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Validation of a new technique to detect *Cryptosporidium* spp. oocysts in bovine feces

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ABSTRACT

Due to its important zoonotic potential, cryptosporidiosis arouses strong interest in the scientific community, because, it was initially considered a rare and opportunistic disease. The parasitological diagnosis of the causative agent of this disease, the protozoan *Cryptosporidium* spp., requires the use of specific techniques of concentration and permanent staining, which are laborious and costly, and are difficult to use in routine laboratory tests. In view of the above, we conducted the feasibility, development, evaluation and intralaboratory validation of a new parasitological technique for analysis in optical microscopy of *Cryptosporidium* spp. oocysts, called *TF-Test Coccidia,* using fecal samples from calves from the city of Araçatuba, São Paulo. To confirm the aforementioned parasite and prove the diagnostic efficiency of the new technique, we used two established methodologies in the scientific literature: parasite concentration by centrifugal sedimentation and negative staining with malachite green (*CSN-Malachite*) and *Nested-PCR*. We observed good effectiveness of the *TF-Test Coccidia* technique, being statistically equivalent to *CSN-Malachite*. Thus, we verified the effectiveness of the *TF-Test Coccidia* parasitological technique for the detection of *Cryptosporidium* spp. oocysts and observed good concentration and morphology of the parasite, with a low amount of debris in the fecal smear.

1. Introduction

Being an important zoonotic pathogen, *Cryptosporidium* spp. infects mammals, birds, reptiles, amphibians and fish (Fayer, 2010; Xiao, 2010). This parasite causes diarrhea in calves (Vargas et al., 2014), with growth retardation, mortality and consequent economic loss (Olson et al., 2004; Santín et al., 2008). In cattle, the most common species are *Cryptosporidium parvum* (Santín et al., 2008), *Cryptosporidium ryanae, Cryptosporidium andersoni* (Xiao, 2010) and *Cryptosporidium bovis* (Feng et al., 2007; Fayer et al., 2008; Rieux et al., 2013).

Laboratory diagnosis of *Cryptosporidium* oocysts in feces can be performed by means of parasite concentration techniques followed by the use of specific and permanent stains as Ziehl-Neelsen (Henriksen and Pohlenz, 1981), Kinyoun (Lennette et al., 1985),

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negative malachite green staining (Elliot et al., 1999), safranin methylene blue (Garcia, 2007, 2009) and trichrome (Garcia, 2007, 2009). Other techniques can also be used for this diagnostic purpose, such as phase contrast microscopy (Teixeira et al., 2011), direct fluorescent antibody test (CDC, 2015) and enzyme-linked immunosorbent assay (CDC, 2015).

Nowadays, the laboratory diagnosis of *Cryptosporidium* spp. oocysts requires the use of specific concentration and permanent staining techniques, which are labor-intensive and costly, and have difficulty in its implementation in the routine of a clinical laboratory (Gomes et al., 2004; Garcia, 2007; Carvalho et al., 2012, 2016).

The precursor technique of this study, called *TF-Test* (*Three Fecal Test*), has been used with practicality for years for fecal parasitological diagnosis in humans, with excellent results (Gomes et al., 2004; Carvalho et al., 2012, 2016). More recently, this technique has been extended to research in animal area by studies involving sheep (Lumina et al., 2006) and canine (Coelho et al., 2013, 2015) species. In these works, new diagnostic procedures were validated with gastrointestinal helminths and protozoa, not taking into account the genus *Cryptosporidium*.

In view of the above, continuing the line of research regarding the above technique, we analysed the feasibility, development, evaluation and intralaboratory validation of a new and practical parasitological technique for analysis in optical microscopy of *Cryptosporidium* spp. oocysts, called *TF-Test Coccidia* (Fig. 3), using fecal samples from calves from the city of Araçatuba, São Paulo.

2. Material and methods

This study was approved by the Animal Ethics Committee of the Faculdade de Odontologia do Campus de Araçatuba – UNESP, with protocol number 2013-00847.

2.1. Harvest and storage of fecal samples

Considering the irregular shedding of oocysts of *Cryptosporidium* spp. (Garcia, 2007, 2009; CDC, 2015), fecal samples were harvested (collected) on three alternate days, directly from the rectum of each calf. This material was divided into three parts: the first part 50 mL was stored under refrigeration for use in the parasite concentration technique by centrifugal sedimentation and negative staining with malachite green, named in our study as *CSN-Malachite*; the second part (5.4 g) was kept in the collecting tubes of *TF-Test Coccidia*; and the third 200 milligrams part was kept frozen at −20 °C until the time of genomic DNA extraction and nested polymerase chain reaction (*nested PCR*).

2.2. Processing of fecal samples

For the confirmation of oocysts and statistical comparison of techniques, besides the new *TF-Test Coccidia* technique, we used two established techniques in the scientific literature: *nested PCR* (confirmation of oocysts) and *CSN-Malachite* (statistical comparison), respectively.

3. Experimental design

3.1. Protocol standardization of the new technique

3.1.1. Study description

This intralaboratory study was conducted with the support of a farm of dairy cattle breeding in the city of Araçatuba, São Paulo, identified with positive samples, during the period of March 2014. For this step, fecal samples (n = 60) were processed only by the *CSN-Malachite* technique. As a result, we obtained five positive samples for *Cryptosporidium* spp. oocysts, which were later divided into 100 aliquots for the development and standardization of the operating protocol of the *TF-Test Coccidia* technique. A total of 60 Holstein calves, 11 males and 49 females, aged from six to 480 days, were involved in this study.

3.2. New technique validation

3.2.1. Study description

This intralaboratory research was conducted with fecal samples in 10 dairy cattle breeding farms in the city of Araçatuba, São Paulo, during the period from March 2014 to October 2015. For this step, we used the *CSN-Malachite* technique to perform the screening of samples to assess the occurrence of *Cryptosporidium* spp. oocysts in the fecal content of cattle. After this screening, we applied the standard protocol of the new *TF-Test Coccidia* diagnostic technique. The diagnostic of fecal samples were confirmed by *nested PCR*. Lastly, a total of 68 calves of Holstein and Girolanda races, 15 males and 53 females, aged between one and 540 days, were examined to assess the presence of the parasite, resulting in 34 positive samples and 34 negative samples for intralaboratory evaluation. These animals were classified according to their age in two groups: ≤ 30 days and ≥ 31 days.

3.3. Centrifugal sedimentation with negative malachite green stain modified technique (CSN-Malachite)

For this procedure, we obtained a pool of three samples for each animal. This material was processed in the laboratory in accordance with the literature of centrifugal sedimentation (Meloni and Thompson, 1996) and staining with malachite green (Elliot et al., 1999).

3.3.1. *New* TF-Test coccidia *technique*

This technique consisted of the following steps:

The three collection tubes (Fig. 3) of *TF-Test Coccidia* (containing formalin) containing 5.4 g of the collected feces were placed in an appropriate shelf. In this material, 25 μL of surfactant organic solvent (colorless neutral detergent) and 3 mL of ethyl acetate pro analysis (Formula $C_4H_8O_2$) were added to each tube. Then, the respective tubes with their caps were closed and shaken vigorously for 30 s with a vortex mixer. After that, the caps of the tubes were removed and connected to the other two parts, called set of filters and centrifuge tube. The union of all these parts was denominated in this study "set processor". Subsequently, this set was centrifuged for two minutes at 500 x G (gravitational force). After this centrifugation, the centrifuge tube was disengaged from the set of filters and collection tubes. Next, the supernatant liquid was decanted from the centrifuge tube, so leaving 500 μL of sediment in the conical bottom of the centrifugation tube. In this sediment, 250 μL of treated water were added and mixed, providing the formation of a fecal suspension. This suspension was redeemed using an automatic pipette, and 150 mL of the fecal suspension were transferred to a *TF-Test Coccidia* collection tube. Then a drop of colorless neutral detergent was added to this material and the whole suspension was manually mixed. After that, 3 mL of neutral formalin solution were added to this material and all material was mixed vigorously for 30 s with a vortex mixer. In sequence, 3 mL of ethyl acetate pro analysis were added, and all material was shaken again for another 30 s on the vortex mixer. Then, the tube was centrifuged at 333 x G for one minute. After this centrifugation, the supernatant was decanted using a pipette, until a small aliquot of suspension on the bottom of the demarcated tube was left, and 25 μL of this material were transferred to a microscope slide. Then, on the slide, 25 μL of the dye developed and standardized in the study (25 μL of modified D'Antoni's iodine solution and 50 μL of modified Masson's

trichrome) were added. Finally, a fecal smear was made on the slide and a coverslip was superimposed for examination by optical microscopy.

3.3.2. Staining and microscopy

The operational protocol of the new technique allowed, even with samples fixed with neutral formalin solution, the preparation of fecal smear using temporary staining composed of modified D'Antoni's iodine solution and modified Masson's trichrome. This new dye allowed the reading of a microscope slide with dry objective lens of 60 x and 100 x magnification in a conventional optical microscope, without the need for immersion oil. It is worth mentioning that these objective lenses for use without immersion oil have good image quality and were recently introduced in the market by Olympus industry, with the following descriptions: objective lens 60 x – UPlan FLN and objective lens 100 x – Plan FLN.

3.4. Nested PCR *targeting the 18S ribosomal RNA gene of* Cryptosporidium *spp*

3.4.1. Fecal DNA extraction

The DNA was extracted from 200 mg of 68 fecal samples "in natura", stored at −20 °C until genomic DNA extraction; for the extraction of samples it was performed heating to 99 °C for 60 min for breaking the wall of the oocyst, using QIAamp® DNA Stool Mini Kit (Qiagen®). The DNA was eluted with 50 μL AE buffer and stored at −20 °C.

3.4.2. Nested PCR

Nested PCR was performed for amplification of a fragment of the 18S ribosomal RNA gene (Xiao et al., 2000). *Cryptosporidium parvum* DNA was used as positive control and ultrapure water was included as a negative control, followed by electrophoresis from a 1.5% agarose gel.

For amplification of the 18S subunit ribosomal RNA gene fragments it was used nested PCR with primers from Sigma-Aldrich® 5 'TTC AGC TAG TAA ATG TAC CG 3′ and 5 'CCC ATT ACA GGA TCC GAA TTC 3 'for the primary reaction, with 1325 base pairs (bp) and 5′-AGG GTT GGA GTA TTT ATT AGA AAT GA 3′ and 5 'AAT GGA AAG GAG ACC ACA TCC 3′ for the secondary reaction (bp 826–840). To perform the following reaction conditions, there was prepared a solution with a final volume of 25 μL from JumpStart Taq™ READYMIX™ reagent from Sigma-Aldrich®, for primary reaction was used: JumpStart Taq™ READYMIX™ 12.5 μL, primer 1 0.5 μL, primer 2 0.5 μL, H_2O 8.25 μL, BSA 0.75 μL and DNA target 2.5 μL. To use secondary reaction: JumpStart Taq™ READYMIX™ 12.5 μL, primer 3 0.5 μL, primer 4 0.5 μL, H_2O 9.0 μL and DNA target 2.5 μL. The samples were subjected to DNA initial denaturation at 94 °C for 3 min followed by 34 cycles each consisting of denaturation for 45 s at 94 °C, 45 s annealing at 55 °C and 60 s extension at 72 °C with extension end at 72 °C for 7 min (Xiao et al., 2000).

3.5. Statistical analysis

The variables (sex, breed and age) were analysed by the chi-square test ($\chi 2$) or *Fisher's* exact test for the association between the variables.

The Kappa statistic was used to measure the agreement between the mentioned techniques.

We used the SAS software (SAS, 2015) for the statistical calculations, with 5% significance level.

4. Results

In the intralaboratory study of the new *TF-Test Coccidia* technique, we observed good sharpness and morphology of oocysts of

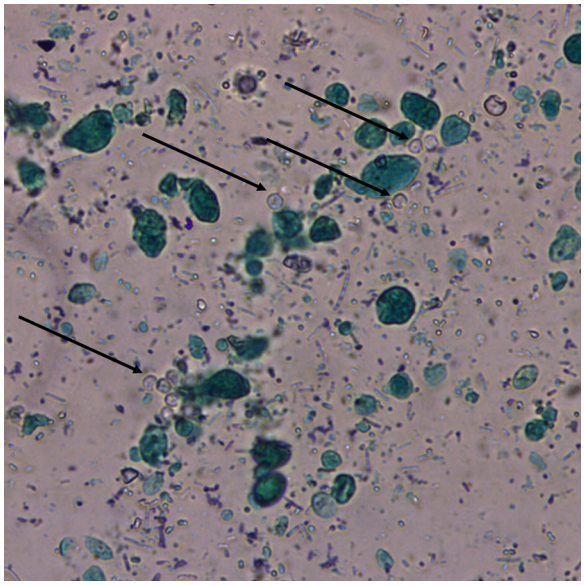

Fig. 1. Image of *Cryptosporidium* spp. oocysts in a sample of calf feces, with the use of a dry objective lens with 60 x magnification.

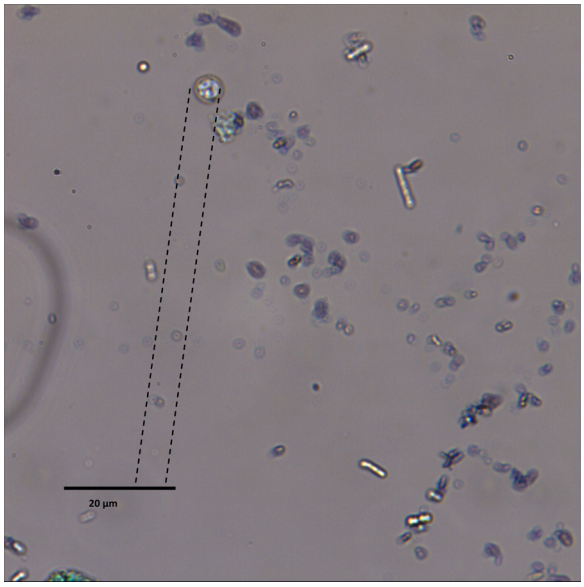

Fig. 2. Image of *Cryptosporidium* spp. oocyst in a sample of calf feces, with the use of a dry objective lens with 100 x magnification.

Cryptosporidium spp. (Figs. 1 and 2). The same could not be demonstrated in most slides processed by the *CSN-Malachite* technique, in accordance with literature reports (Coelho et al., 2015).

Furthermore, the new technique presented fecal smear slides with low quantity of debris, good concentration of parasite on a microscope slide (Fig. 1) and demonstrated the preparation of fecal smears with the use of a new temporary stain. Unlike conventional staining, the dye of the new technique allowed to highlight the sporozoites of oocysts with a purple hue (Fig. 2), and the fecal debris with a green color.

The concentration adjustment between an iodine solution and Masson allowed this new composition dye work with same accuracy in fecal samples collected in neutral formalin solution, unlike literature indications (Garcia, 2007, 2009).

In this intralaboratory study, the same examiner detected 34 positive samples in 68 calves examined by both *CSN-Malachite* and

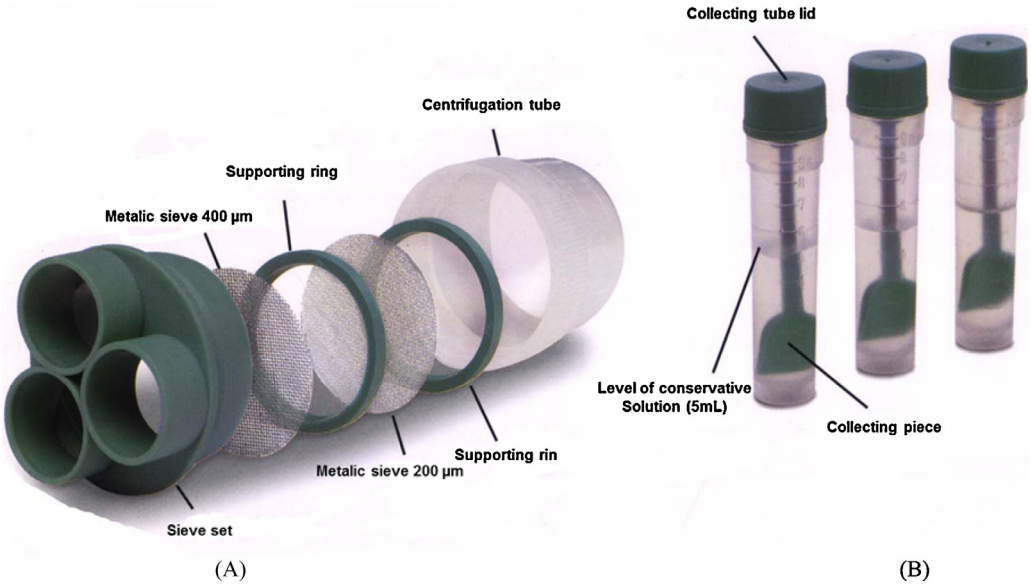

Fig. 3. Illustration of the set of centrifugation (A) and collectors tubes (B) of *TF-Test Coccidia.*

TF-Test Coccidia techniques, with full statistical correlation between these results (Kappa = 1.000). However, with *nested-PCR* we confirmed 24 positive and 10 false negative samples.

Regarding the negative samples (n = 34), there are statistic agreement between the *CSN— Malachite* and *TF —Test Coccidia* and *nested PCR* techniques.

By the chi-square test ($\chi 2$), a higher frequency of infection by *Cryptosporidium* spp. was observed in younger calves in relation to other age groups ($P<0.0001$), and with respect to sex and breed, there was no significant difference in parasitic infection rate ($P>0.05$).

5. Discussion

Through the data shown in this intralaboratory study, we observed good efficacy in oocyst detection of the protozoan *Cryptosporidium* spp. by using the *TF-Test Coccidia* technique, which favors the use of this new technique in laboratory routine.

For this research, we prefer to take fecal samples of newborn calves (Fayer and Santín, 2009; Silverlås et al., 2010) because they present higher frequency of cryptosporidial infection, which was confirmed by our results that showed a significant difference for this age group compared to animals of other ages.

The new technique clearly showed fecal smears with good parasitic concentration, and largely free of debris (Figs. 1 and 2). In many slides it was possible to view a concentration of more than seven oocysts per field, with 60 x magnification objective lens without immersion (Fig. 1). This should favor the detection of oocysts of this parasite in microscope reading practiced in laboratory routine, reducing fatigue and diagnostic interpretation error practiced by humans.

The use of the new dye preserved the morphological integrity of *Cryptosporidium* spp. oocysts, always dyeing the sporozoites in lilac color (Fig. 2) and almost all of the impurities in green. This condition should facilitate the microscopy reading, significantly reducing the diagnostic interpretation error practiced by humans, which can occur with a false positive or false negative result.

Unlike literature recommendation (Garcia, 2007, 2009), the *TF-Test Coccidia* technique allowed the preparation of slides with temporary coloring and microscopy reading with dry objective lens. This eliminated the limitations provided by specific techniques using permanent dyes, for example, those derived from carbol fuchsin, which despite demonstrating excellent diagnostic performance, are rarely used in routine laboratory because they are labor-intensive and costly.

For the *CSN-Malachite* and *TF-test Coccidia* techniques, the results were concordant for the presence of the protozoan *Cryptosporidium* spp., that is, the same animals showed positive (n = 34) and negative (n = 34) samples. PCR was used only for confirmation of fecal samples, to indicate that *TF-Test Coccidia* detected the oocysts in the samples correctly.

The same examiner performed the processing of stool samples with the negative staining with malachite green (*CSN-Malachite*) and *TF-Test Coccidia* techniques, which were later confirmed by *Nested PCR*. The samples were processed randomly in each technique, without a sort order for the reading of the fecal samples, and thus the examiner was not suggestible when interpreting the data.

The proper selection of an extraction and purification method is crucial to ensure reliable results (Paulos et al., 2016), and is widely known that molecules found in fecal samples, or in the composition of buffers used for DNA extraction, can inhibit the enzymes used in amplification (Monteiro et al., 1997; Schrader et al., 2012; Paulos et al., 2016). Therefore, the detection efficiency of the molecular test may have been influenced by the possible presence of inhibitory agents in the material (Ward and Wang, 2001), or the vegetable intake by the animals (Monteiro et al., 2001). There are differences in the performance of kits to extract DNA from oocysts depending on the pathogen, and the intensity of the infection (Paulos et al., 2016). The justification for the lower sensitivity of PCR is because the stool samples were used "in natura", without purification and in a small amount (200 mg) when compared with *TF-Test Coccidia*, which was processed with 5.4 g of feces. It is noteworthy that, despite the comments above, the use of *Nested-PCR* in this study was extremely important, especially for the molecular confirmation of the parasite *Cryptosporidium* spp.

TF-Test Coccidia was considered an efficient technique and could be applied in the veterinary medicine field for the fecal diagnosis of *Cryptosporidium* spp. oocysts.

The preliminary results of this study allow us to move forward to the next research step: the interlaboratory validation of the *TF-Test Coccidia* technique. With this, we can begin with the study of computerized image analysis of *Cryptosporidium* spp. oocysts, which

should result in advances in this type of diagnosis in public and private laboratory routines, as well as government programs.

6. Conclusion

In this study, we verified the effectiveness of the *TF-Test Coccidia* parasitological technique for the detection of *Cryptosporidium* spp. oocysts and observed a good concentration and morphology of the parasite, with a low amount of debris in the fecal smear.

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