Table 1
The 8 different detection methods tested using directly conjugated PNA (PNA-rhod or PNA-FITC) or biotin-conjugated PNA (B-PNA).

Method Abbreviation	Fluorochrome	Staining Technique and Concentrations
Rhod	Rhodamine (PNA-Rhod)	PNA-Rhod at 30 µg/ml
FITC	Fluorescein isothiocyanate (PNA-FITC)	PNA-FITC at 30 µg/ml
SaAF546	Streptavidin Alexa Fluor 546 (SaAF546)	Biotinylated PNA (BPNA) at 30 µg/ml then SaAF546 at 3 µg/ml
SaAF488	Streptavidin Alexa Fluor 488 (SaAF488)	BPNA at 30 µg/ml then SaAf488 at 10 µg/ml
SaAF488 2X	SaAF488 before and after incubation with biotinylated anti-streptavidin antibodies (B anti-SaA)	BPNA at 30 µg/ml then SaAf488 at 10 µg/ml then biotinylated anti-streptavidin at 20 µg/ml then SaAf488 10 µg/ml
SaAF405	Streptavidin Alexa Fluor 405 (SaAF405)	BPNA at 30 µg/ml then SaAf405 at 20 µg/ml
SaAF405 2X	SaAF405 before and after incubation with B anti-SaA	BPNA at 30 µg/ml then SaAf405 at 20 µg/ml then biotinylated anti-streptavidin at 20 µg/ml then SaAf405 at 20 µg/ml
2XSaAF405	Streptavidin Alexa Fluor 405	BPNA at 60 µg then SaAf405 at 20 µg/ml

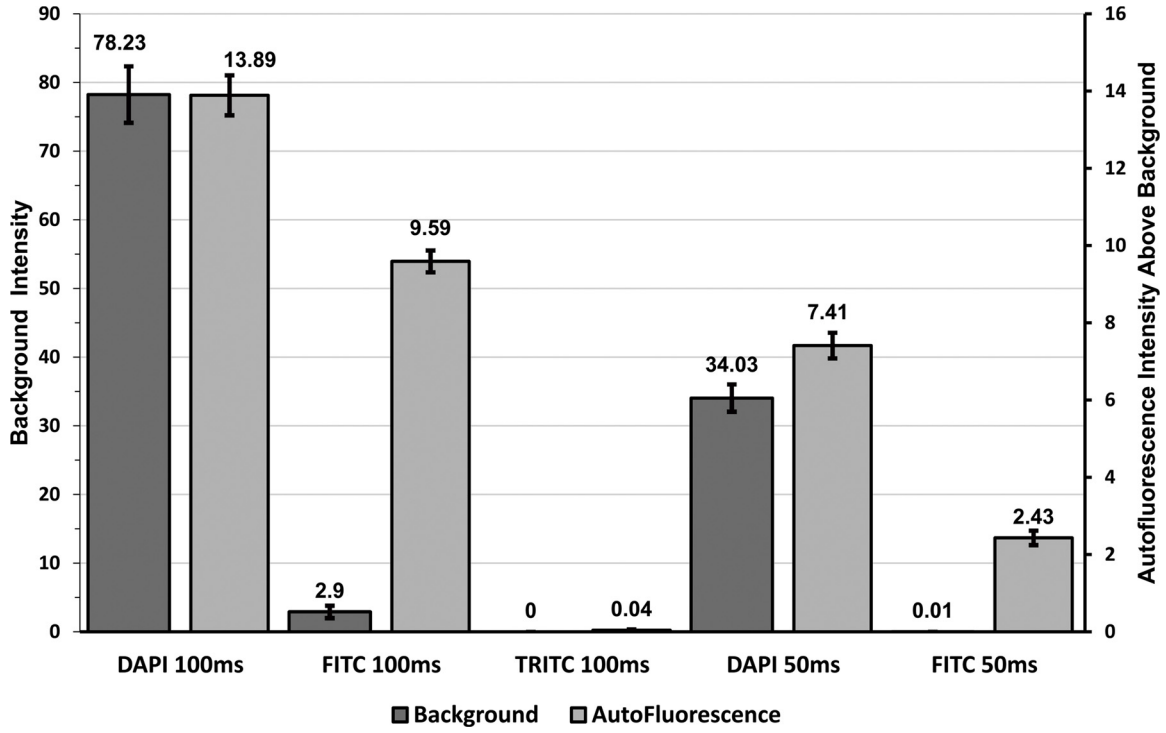

Fig. 1. Background pixel intensities (left axis) and egg autofluorescence pixel intensities above background (right axis) for *H. contortus* eggs photographed for 50 and 100 ms with DAPI, FITC and TRITC-B filters. Mean pixel brightness intensities were calculated only using the 8-bit color channel associated with each of the appropriate filters. Error bars represent the standard error of the mean (SEM).

times in 10 mM HEPES buffer, and the PNA conjugate was added to the final HEPES egg suspension according to the concentrations reported in Table 1. Two different lectin binding methods were evaluated: a single-step approach involving PNA that was directly conjugated to a fluorochrome, and a multiple-step approach involving biotinylated-PNA detected through the addition of streptavidin conjugated to a fluorochrome. Eggs and lectins were incubated in the dark at room temperature for 1 h with regular vortexing to insure equal distribution of the lectins. Eggs were then washed twice with the HEPES buffer via centrifugation at 2000 x *g* for 10 min. Eggs incubated in directly conjugated PNA were processed immediately for fluorescence microscopy as described below. Washed eggs incubated in the biotin-conjugated PNA were again incubated in a 1 ml solution containing one of 3 Streptavidin Alexa Fluor fluorophores: 405, 488 and 546 (Thermo Fisher Scientific, Waltham, MA) at concentrations recommended by the manufacturer for 1 h at room temperature. In an attempt to amplify the fluorescence, half of the eggs incubated in the streptavidin solution were

additionally incubated for 1 h in a solution containing biotinylated anti-streptavidin antibodies (B anti-SaA) and then another 1 h incubation in the appropriate streptavidin Alexa Fluor conjugates. All incubations were followed by 2 washing steps in HEPES buffer as described above. All concentrations used for each of these steps were based upon are the manufacturer's recommendations and as reported in Table 1. At the completion of the final step for each of the groups, the concentrated eggs were added to a drop of Vectashield mounting medium, cover-slipped on a glass slide and photographed as described above for the autofluorescence study, except that the black background was defined for an area of each slide not containing eggs. The filter cubes used for each group were appropriate for the chosen fluorochrome (i.e. the TRITC-B filter for rhodamine and Alexa Fluor 546; GFP/FITC filter for FITC and Alexa Fluor 488; DAPI filter for Alexa Fluor 405). Exposure times for those lectins viewed with the DAPI and GFP/FITC filters was 30 ms, and 100 ms for lectins viewed with the TRITC-B filter.

2.4. Statistical analysis

Mean fluorescence intensities were compared among the various filters and protocols using a one-way ANOVA followed by the Turkey multiple comparisons test from GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.

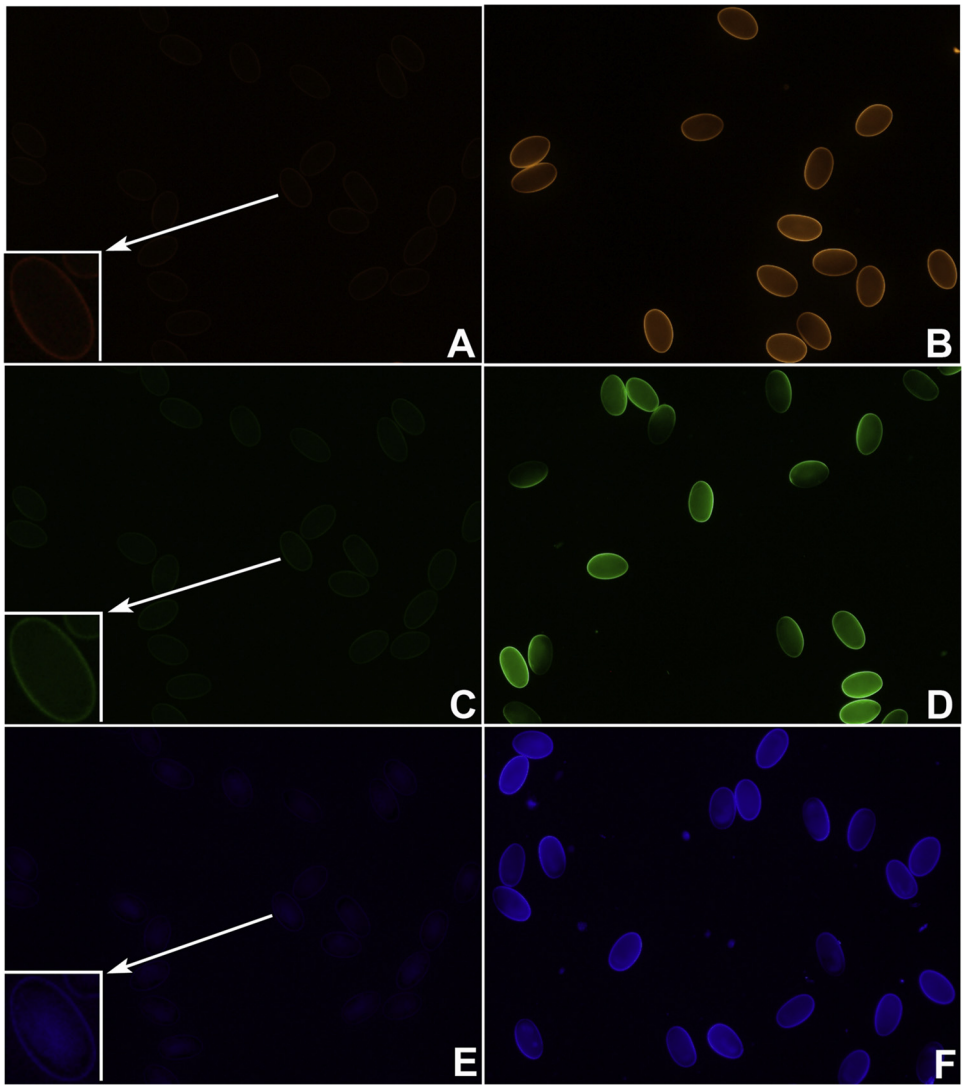

Fig. 2. *Haemonchus contortus* eggs either unstained (A, C and E) or stained with PNA-conjugated to 3 of the detection methods tested (B, D and F). Image B was stained with PNA directly conjugated to rhodamine, and images A and B were captured with the TRITC filter at 30 ms; Image D stained with PNA conjugated to FITC, and Images C and D captured with the GFP/FITC filter at 30 ms; image F was stained using PNA indirectly Alexa Fluor 405 via a biotin-streptavidin linkage; images E and F were captured with the DAPI filter at 200 and 100 ms, respectively. Inserts for each unstained photograph shows a digital blowup of the end at the back end of each arrow. The brightness of the inserts were increased digitally to allow for the distribution of the autofluorescence to be seen. The increased intensities are not related to their actual brightness levels.

com.

3. Results

3.1. Autofluorescence analysis

The brightness intensities of the background areas varied depending on the filters being used (Fig. 1). This intensity was lowest with the TRITC-B filter, and therefore it was used to set the black balance setting on the camera; for this reason, the mean background intensity for this filter in Fig. 1 was 0 for the 100 ms exposure. Mean background intensity for the FITC filter was slightly higher, but it was significantly higher (i.e. more than 78 intensity units, $p < 0.0001$) for the DAPI filter (Fig. 1). These background differences can be eliminated for camera use by setting the black balance for each image and filter, but high backgrounds can make visual interpretations of fluorescence more difficult.

The intensity and location of autofluorescence in the *H. contortus* eggs also varied depending on the filter used. The lowest ATFs occurred with eggs viewed through the TRITC/rhodamine filter (Fig. 1 and 2 A), and the small amount of autofluorescence that was present in each egg was confined to the egg shell (Fig. 2A insert). Mean autofluorescence with the FITC filter was significantly higher ($p < 0.0001$) with a mean of almost 10 intensity units (Fig. 1), but the brighter pixels were generally distributed throughout the egg, but especially the shell (Fig. 2C and insert). The DAPI filter showed the highest AFIs, averaging almost 14 intensity units; brighter pixels were found in the shell, but especially throughout the internal blastomere cells (Fig. 2 E and insert). Decreasing the exposure time from 100 ms to 50 decreased background by 56.5% for the DAPI filter, but autofluorescence by only 46.7%. The decrease was much more significant for the FITC filter, decreasing background to almost 0, and autofluorescence by almost 75%.

3.2. Detection of PNA binding

Mean FIs for the nine PNA-detection methods are shown in Fig. 3. Specific PNA-binding was clearly detected for all nine detection methods relative to the negative controls, with the lowest PNA-fluorescing egg for each method being significantly higher than the highest auto-fluorescing egg for each filter. (Fig. 2 and 3). While the FIs (over background) for the two fluorophores directly conjugated to PNA (method Rhod and FITC in Table 1) were sufficient for detection, the use of biotinylated PNA along with any of the three streptavidin-conjugated fluorophores significantly increased the fluorescence intensity over that of the direct conjugates. FIs were not statistically different ($p > 0.05$) among the three streptavidin-conjugated fluorochromes. Additionally, increased FIs were achieved by re-incubating eggs already containing streptavidin-conjugates in B anti-SaA that were then incubated in the appropriate streptavidin-conjugated fluorophores. Fluorescence intensity was highest in the AF405 amplified system.

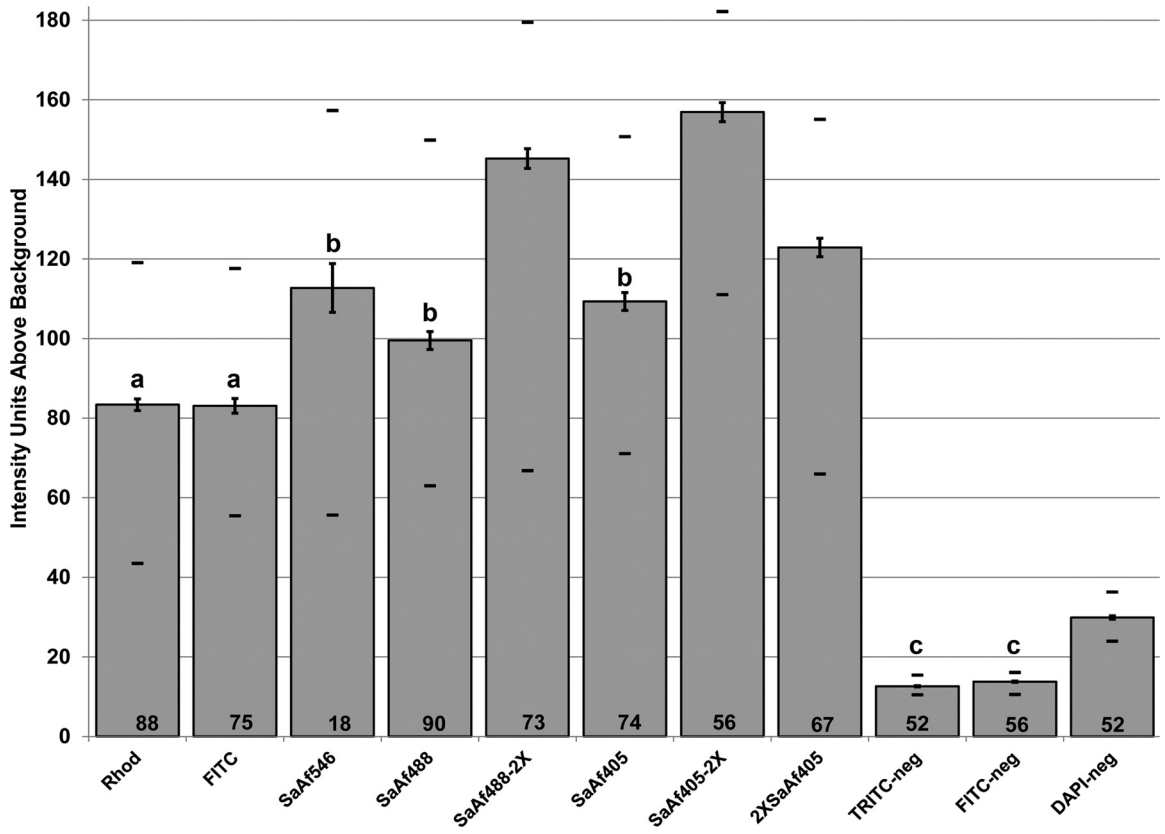

Fig. 3. Mean fluorescence intensity values for the eight techniques (first eight bars) for detecting PNA binding to *Haemonchus* eggs and the three negative non-stained controls for TRITC, FITC and DAPI filters (last three bars). The numbers at the bottom of each bar represents the number of eggs measure. Error bars associated with each sample bar show the standard error of the mean. The fluorescence value for the egg showing maximum fluorescence intensity is shown by the dash about each sample bar; the dash below each sample bar indicates the egg with the minimum fluorescence intensity. Mean sample bars with a similar letter are not statistically different at $p < 0.05$.

Increasing the biotinylated PNA concentration from 30 µg/ml to 60 µg/ml increased the FIs at least for the 405 nm fluorophore, but not as much as the use of the anti-streptavidin amplification approach.

Fluorescence patterns among the various stained eggs were similar for all of the detection methods (Fig. 2). Fluorescence associated with many of the eggs was uniformly high along the margin, and decreased toward the center of each egg, however, in some eggs, FIs were significantly lower along one side. This uneven binding was responsible for a significant portion of the variability observed in the mean FIs for each detection method.

4. Discussion

This study demonstrated that all of the PNA detection methods tested with *H. contortus* eggs generated FIs that were significantly above the autofluorescence generated by the eggs. Autofluorescence is a very common characteristic among many plant components, and is occasionally present in some animal tissues (Monici, 2005). Significant autofluorescence was observed in the present study among unstained *H. contortus* eggs with the DAPI filter, but it was much lower with the FITC filter and was virtually absent with the rhodamine filter. Autofluorescence has already been described from UV excitation (340–380 nm) among nematode eggs and coccidian oocysts in swine feces (Daugschies et al., 2001). The fluorescence pattern for the trichostrongyle nematode egg included in their study, *Oesophagostomum dentatum*, was similar to the DAPI filter autofluorescence observed for *H. contortus* in the present study. Autofluorescence was not mentioned in any of the other lectin studies of *H. contortus* eggs even though a FITC or rhodamine filter was used in their protocol, and autofluorescence would have likely been seen within the negative controls for these studies (Hillrichs et al., 2012; Jurasek et al., 2010; Palmer and McCombe, 1996; Umair et al., 2016). Future studies are needed to determine the AFIs among the various emission and excitation wavelengths for other species, but it is very likely that autofluorescence will be a characteristic for all trichostrongle eggs.

Fluorophores directly conjugated to PNA have only been commercially available for FITC and rhodamine fluorophores, and these products provide the option for a one-step protocol that has been popular for many of the previous lectin studies involving *Haemonchus*. All lectins in these previous one-step studies were conjugated to FITC (Colditz et al., 2002; Jurasek et al., 2010; Ljungstrom et al., 2017; Palmer and McCombe, 1996), but the present study found that both FITC- and rhodamine-conjugated PNA provided equal levels of FIs, and would be useful for a dual-color, dual-genera protocol for diagnosing two genera of trichostrongyle eggs. This protocol would likely include two-steps, but it is possible that both lectins could be combined into a one-step incubation protocol. Due to the lower autofluorescence with rhodamine/Alexa 546-type filters, it would be more effective in a dual-staining protocol to use the rhodamine-lectin conjugate for the lectin which binds less intensely to eggs of a particular species. For example, Umair et al. (2016) scored the binding of PNA to *Haemonchus* eggs as a +4, but the binding of LCA to *Teladorsagia* sp. eggs as a +3; therefore assuming a similar level of autofluorescence, it would be more effective to use FITC-conjugated PNA and rhodamine conjugated LCA in a dual-genera protocol.

In the initial lectin study, Palmer et al. (1996) found that a three-step staining protocol involving unconjugated PNA detected with anti-lectin primary antibodies and FITC-conjugated secondary antibodies provided higher FIs than PNA directly conjugated to FITC; yet, when available, they used directly conjugated lectins for subsequent parts of

the study because they were more convenient. More recently, the avidin-biotin detection systems have become much more popular for most immunofluorescence studies, and were used in a two-step protocol involving 19 biotinylated lectins and avidin Alexa Fluor® 546 for two of the more recent lectin-screening studies for trichostrongyle eggs (Hillrichs et al., 2012; Umair et al., 2016). Alex Fluor® fluorophores are a family of fluorescent dyes developed by Molecular Probes™, and sold under the Invitrogen™ brand name. Under the experimental conditions of the present study, biotinylated PNA coupled with Alexa Fluor 488 or 546 conjugated to streptavidin provided higher FIs than directly conjugated FITC and rhodamine respectively. Fluorescence intensity with Alex Fluor 405 was similar to Alexa Fluor 488 or 546. These increased FIs with the avidin-biotin detection methods requires an extra incubation and extra group of wash steps, and would not be necessary for spectra ranges where directly conjugated lectins are available and autofluorescence is low (i.e. for Alexa Fluor 488 or 546). However, the increased FI for Alexa Fluor 405 would be useful for overwhelming the increased autofluorescence in the DAPI filter. Therefore, it would be possible to create a tri-color, tri-genera protocol for diagnosing three genera of trichostrongyle eggs by adding the streptavidin Alexa Fluor 405 detection method to the two directly conjugated lectins commercially available. Given the intensity of PNA binding to *Haemonchus* eggs, PNA would be the most likely candidate for the Alexa Fluor 405 method.

Results from the present study also demonstrated that an additional level of FI could be achieved by incubating eggs containing the streptavidin-Alexa Fluor complex with biotinylated anti-streptavidin antibodies conjugated to biotin, and then re-incubating them in the same streptavidin-Alexa Fluor complex used previously. This amplification approached seemed to work better for the Alexa Fluor 405 system than for the AF488 system. Doubling the concentration of PNA also significantly increased the FI, at least for the AF405 detection systems, but not as much as using the B anti-SaA FI amplification additional steps.

In conclusion, there are several commercially available options that provide acceptable levels of fluorescence above background for the detection of PNA-binding to *Haemonchus* eggs. Multiple companies sell PNA and other lectins that are directly conjugated to either FITC and/or rhodamine fluorophores, and protocols using them provide large time and cost advantages because they only require a single incubation for each lectin followed by wash steps. The relatively low FI from directly conjugated PNA should not be a problem at least for *Haemonchus* eggs because their natural AF is low for the FITC and rhodamine-type filters. Assuming that other trichostrongyle species have similar levels of AF, it should be possible and practical to simultaneously detect eggs from *Haemonchus* and another genus of trichostrongyle using directly conjugated lectins. Detecting a third genera with a third lectin presently requires the use of an avidin-biotin detection system because directly-conjugated lectins are not available in the blue emission spectral range. This system requires two incubation steps, but should provide a higher level of FI which would be useful in overwhelming the high natural AF present in these eggs for DAPI-type filters. Future studies are needed to measure AF levels among other trichostrongyle eggs, and to verify that lectins don't interfere with each other's binding potentials when used sequentially in a series of incubations or when combined into a single incubation.

Declaration of interests

None.

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