Further Comparison of Centrifugation Versus Passive Fecal Flotation for the Recovery of *Toxocara canis*, *Trichuris vulpis* and *Ancylostoma caninum* Eggs.

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ABSTRACT

Between 2010 and 2018, 239 individuals in 29 classes participated in a week-long clinical parasitology training program. Participants were either veterinarians (233) or persons with advanced training in parasitology (6). As part of the course, attendees participated in a fecal diagnostic wet lab. Fecal samples were collected from dogs at the local humane society and verified as positive in the Kansas State University Parasitology Diagnostic Laboratory for various parasite diagnostic stages. While samples contained diagnostic stages of several different species of parasites, all classes evaluated samples that contained *Ancylostoma caninum*, *Toxocara canis* and *Trichuris vulpis* eggs. Each participant conducted a direct smear, a passive floatation (Ovassay® Plus) with 1.18 sp. gr. ZnSO4 solution, a centrifugation procedure using 1.18 sp. gr. ZnSO4 solution and a centrifugation procedure using 1.27 sp. gr. sugar solution. Participants recovered *A. caninum*, *T. canis*, and *T. vulpis* eggs from 71.9% (172/239), 61.1% (146/239), and 37.7% (90/239) of the samples, respectively when using the Ovassay® device. When comparing centrifugation techniques, participants were more likely to recover *T. vulpis* eggs using the higher sp. gr. sugar solution, 96.7% (231/239), than using ZnSO4, 80.3% (192/239). Recovery of *A. caninum* and *T. canis* did not significantly differ between the centrifugation methods. Reliability of the Ovassay® technique was poor compared to

centrifugation. *Trichuris vulpis* eggs were recovered by every participant in the class only 3.4% (1/29) of the time by Ovassay® passive flotation, compared to 34.5% (10/29) and 75.9% (22/29) of the time by ZnSO4 or sugar centrifugation, respectively. These data provide further evidence that passive fecal flotation is an inferior fecal technique and should not be considered as a reliable diagnostic test in practice, especially in areas where *T. vulpis* infections are common.

INTRODUCTION

Routine fecal examinations for recovery of parasite eggs, oocysts, & cysts are part of the daily routine for most veterinary practices. They are used as part of annual examination protocols and to aid in the diagnosis of a number of intestinal and respiratory parasites, where diagnostic stages are passed in the feces. The validity of a fecal examination is dependent on the procedure, the type of flotation solution, the specific gravity of the flotation solution, and the training of the person conducting the test.[1,2]

Various techniques are utilized, including direct smears, passive flotation, centrifugation, Baermann technique and sedimentation.[2] For routine parasite diagnostic screening generally passive flotation and centrifugation are performed, however, some practices still conduct quick screening with a direct smear, especially for Giardia.[2,3]

Even with the recent development of fecal antigen testing, fecal flotations (passive or centrifugation) are still the primary parasite diagnostic tools utilized by veterinarians (aData on file at IDEXX Laboratories, Inc). Fecal flotations are used to concentrate parasite diagnostic stages out of a quantity of fecal matter. Flotations are based on differential specific gravity of parasite eggs/oocysts/cysts, fecal debris and flotation solution, where specific gravity refers to weight of object compared to equal volume of water. Given that many of the common nematode and cestode eggs, have a mean specific gravity between 1.1 and 1.234, routine fecal flotations for the recovery of common parasite eggs should be conducted with a solution having a specific gravity > 1.24.[5]

Several studies have been conducted demonstrating that centrifugation techniques recover more eggs and find more parasitized animals than passive flotation.[1,3,5-9] However, passive flotation techniques are still being widely utilized.[10] Therefore, additional education and research efforts are needed to change historic use of passive flotation systems.

The data contained within this publication were generated by 239 individuals that participated in fecal wet-labs at Kansas State University. The results document the reliability of direct smears, passive flotation and centrifugation techniques when conducted by a large number of individuals over an 8-year period.

MATERIALS AND METHODS

From 2010 to 2018, 239 individuals in 29 separate classes participated in week-long small animal clinical parasitology short courses held at the College of Veterinary Medicine at Kansas State University. As part of the course, each individual participated in a fecal diagnostic wet-lab. The class participants included 233 veterinarians (including veterinarians in industry, private practice, parasitology residencies, post-doctoral programs, and parasitology teaching faculty) and six non-veterinarians with previous training in parasitology (post-doctoral students in parasitology programs and additional non-parasitology teaching faculty).

Fecal samples were collected by personnel at local animal shelters from naturally infected dogs and verified as positive for parasite diagnostic stages by diagnostic laboratory technicians for various parasite diagnostic stages in the Kansas State University Parasitology Diagnostic Laboratory using standard double centrifugation methodology using 1.27 specific gravity Sheather's sugar solution. Feces were then thoroughly mixed to form a composite sample and then rechecked using the same methodology to verify that all parasite diagnostic stages were present in the composite sample.

During these classes various parasite diagnostic stages were recovered including eggs/oocysts/cysts from *Alaria spp*., *Ancylostoma caninum*, *Baylisascaris procyonis*, *Cystoisospora spp*., *Dipylidium caninum*, *Eucoleus spp*., *Giardia sp*., *Taenia spp*., *Toxocara. canis*, & *Trichuris vulpis*. While the range of different parasite diagnostic stages varied from class to class, each of the 29 short-course composite samples contained eggs of *A. caninum*, *T. canis* and *T. vulpis*. No attempt was made to standardize the number of eggs between classes.

Each participant within a specific class conducted their fecal examination procedures on the same composite sample. However, at the time of conducting the examinations, participants were given separate aliquots from the composite and remained blinded to the fact that each aliquot came from a single composite sample.

Prior to the wet-lab a 45-minute tutorial was presented to each class on fecal diagnostic procedures, and participants were shown images of parasite diagnostic stages that "might" be contained within the samples. During the wet-lab participants had access to parasitology diagnostic reference manuals and could ask instructors for assistance in identifying parasite diagnostic stages.

Each participant was asked to conduct a direct smear, a passive flotation with 1.18 sp. gr. ZNSO4 using the OVASSAY® PLUS fecal flotation device (Zoetis, 10 Sylvan Way, Parsippany, NJ), a centrifugation technique using 1.18 sp. gr. ZNSO4 and a centrifugation technique using 1.27 sp. gr. Sheather's sugar solution. For the direct smear participants collected a small amount of feces on the end of a wooden applicator stick and mixed on a glass slide with a drop of saline. Although samples were not weighed, participants were asked to use approximately equivalent sized samples, between 2 to 5 grams of feces, for the passive and centrifugation techniques. The approximate sample size was visually demonstrated to the class. This was done to approximate standard technique in private veterinary practice, where samples are almost never weighed. For the passive flotation technique participants were provided with and asked to follow directions from the package insert from the manufacturer. In our wet-lab the instructions were to leave the cover-slip on the meniscus of the device for 10 minutes and not the 5 minutes that is currently in the directions (https://www.zoetisus.com/products/diagnostics/ovassay-plus-kit-fecal-flotation-devices.aspx). Older versions of instructions for this device did specify a 10-minute standing wait time. For centrifugation a standard single-centrifugation technique using a swinging head-rotor was used where samples were centrifuged for 5 minutes at approximately 280xG, and then the cover-slip was allowed to remain on centrifuge tube for an additional 10 minutes before being removed for examination.5 Once coverslips were removed and placed on the glass slide participants were instructed to first examine systematically under 100X, then verify parasite identity using 400x magnification and record results for each parasite.

For comparisons of techniques two questions were asked.

1) How often did every participant in a class find the parasite diagnostic stages? This was looking at the probability that everyone within a single class using a specific technique will find the diagnostic stages (eggs) in that sample.

2) What percentage of the samples were found to be positive by the participants?

Question 1 was addressed by comparing the proportion of classes in which all eggs were discovered, broken down by species of parasite and method of discovery. Question 2 was addressed using a chi-square test for difference in proportions at a significance level of 0.05, adjusted for multiple comparisons using the Holm-Bonferroni method.

RESULTS

How often did every participant in a class

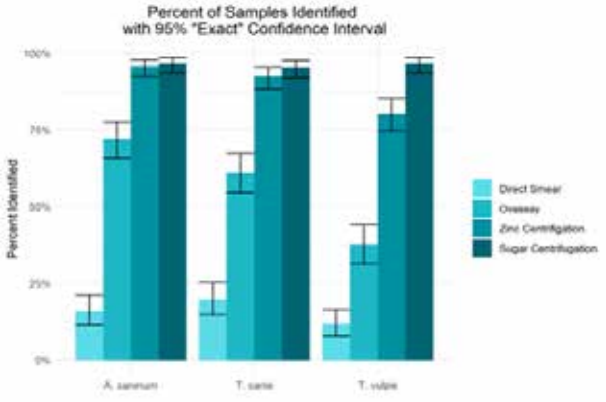
find the parasite diagnostic stages?

Using the direct smear technique, only one class (1/29) had every participant recover *A. caninum* eggs, and no classes (0/29) had every participant in a class recover eggs of *T. canis* or *T. vulpis* (Table 1). When participants used the Ovassay® Plus device with 1.18 sp. gr. zinc sulfate, every participant in the class recovered eggs of *A. caninum*, *T. canis* and *T. vulpis* 31.0% (9/29), 17.2% (5/29) and 3.4% (1/29) of the time, respectively (Table 2). However, when they used the same zinc sulfate flotation solution in a centrifugation technique every participant in the class recovered eggs of *A. caninum*, *T. canis* and *T. vulpis* 75.9% (22/29), 58.6% (17/29) and 34.5% (10/29) of the time, respectively (Table 3). Using the higher specific gravity Sheather's sugar solution (1.27 sp. gr) in the centrifugation technique every participant in the class recovered eggs of *A. caninum*, *T. canis* and *T. vulpis* 82.8% (24/29), 79.3% (23/29) and 75.9% (22/29) of the time, respectively (Table 4).

What percentage of the samples were found to be positive by the participants?

Participants recovered *A. caninum*, *T. canis*, and *T. vulpis* eggs from 72.0% (172/239), 61.1% (146/239), and 37.7% (90/239) of the samples, respectively, using the Ovassay® Plus device (Figure 1). When comparing centrifugation techniques, participants were more likely to recover *T. vulpis* eggs using the higher sp. gr. sugar solution, 96.7% (231/239), than using ZnSO4, 80.3% (192/239) ($p<0.001$) (Figure 1). Recovery of *A. caninum* and *T. canis* did not significantly differ between the centrifugation methods (pAC=0.81; pTC=0.25). Participants using either the ZnSO4 or sugar solutions in the centrifugation technique recovered *A. caninum* eggs in 95.8% and 96.7% of the samples, respectively. Similarly, *T. canis* eggs recoveries for both solutions using the centrifugation technique were 92.5% and 95.4% for ZnSO4 and sugar, respectively (Figure 1). Egg recovery rates of the Ovassay® technique was significantly inferior to centrifugation for every parasite (pTCZ<0.001, pTCS<0.001, pACZ<0.001, pACS<0.001, pTVZ<0.001, pTVS<0.001). The direct smear technique was uniformly poor in egg recovery, with only 11.7 – 19.7% of samples determined to be positive for any parasite (Figure 1).

Figure 1. *Percent of composite fecal samples recorded as positive for Ancylostoma caninum (hooks), Toxocara canis (rounds) or Trichuris vulpis (whips) by attendees at fecal wet-labs using 4-different techniques.*



DISCUSSION

A number of studies conducted over the past two decades have repeatedly demonstrated the poor recovery rate of eggs, cysts or oocysts using passive flotation methodologies.[1,3,5-9]

These current data further clarify that passive flotation struggles to recover parasite eggs as the specific gravity of those eggs increases. The mean specific gravity of the eggs of *A. caninum*, *T. canis*, and *T. vulpis*, are 1.0559, 1.0900, and 1.1453, respectively.4 In this current study the Ovassay ® Plus passive flotation fecal system decreased in percent positive samples from 72% with *A. caninum* down to 37.7% with *T. vulpis*. This was even more dramatically highlighted when looking at the percent of an individual class where every attendee found eggs in a known positive sample. In the 29 classes

Table 2. *Percent of attendees in each of 29 wet-lab classes recording as positive composite fecal samples containing eggs of Ancylostoma caninum (hooks), Toxocara canis (rounds) or Trichuris vulpis (whips) using a passive flotation system (OVASSAY® PLUS fecal flotation device) and a 1.18 sp. gr. $ZNSO_4$ fecal flotation solution.*

	Passive Flotation – 1.18 sp. gr. $ZNSO_4$ fecal flotation solution: % of samples positive[1]																												
Year	2010			2011			2012				2013				2014			2015			2016			2017			2018		
# of Participants in a class[2]	5	5	4	7	9	6	8	8	4	7	9	11	5	7	2	7	11	10	10	8	11	11	10	11	9	12	11	9	12
Hooks	100	60.0	100	71.4	100	83.3	87.5	100	75.0	14.3	11.1	54.6	60.0	28.6	100	57.1	63.6	50.0	100	50.0	100	100	100	54.6	55.6	58.3	91.0	77.8	91.7
Rounds	100	100	100	57.1	55.6	16.8	75.0	0.0	50.0	71.4	22.2	72.7	80.0	57.1	50.0	71.4	36.4	40.0	30.0	37.5	45.5	90.9	100	100	66.7	66.7	54.6	55.6	83.3
Whips	20.0	0.0	0.0	42.9	0.0	83.3	100	62.5	25.0	14.3	44.4	63.6	80.0	85.7	0.0	14.3	27.3	0.0	50.0	37.5	27.3	9.1	20.0	63.6	55.6	25.0	36.4	44.4	33.3

1 Different composite samples used for each class and all demonstrated positive in the Parasitology Diagnostic Laboratory at Kansas State University
2 239 individuals in 29 separate classes (233 Veterinarians and 6 non-veterinarians with previous experience/training in parasitology)

Table 1. *Percent of attendees in each of 29 wet-lab classes recording as positive composite fecal samples containing eggs of Ancylostoma caninum (hooks), Toxocara canis (rounds) or Trichuris vulpis (whips) using a direct smear technique.*

	Direct Smear: % of samples positive[1]																												
Year	2010			2011			2012				2013				2014			2015			2016			2017			2018		
# of Participants in a class[2]	5	5	4	7	9	6	8	8	4	7	9	11	5	7	2	7	11	10	10	8	11	11	10	11	9	12	11	9	12
Hooks	100	0.0	50.0	28.6	22.2	33.3	0.0	37.5	50.0	0.0	11.1	0.00	20.0	0.0	0.0	0.0	9.1	10.0	0.0	0.0	0.0	18.2	0.0	0.0	0.0	0.0	36.4	33.3	33.3
Rounds	80.0	60.0	25.0	28.6	55.6	0.0	25.0	0.0	25.0	42.9	22.2	27.3	20.0	0.0	0.0	14.3	9.1	0.0	0.0	12.5	0.0	45.5	40.0	36.4	11.1	0.0	0.0	11.1	16.7
Whips	0.0	0.0	25.0	28.6	22.2	33.3	87.5	0.0	0.0	0.0	11.1	27.3	0.0	14.3	0.0	14.3	0.0	0.0	10.0	0.0	27.3	9.1	10.0	0.0	33.3	16.7	0.0	0.0	0.0

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Table 3. *Percent of attendees in each of 29 wet-lab classes recording as positive composite fecal samples containing eggs of Ancylostoma caninum (hooks), Toxocara canis (rounds) or Trichuris vulpis (whips) using a centrifugation technique and a 1.18 sp. gr. $ZNSO_4$ fecal flotation solution.*

	Centrifugation – 1.18 sp. gr. $ZNSO_4$ fecal flotation solution: % of samples positive[1]																											
Year	2010			2011			2012				2013				2014			2015			2016			2017			2018		
# of Participants in a class[2]	5	5	4	7	9	6	8	8	4	7	9	11	5	7	2	7	11	10	10	8	11	11	10	11	9	12	11	9	12
Hooks	100	100	100	100	100	100	100	100	100	100	77.8	72.7	100	85.7	100	100	90.9	100	100	87.5	90.1	100	100	100	88.9	100	100	100	100
Rounds	100	100	100	100	100	83.3	100	75.0	100	100	88.9	90.9	100	100	100	100	90.9	70.0	70.0	100	81.8	100	100	100	88.9	100	91.0	88.9	91.7
Whips	80.0	60.0	100	71.4	100	100	100	87.5	50.0	28.6	100	90.9	100	100	100	71.4	45.5	90.0	90.0	100	54.6	72.7	70.0	81.8	88.9	75.0	81.8	100	75.0

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Table 4. *Percent of attendees in each of 29 wet-lab classes recording as positive composite fecal samples containing eggs of Ancylostoma caninum (hooks), Toxocara canis (rounds) or Trichuris vulpis (whips) using a centrifugation technique and a 1.27 sp. gr. Sheather's sugar fecal flotation solution.*

	Centrifugation – 1.27 sp. gr. Sheather's Sugar solution: % of samples positive[1]																											
Year	2010			2011			2012				2013				2014			2015			2016			2017			2018		
# of Participants in a class[2]	5	5	4	7	9	6	8	8	4	7	9	11	5	7	2	7	11	10	10	8	11	11	10	11	9	12	11	9	12
Hooks	100	100	100	100	100	100	100	100	100	100	77.8	72.7	100	100	100	100	90.9	100	100	100	100	100	100	100	88.9	91.7	100	100	100
Rounds	100	100	100	100	100	100	87.5	87.5	100	100	100	100	100	100	100	100	90.9	60.0	70.0	100	100	100	100	100	88.9	100	100	100	100
Whips	100	100	100	100	100	100	100	100	100	85.7	100	90.9	100	100	100	100	90.9	100	90.0	87.5	81.8	90.9	100	100	100	100	100	100	100

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31.0% of the time every attendee in a class found *A. caninum* eggs using the passive flotation system whereas that only happened once (3.4%) with *T. vulpis*. This was contrasted with the Sheather's sugar solution centrifugation technique where 82.8% of the time every attendee in a class found *A. caninum* eggs and 75.9% of the time every attendee in a class recovered eggs of *T. vulpis*. This does not reflect a reduction in parasite eggs found on passive flotation techniques, but a complete absence of eggs detected. These data present evidence of not only the poor sensitivity of the passive technique but also on its lack of consistency or reliability.

Previous studies have provided similar results. In one study where veterinary students used a single composite canine fecal sample, a commercial passive flotation system only detected *T. vulpis* eggs in 68% of positive samples, whereas those same students found 95% of the samples positive using centrifugation.[5] Another study found that in a comparison of centrifugation versus passive flotation the most pronounced differences also occurred with recovery of *T. vulpis* infections in shelter dogs.[3] Centrifugation using zinc sulfate or Sheather's sugar solution found 1.5 to 1.7 times more positive dogs than the commercial passive flotation system. In the current study participants found more positive *T. vulpis* samples (96.7%) using centrifugation with 1.27 sp. gr. Sheather's sugar solution than when they used 1.18 sp. gr. ZNSO4 (80.3%). This difference was likely due to the increased specific gravity of the Sheather's sugar solution and the variable eggs numbers in the 29 different composite samples. While passive flotation did poorly in recovering *T. vulpis* eggs, participants also failed to recover *T. canis* eggs over one-third of the time (38.9%). This is particularly troubling given the serious zoonotic implication of visceral larval migrans and ocular larval migrans caused by *T. canis*. Even though several studies have demonstrated the poor egg recovery rate of passive flotation systems, surveys have documented that such passive flotation is still commonly employed in private practices in the United States. In a 2010 study where over 450 veterinarians responded to a survey of parasitological techniques the authors state that based on the data, benchtop simple fecal flotation and the direct smears are the most common techniques performed in practice.[10] Then in a survey conducted by IDEXX Laboratories between 2016 - 2018 of 1001 veterinary practices in the United States they found that 75% of practices were still conducting some or all of their fecal examinations "in-clinic".[a] In another survey of 301 veterinary practices in the United States they found that if practices were conducting fecal examinations in-clinic, 68% were using simple passive flotation methodologies ([a]Data on file at IDEXX Laboratories, Inc).

Given the clinical and/or zoonotic implications of veterinarians failing to detect gastrointestinal parasitism in dogs (or cats) it is surprising that inferior diagnostic techniques are utilized. Rationales such as technician time constraints and expense cannot be medically justified given the poor sensitivity and reliability of passive flotation systems. Veterinarians using passive flotation techniques need to address the issue that they are charging clients for a test with a sensitivity that is likely 38 – 72% for some of the most common intestinal parasites seen in-clinic, and that they are leaving patients under-diagnosed and likely under-protected as well. This is why the Companion Animal Parasite Council recommends that veterinarians "Conduct fecal examinations by centrifugation at least four times a year, depending on patient health and lifestyle factors." (https://capcvet.org/guidelines/general-guidelines/)

Reliability and consistency are also extremely important given that in many practices several different individuals may be conducting the fecal examinations, especially over a period of several years. Examination of tables 2 and 3 while focusing on the number of times 100% appears in a row provides quick visual evidence of the poor reliability and consistency of the passive flotation system as compared to centrifugation

when using the same flotation solution.

Historically, there have been many reasons for a veterinarian's reluctance to utilize centrifugal flotation techniques, including the expense of buying a centrifuge to conduct fecal examination, limited space for the centrifuge, or concerns of time spent running flotations by technicians. These concerns can be avoided for clinics that do not wish to do high-quality fecal flotations in-clinic by sending fecal samples to one of many commercial or state diagnostic laboratories that are available to practitioners.

Visual egg identification, even when conducted by an expert, is an imperfect gold standard and thus some uncertainty of egg presence was inherent in all samples in this study. While the samples provided for each class were a pooled group of samples thought to contain eggs from *T. canis*, *A. caninum*, and *T. vulpis*, the aliquots of sample provided to each participant were not assured to contain each of these. However, each sample was mixed thoroughly as evidenced by > 95% of all 239 participants being able to recover and identify eggs of all three parasite diagnostic stages in their aliquot when using the higher specific gravity sugar solution by the centrifugation method. It is important to note that the pooled samples provided to different classes differed in their egg-density introducing some class-to-class variability. Parasite diagnostic stage density in feces clearly varies from one parasitized animal to the next. Additionally, the technical expertise and experience of conducting fecal examinations of individual participants did vary and likely also introduced some variability. These factors are limitations of the current study, but are also represent general limitations to fecal flotation, as egg-dispersal, is likely to be non-homogenous in samples collected in the field. Additionally, training and experience of persons conducting fecal examinations in private veterinary practice varies considerably. Given these issues, utilization of the most effective technique would likely minimize recovery and detection errors as was demonstrated in this investigation.

CONCLUSION

These data provide further evidence that a passive fecal flotation methodology is an inferior fecal technique and should not be considered as a reliable diagnostic test in practice, especially in areas where *T. vulpis* infections are common.

CONFLICTS OF INTEREST

These authors have no conflict of interest to declare.

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Primary research conducted with U.S. veterinarians in 2016, 2017, 2018 with data on file at IDEXX Laboratories, Inc.

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