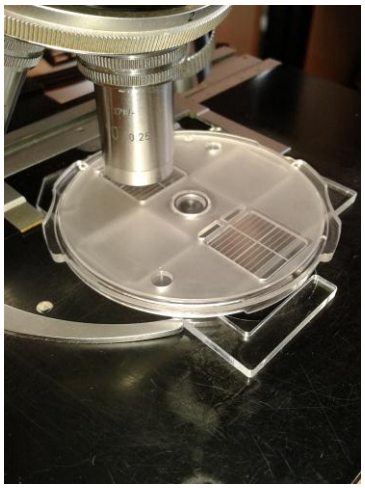
The mini- FLOTAC, a comparison with the centrifugal sedimentation/flotation, McMaster and the passive flotation technique for coproscopical examination of dog feces

Research report 2013



Caroline Palmbergen

Supervisor: Drs. Rolf Nijsse

Utrecht University

Utrecht University, Faculty of Veterinary Medicine
Department of Clinical Infectiology
Yalelaan 1, 3584 CL Utrecht
The Netherlands

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1. Abstract

The canine gastrointestinal parasite *Toxocara canis* forms a threat to the public health and this parasite forms the foundation for the European Scientific Counsel Companion Animal Parasites (ESCCAP) to advice dog owners to treat their dogs at least 4 times a year. Anthelmintic therapy is usually carried out without coproscopical examination, which could result in unnecessary shedding of anthelmintic residues that could enhance anthelmintic drugs resistance. As anthelmintic resistance is a growing problem in different veterinary fields, an easy to use and sensitive coproscopical examination technique is needed. Recent studies about the mini-FLOTAC technique, from the FLOTAC family, indicate that this technique could improve canine coproscopical examination in both the veterinary practice and the laboratory. The aim of this study is to examine if the mini-FLOTAC is a suitable alternative to use in laboratories and/or veterinary practices for the coproscopical examination of dog feces. To answer this question the sensitivity, repeatability, user-friendliness and the costs made for the CSF (labelled as the golden standard), were compared to the mini-FLOTAC, the passive flotation and the McMaster technique. The results showed no significant difference in the detectionlimit of the mini-FLOTAC compared to the CSF and the mini-FLOTAC showed an equal repeatability. The findings of this study indicate that the mini-FLOTAC is a promising technique for canine coproscopical examination in the veterinary practice or low-budget laboratory.

2.Introduction

All over the world animals can become infected with different kinds of gastrointestinal parasites. There are several gastrointestinal parasites in the Netherlands that can infect dogs. To give a compact overview of some of these gastrointestinal parasites that can be found by coproscopical examination table 1 provides some basic information. A study by Overgaauw et al. presented that *Giardia intestinalis* is the most commonly found gastrointestinal parasite, follow by *Cryptosporidium* spp. and *Toxocara canis*.(1, 2)Because of the potential zoonotic behavior of some of the gastrointestinal parasites and to ensure the health of the dog, regularly blindly anthelmintic treatment is advised by the European Scientific Counsel Companion Animal Parasites (ESCCAP), namely at least 4 times a year.(3)

Parasite	**Taxonomy**	**Size of egg or oocyst (µm)**	**Clinical signs dog**	**Shedding**	**Zoonotic**
Toxocara canis [3 - 7]	Nematode (Ascaridida)	85-90 x 75	Almost never any clinical signs, with puppies swollen bellies and cachexia can occur	Continuous	Yes
Uncinaria stenocephala [4, 5,8, 9]	Nematoda (strongylida)	72 x 45	Almost never any clinical signs, in heavy infection protein loosing enteropathy and dermatitis can occur	Intermittent	No
Giardia intestinalis[4, 5, 6, 8]	Protozoa (flagellate)	9-13 x 7-9	Sometimes clinical signs of acute or chronic diarrhea	Intermittent	Potential
Trichuris vulpis [3,5,8]	Nematode (Enoplida)	72-90 x 32-40	Almost never clinical signs, in heavy infection bloody diarrhea can occur	Continuous	No
Capillaria sp. [4,5, 10]	Nematode (Enoplida)	54-79 x 29-40	Usually subclinical infection and rarely can give polyuria, dysuria and cystitis	Intermittent	No
Isospora spp. [4,5]	Protozoa (coccidia)	24-36 x 21-30	Usually subclinical infection but can also cause diarrhea in puppies	Continuous	No
Neospora caninum [4,5, 11, 12]	Protozoa (coccidia)	11-14 x 9-11	Usually subclinical infection but can cause paralysis of rear limbs and death in puppies	Uncertain	No

Table 1. Gastrointestinal parasites in dog feces. (3-12)

Toxocara spp. is an important zoonotic gastrointestinal parasite that is found regularly in coproscopical examination of dog feces. Because of the risk for public health and the health of the dog, *Toxocara canis* forms the foundation for the advice of canine anthelmintic drugs use of at least 4 times a year. For the above mentioned reasons it is discussed below in more detail.

Toxocariosis is an infection that can occur in humans after ingesting infectious *Toxocara* spp. eggs. Research of toxocariosis in humans showed that 4% of the children in the Netherlands in the age category of 1-4 years old and 39% of the adults in the age category of 75-79 years old are seropositive for *Toxocara* spp.. These numbers clearly show an increase in the seropositivity correlating with the age of humans.(13, 14) This high number of seropositive adults is not very surprising as the adult females of *Toxocara canis* can produce up to 200.000 eggs per day.(15)

After the *Toxocara canis* eggs are ingested by a human, they reach the gastrointestinal tract where they hatch and the larvae penetrate the intestinal wall after which they start to spread to various parts of the body, like retina and liver. This spread in the body is through the blood and lymphatic system. In most cases toxocariosis in humans is asymptomatic, but with severe infections the migration of the larvae can cause damage that is characterized with a diverse scale of clinical signs. When the larvae cause the syndrome visceral larva migrans, the patient suffers from eosinophilia, fever, malaise, hepatomegaly and respiratory distress. Another syndrome that can be caused by the larvae of *Toxocara canis*, when they migrate to the eye, is

called ocular larva migrans. Within the eye the migrating larvae can cause a granulomatous reaction in the retina, which could result in blindness of the affected eye. (13, 16)
When a human is suspected from toxocariosis the diagnosis is mainly based on serology, not on coprological examination. This indirect diagnosis differs from dogs because the human is a paratenic host where the larvae cannot mature into their adult stage and therefor no egg shedding will occur. Nevertheless the larvae can still survive for many years, and an antigen produced by the larvae can be detected by both ELISA and Western Blot techniques. (17) As the diagnosis is indirect, caution should be taken when assuming that the seropositivity accounts for the clinical signs, other conditions could also be the cause of the symptoms.(17)
When a human is diagnosed with toxocariosis, anthelmintics are prescribed in combination with a corticosteroid therapy to minimize the inflammatory reaction that can be caused by the presence of the dead larvae.(13, 16)
Concerning the lifecycle (see figure 1) and the zoonotic potency, *Toxocara canis* is a parasite that spreads successful through dog populations and forms a big risk for the public health. Quick diagnosis with a high sensitivity of this specific parasite in dog feces is important for the reduction of this risk for both humans and dogs.(3) Therefor a simple yet precise coproscopical technique should be available in every veterinary practice.

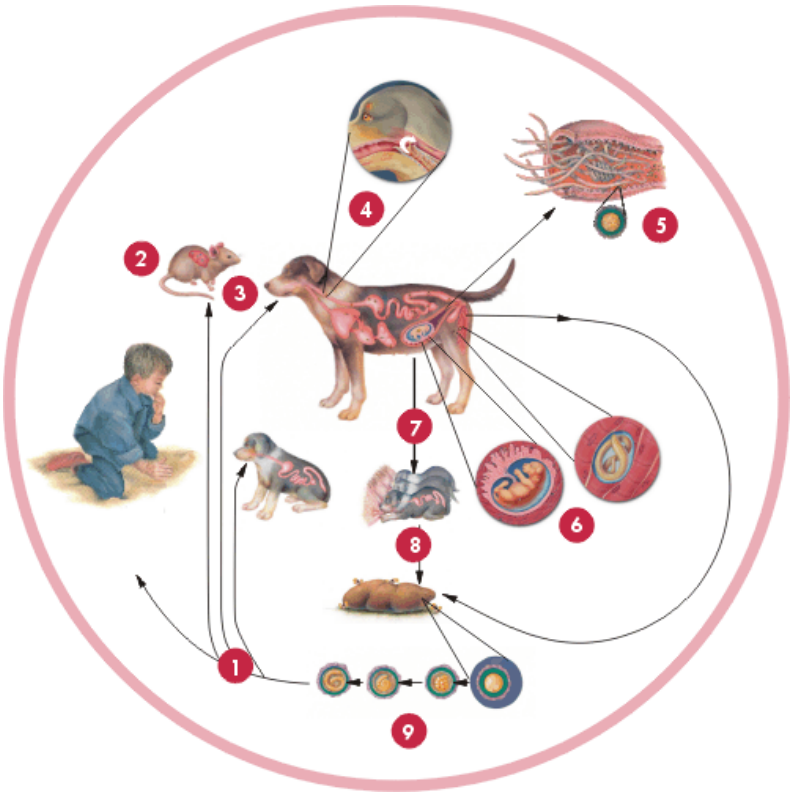

Figure 1. The lifecycle of toxocara canis.
1. Contact with infectious larvae by human, intermediate host, puppy or adult dog from the environment; 2. Development of larvae in intermediate host; 3. Ingestion of infectious larvae through predation on the intermediate host; 4. Ingestion and start of gastrointestinal migration of infectious eggs in dogs; 5. Eggs hatch in the intestine and larvae can complete a transtracheal migration after which egg shedding adults are present in the intestine or they can migrate to surrounding tissues; 6. With gravid bitches transplacental infection can occur from the 42th day of gravidity when activation of the resting larvae in the tissues occurs; 7. Puppies can get infected through drinking larvae infected milk; 8. Infectious eggs or larvae present in the puppies also become egg shedding adults after transtracheale migration; 9. In the environment the non-infectious eggs develop into infectious eggs within a few weeks.(13) Bayer®

Another reason for a high sensitive and quick coproscopical examination is that it can form an alternative for blind treatment and therefore could reduce some of the threat of anthelmintic

resistance of the parasites. This threat of anthelmintic resistance development decreases when as less as possible anthelmintic residues are contaminating the environment. In studies anthelmintic resistance is already detected with different types of gastrointestinal parasites. A study by Blagburn in the United States was conducted to test the efficiency of different types of ivermectine against *Dirofilaria immitis*, with the conclusion that not all of the available products were effective against a certain strain of the heartworm. (18)
A study by Kopp et al. about pyrantel, an anthelmintic drug, in small animal medicine revealed that at least one of the canine nematodes, the hookworm *Ancylostoma caninum*, has developed a resistance to pyrantel.(19) Pyrantel is still a popular drug to treat infections with gastrointestinal parasites, but the emerging resistance in some regions, mostly in Australia, now threatens the effectiveness of it. Besides the pyrantel resistance of *Ancylostoma caninum* in small animals, reports of pyrantel resistance in horses also indicate the emergence of pyrantel resistance. These reports are mainly in cyathostomes and also in large strongyles. In ovine medicine the drug morantel, a related anthelmintic, also suffers from the development of resistance by gastrointestinal parasites.(19-22)
All these studies indicate that anthelmintic drugs resistance is growing in different fields of anthelmintic therapy. Therefor it is very important that anthelmintic therapy is used in the most accurate manner, with as little anthelmintic residues as possible shed in the environment. To achieve that, the first important phase is to perform a coprological examination with high sensitivity and repeatability. The second phase is to interpret the results from that examination and determine whether anthelmintic therapy is indicated and if so, which drug would be the most effective for the individual patient. The third phase is to repeat the coprological examination after treatment to determine the effectiveness of the anthelmintic therapy. (19) For a good indication of effectiveness a quantitative repeatable technique is desirable.

Coproscopical examination of dog feces can be executed by the centrifugal sedimentation/flotation (CSF), passive flotation and the McMaster technique. Further explanation about these commonly used techniques follows in chapter 3.2. In coproscopical examination the CSF is frequently considered the “golden standard” to diagnose a gastrointestinal parasite infection, but this is not completely accurate, for necropsy is the most reliable way to diagnose such an infection. The CSF technique can only diagnose an infection when egg shedding occurs, and it is realistic that the latter is not always the condition. It is important to compare these more commonly used coproscopical techniques to create a clear view about the possibilities and limitations of the usage of them. Studies show that all techniques have their own specific positive and negative characteristics. Dryden et al. (2005) revealed that the centrifugal method has a significant higher fecal egg count in comparison with the passive flotation. Another result was that it did not make a significant difference for the EPG result of passive flotation whether the waiting time before microscopical examination was prolonged or not. In this study practitioners were asked why they used commercial fecal kits or simple flotation. The reasons for using such techniques were the low costs and also the small amount of time needed to execute the technique. (23) A disadvantage of the McMaster is that multiplication factors for extrapolation are needed and would give less precise EPG counts. Furthermore the McMaster has a lower sensitivity and repeatability in comparison with the centrifugal sedimentation/flotation technique.(24) Unless the disadvantages, the McMaster technique is the recommended coproscopical technique by the World Association for the Advancement of Veterinary Parasitology to examine livestock feces for eggs of gastrointestinal nematodes. (25) With such a quantitative technique the Fecal Egg Count Reduction Test (FECRT) is performed. The FECRT is the main method to detect any anthelmintic resistance in livestock.(26)

The studies about the different commonly used coproscopical techniques for diagnosis of gastro-intestinal infections indicate an interest for another coproscopical technique. This in combination with the need for a technique with regards to the threat to public health and to antihelmintic resistance, a new technique is desirable with a high sensitivity and repeatability and should also be inexpensive and easy to use. Conceivably such novel technique is recently invented in Italy. Proffesor Giuseppe Cringoli at the University of Naples developed the FLOTAC and the mini-FLOTAC techniques, two innovative techniques for the detection of gastro-intestinal parasite eggs and cysts in human and animal feces. Detailed operator information about the mini-FLOTAC technique can be found in chapter 3.2.4. There have been different studies about the FLOTAC, the technique where the mini-FLOTAC is derived from and a few studies have followed in which the mini-FLOTAC has been studied. Because of a lot of similarities between the FLOTAC and the mini-FLOTAC and the smaller amount of information that has been gathered about the mini-FLOTAC, studies of both techniques are significant for this study.

Recent studies about the mini-FLOTAC have shown that it is a unique and promising apparatus.The study by Barda et al. 2013 remarks that the mini-FLOTAC was created to combine a good sensitivity with a low cost technique. In this study of human infections with e.g. hookworms, the mini-FLOTAC was compared with the WHO advised golden standard, the Kato Katz technique. This study had positive results for the mini-FLOTAC because there was no significant difference in the EPG for both techniques. It also suggested that the mini-FLOTAC is safe in use for the operators because it is a semi- closed system.(27) The latter is also observed in the study by Cringoli et al. in which the mini-FLOTAC is mentioned as an innovative diagnostic tool which could participate in better human and veterinary diagnostics regarding gastro-intestinal parasites.(28)
Another study by Barda et al. 2013 was performed in different countries to examine human stools for intestinal parasites. The mini-FLOTAC was compared with the direct smear and the formal-ether concentration method. The mini-FLOTAC had the highest sensitivity for helminth diagnosis, but relative poor results for the diagnosis of infections by protozoa. This study also revealed that the 400x amplification with the mini-FLOTAC is not very clear, which make it more difficult to have a flawless visibility of the internal structures. With some improvement of the apparatus, it might also become useful for the diagnosis of protozoa infections. Furthermore Barda et al. agree that it is a less expensive technique because the mini-FLOTAC apparatus is re-usable after careful cleaning with the result that only the flotation solution needs to be purchased regularly. (29)
Silva et al. 2013 performed a study about fecal samples of goats containing *Eimeria* spp. with a comparison between the McMaster and the mini-FLOTAC. In this study the mini-FLOTAC was also regarded as a low-cost and sensitive technique and they suggest that this technique could be a good alternative for the McMaster technique.(30)

As mentioned above, more studies have been performed about the FLOTAC in comparison to the mini-FLOTAC and results from studies about the FLOTAC are also important for the understanding of the mini-FLOTAC. In a study by Rinaldi et al. (2010) the passive flotation, McMaster and FLOTAC for parasite egg count in sheep were compared. Passive flotation gave very low egg counts for *Moniezia expansa*, *Dicrocoelium dendriticum* and gastrointestinal strongyles. Furthermore the EPG results for passive flotation were very variable. The McMaster was, depending on the number of counting chambers, less precise than methods that do not need extrapolation (calculation of the EPG) like the passive flotation. It has a lower sensitivity, specificity and repeatability. The result of this experiment is that the best sensitivity and repeatability was obtained by the FLOTAC technique. (31)

A study by Cringoli et al. (2010) made for different parasites of veterinary and human importance also rendered the best results with the FLOTAC technique compared to the McMaster, Wisconsin, Kato-Katz and ether based concentration techniques.(32) Levecke et al (2012) also did a study about the FLOTAC, where they compared it with other coproscopical techniques, namely the McMaster and the passive flotation, with fecal samples of cattle. The FLOTAC was the technique with the highest EPG values in comparison with both the McMaster and the passive flotation.(33) An important difference between the mini-FLOTAC and the FLOTAC is that with the mini-FLOTAC less feces is used for coprological examination, therefor the sensitivity of this technique is probably slightly reduced in comparison with that from the FLOTAC technique. Another difference that could result in a reduced EPG result is that the mini-FLOTAC is executed without any centrifuging.

As previously mentioned above, there are multiple reasons for the need of an improved coproscopical examination technique. Research about the FLOTAC and mini-FLOTAC seem very optimistic about the usage of both techniques for fecal examination, but there is no report present in which the FLOTAC or the mini-FLOTAC is compared with all of the commonly used coproscopical techniques. For the examination of dog feces, the CSF technique is generally used in laboratories and the passive flotation in veterinary practices. Both techniques will be compared with the mini-FLOTAC to assess the usage of the technique in laboratories as well as in the veterinary practice. Because the CSF and the passive flotation are qualitative coproscopical techniques, a quantitative technique, the McMaster, is included in this study to compare not only the qualitative but also the quantitative qualities of the mini-FLOTAC. To broaden the comparison, for both the CSF and the passive flotation calculations for eggs per gram values were made. Furthermore, to make the comparison to the laboratory and veterinary practice as accurate as possible, the typically used flotation solutions are used. This results in the usage of 3 different flotation solutions, more information about this is below in chapter 3.2. During each experiment, all the techniques were performed 4 times with the same dog fecal sample and the averages of these results were ultimately compared by regression analysis.

Concerning the above mentioned, this study should give a good indication of the usability of the mini-FLOTAC in either laboratory or veterinary practice environments. The central question that should be answered is as follows: Is the mini-FLOTAC a suitable alternative to use in laboratories or veterinary practices for the coproscopical examination of dog feces? To answer this question, this study emphasizes on the sensitivity, repeatability, user-friendliness and the costs made for the mini-FLOTAC in relation to the CSF, passive flotation and the McMaster technique. The hypotheses that are formulated to answer the central question are:

Hypothesis$_1$:
The mini-FLOTAC yields the highest EPG values compared to the CSF, passive flotation and the McMaster technique

Hypothesis$_2$:
The mini-FLOTAC has the best repeatability compared to the CSF, passive flotation and the McMaster technique.

3. Materials and Methods

3.1. Materials

The material used in this experiment was dog feces that was send by mail to the faculty of veterinary medicine, Utrecht University. Participating dogs, around 300 to 500 dogs older than 6 months of age, were from both sexes and different breeds. These dogs were not treated for enteric parasites, until an enteric parasitical infection was diagnosed at Utrecht University. The different specimens used were first examined for an ongoing experiment taking place at Utrecht University, a coprological microscopic examination to diagnose possible enteric parasitical infections. Monthly, the participating dog owners had to send an amount of feces to the Utrecht University, where it was examined with the centrifugal sedimentation/ flotation technique.
Regarding the outcome of the first examination some of the fecal samples were used in this experiment to compare the different diagnostic techniques. The most important zoonotic parasite in this experiment has been *Toxocara canis*, therefor most of the fecal samples used were determined positive for that specific parasite. Furthermore feces containing strongyle type of eggs, *Giardia intestinalis, Trichuris* sp., *Capillaria* sp. and *Cyniclomyces guttulatus* were examined.

3.2. Methods

In this study 4 techniques of coprological examination are utilized, all the techniques rely on the differences of the specific gravity of the eggs, fecal debris and the flotation solution that is used. These 4 different techniques are the centrifugal sedimentation/ flotation, McMaster, passive flotation and the mini- FLOTAC technique and are described in more detail below.

3.2.1. Centrifugal sedimentation flotation technique

For this technique 3 gram of feces and 55 ml of water were put into a pestle and with a mortar a suspension was produced. This suspension was ran through a sieve, separating the fluid from large solid particles. After swirving the collecting tube the remaining fluid was poured into a test tube, filling the test tube with 5,5 ml suspension. After that it was centrifuged for 2 minutes with a rotation speed of 3000 rotations per minute (RPM) in a Hettich Rotofix 32® centrifuge. The test tube was taken out of the centrifuge and the supernatant was decanted, leaving the sediment in the test tube. 1 ml of flotation solution was poured into the test tube, where the sediment merged with it on the vortexer until a homogenous suspension was formed. The flotation solution used with this technique was a sugar solution with a specific gravity of 1.27 - 1.30 g/cm^3, see attachments. The test tube was filled with more saturated sugar solution until a slight meniscus appeared. A coverslip was applied on top and the test tube was again centrifuged with 3000 RPM after which the coverslip was perpendicular removed and put on to a slide. The slide was examined by light microscopy with an amplification of 100x, 250x and 400x.

Analytic sensitivity calculation:
58 ml of suspension contained 3 gram of feces, 5,5 ml of suspension was put into the test tube, containing 0,28 gram of feces. Assuming all eggs in this 0,28 gram of feces floated to the surface, one egg found is equivalent to 3,5 eggs per gram of feces. If no eggs were found, the feces contained 0 < 3,5 EPG.

EPG_{CSF}= amount of eggs found x 3,5

3.2.2. McMaster technique
For this technique 3 gram of feces and 42 ml of flotation solution were combined in a pestle and with a mortar a suspension was formed. The flotation solution used with this technique was a saturated salt solution with a specific gravity of 1.18 - 1.20 g/cm³, see attachments. The fluid was separated from the large solid particles by a sieve and it was collected in a container. The container was swerved, after which a small amount was pipetted into both the counting chambers of the McMaster. The McMaster was left in a horizontal position for at least 10 minutes, after which it was ready to be examined by light microcopy at an amplification of 100x.

Analytic sensitivity calculation:
45 ml of suspension contained 3 gram of feces, regarding both filling chambers, two times 0,15 ml of suspension was examined containing two times of 0,01 gram feces. Assuming all eggs in this 0,02 gram of feces floated to the surface, one egg found is equivalent to 50 eggs per gram of feces. If no eggs were found, the feces contained 0 < 100 or 0< 50 EPG when respectively one or both counting chambers were examined.

$EPG_{McMaster}$= amount of eggs found x 50

3.2.3. Passive flotation technique
For this technique 1 gram of feces was put into the container of the Parasieten diagnosesysteem van Janssen®, which was filled for one third with flotation solution. The flotation solution used with this technique was a sodium nitrate solution with a specific gravity of 1.20 g/cm³, see attachments. A suspension was formed using a spatula, after which the upper part, the sieve, was placed firmly onto the container. More flotation solution was poured into the container, until a slight meniscus was formed on which a coverslip was placed. The container was left in a vertical position for at least 10 minutes, after which the coverslip was placed on a slide where after it was examined by light microscopy at an amplification of 100x, 250x and 400x.

Analytic sensitivity calculation:
The Passive flotation technique contained 1 gram of feces and 22,2 ml of suspension when it was completely filled with a bulging disc at the top. Therefor 23,2 ml of suspension contained 1 gram of feces. Assuming all eggs from this gram of feces floated to the surface, the counted eggs are equivalent to the amount of eggs per gram of feces.

$EPG_{Ovassay\ Plus}$= amount of eggs found x 1

3.2.4. Mini- FLOTAC technique
Preparing the mini- FLOTAC for use; first the two discs were placed on top of each other and the key was attached. The key was turned for 90° degrees whereby the filling chambers were in the position ready to be filled with suspension.

For this technique 2 grams of feces and 38 ml of the flotation solution were put into a pestle and with a mortar a suspension was produced. The flotation solution used with this technique was a sugar solution with a specific gravity of 1.30 g/cm³, see attachments. The created suspension was ran through a sieve, after which the remaining fluid was collected in a container.

The container was swerved a couple of times after which immediately with a pipette an amount of the suspension was taken from the container. The mini- FLOTAC was held at a slope and the first counting chamber was filled with the suspension until a slight mensicus was formed. For the second counting chamber the procedure was repeated, from the swerving of the container until the the bulging disk was formed. After filling both counting chambers the bulging discs disappeared after a minute, therefore at that moment a refill was performed by adding 2 drops of suspension to both filling chambers until new slight meniscuses were formed. Then the mini- FLOTAC was left for at least 10 minutes in a horizontal position.

When the 10 minutes were passed, equal pressure was applied on the key of the mini-FLOTAC and it was turned for 90° degrees. Hereby the upper part of the counting chambers was separated from the bases and both top layers of the counting chambers were examined by light microscopy at a 100x or 400x amplification. Before the mini-FLOTAC could be placed underneath the microscope, an adaptor had to be placed.

Analytic sensitivity calculation:
40 ml of suspension contained 2 gram of feces, one filling chamber contained 1 ml of suspension, containing 0,05 gram of feces. Two filling chambers contained 0,10 gram of feces together. Assuming all eggs in this 0,10 gram of feces floated to the surface, one egg found is equivalent to 10 eggs per gram of feces. If no eggs were found, the feces contained 0 <10 EPG.

$EPG_{Mini\text{-}FLOTAC}$= amount of eggs found x 10

4. Results

During this study different criteria of the 4 coproscopical examination techniques were observed. These facets include the time needed to prepare and examine the samples, but also how much feces was needed per technique. These criteria and other aspects like user-friendliness and the possibilities of the diverse magnifications underneath the light microscope are presented in table 2.

Criteria	**CSF**	**McMaster**	**Passive Flotation**	**Mini-FLOTAC**
Time needed to prepare slide for microscopical examination	±11 minutes, many steps needed to prepare slide	±5 minutes, few steps are needed to prepare McMaster for examination, time excludes waiting time of 10 minutes	±3 minutes, few steps are needed to prepare slide, time excludes waiting time of 10 minutes	±5 minutes, few steps are needed to prepare mini-FLOTAC for examination, time excludes waiting time of 10 minutes
Amount of feces needed	3 grams	3 grams	1 gram	2 grams
Time needed for microscopical examination	±8 minutes	±4 minutes	±8 minutes	±5 minutes
User-friendliness	Because of the many steps less user-friendly	Quick and easy	Quick and easy, but a higher chance of spilling when removing coverslip when the regular sized coverslips are used instead of the larger ones	Quick and easy
Importance of securing waiting time in advance of microscopical examination	-	+	+	+
Chance of impaired view	Not big, sometimes there is a thick layer of particles covering the view	Big, if there are many large, dark particles in the counting chamber, recognition of eggs is more difficult	Not big, usually there are less particles compared to the CSF technique	Intermediate, abrasions or spilled sucrose solution droplets on top of the counting chamber impair view, and a chance of air bubble creation in the counting chamber during filling and turning
Life span of apparatus	Long life span of centrifuge	Long life span of McMaster	Short life span of container and top	Quite long life span of mini-FLOTAC
Expensiveness	Quite expensive	Not expensive	Not expensive	Not expensive
View at 40x	+++	+++	+++	+++
View at 100x	+++	+++	+++	+++
View at 400x	+++	-	+++	+

Table 2. The different coproscopical techniques are compared using specific criteria.

The different coproscopical examination techniques displayed a diverse scale of possibilities to diagnose gastro-intestinal parasites. The CSF was the only technique that could detect all of the gastro-intestinal parasites used in this study. To ensure the latter, 4 times feces that was declared gastro-intestinal parasite free by the CSF had been examined by all 4 techniques,

with every technique completed 4 times. Table 3 displays the techniques and the gastro-intestinal parasites that they can detect.

Type of eggs/ oocystes	**CSF**	**McMaster**	**Passive flotation**	**Mini-FLOTAC**
Toxocara canis and *cati*	+	+	+	+
Strongyle type of eggs	+	+	+	+
Giardia intestinalis	+	-	-	-
Cyniclomyces guttulatus	+	+/-	+	+/-
Isospora	+	+	+	+

Table 3. Microscopic visibility (+), no visibility (-) and variable visibility (+/-) of eggs and oocystes in dog feces after diagnosis of the specific enteric parasites with the CSF.

Another aspect that was examined during this study was if all the eggs were collected and examined during the CSF examination. After the normal CSF procedure was completed, the test tube was re-filled with the sucrose flotation solution until a bulging disc was formed. Then again a coverslip was put on the testtube and the centrifugation was repeated. These results show that again some *Toxocara canis* eggs were collected the second time the same test tube was refilled and centrifugated, see figure 2. The average of the eggs (EPG) that were collected the second time was 14,3% of the first collection. In this study also some fecal samples were examined with all the techniques whilst they were first labeled negative with the CSF. The results from those fecal samples were negative for all of the 4 techniques, performed 4 times.

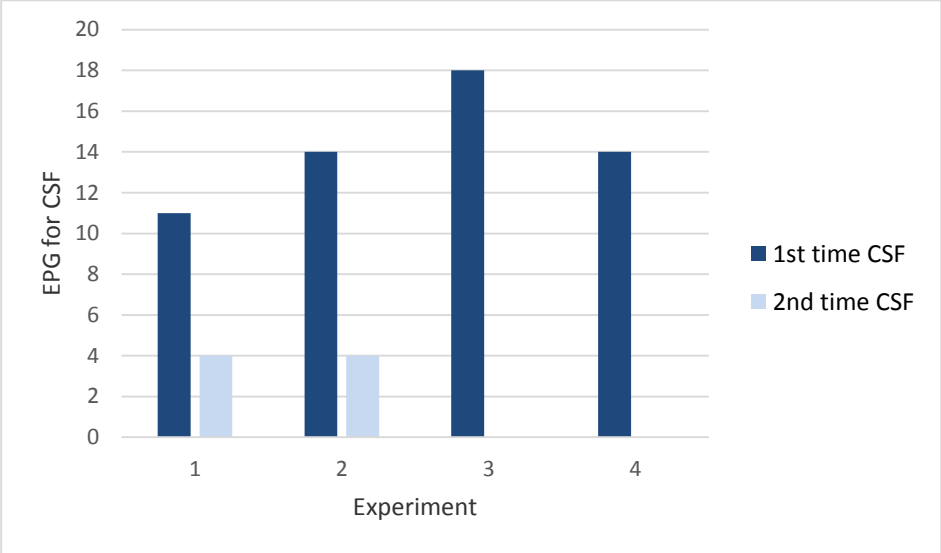

Figure 2. *Toxocara canis* positive samples that were centrifuged twice

This study also examined the influence of time on the turning of the mini-FLOTAC counting chamber from the bases. As the mini-FLOTAC does not have a centrifugal step in its procedure, time for the eggs to float to the surface of the apparatus before the counting chambers are turned from the bases seemed essential. This was examined by examination of one feces sample with different times before the top was turned from the bases. This experiment was performed 4 times for every different waiting time. Figure 3 show the results of this experiment.

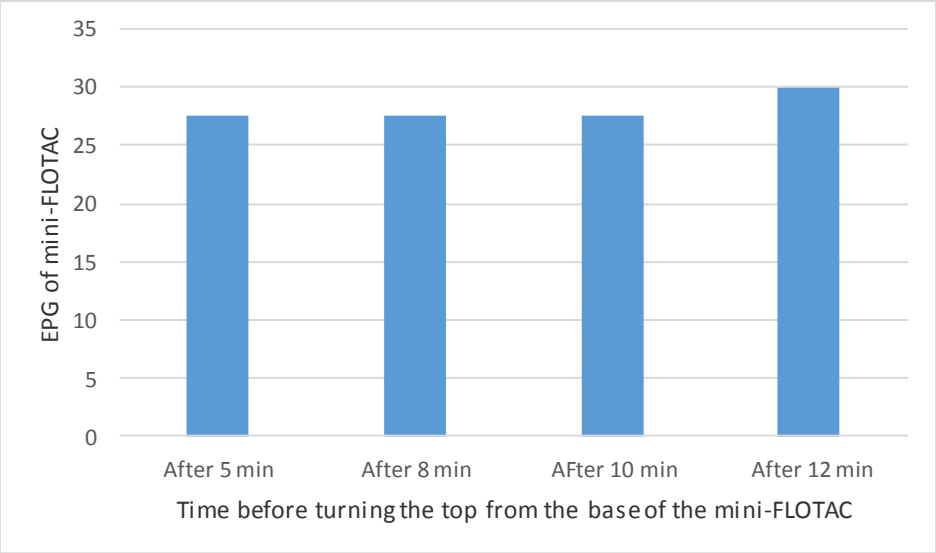

Figure 3. Different waiting times before turning the top of the mini-FLOTAC show different results of *Toxocara canis* egg counting

The mean results, of the 4 examinations per technique, of the *Toxocara canis* positive fecal samples were integrated into different figures where the CSF technique was compared to every individual technique. The gradients of the comparisons with the McMaster and the mini-FLOTAC were close to 1 and both had 0 in their 95% confidence interval. The gradient of the comparison with the passive flotation results in a gradient close to 0 with the 0 in the 95 confidence interval. See figure 4 - 6. Other results from these examinations show that the passive flotation has a very low EPG value compared to the EPG from the CSF technique. The EPG values of the CSF, McMaster and mini-FLOTAC technique were far more similar. Furthermore, the McMaster showed in 18,3% of the cases a false negative diagnosis, followed by the passive flotation with 6,7%, the mini-FLOTAC 3,3% and with the CSF 0% of false negative diagnoses.

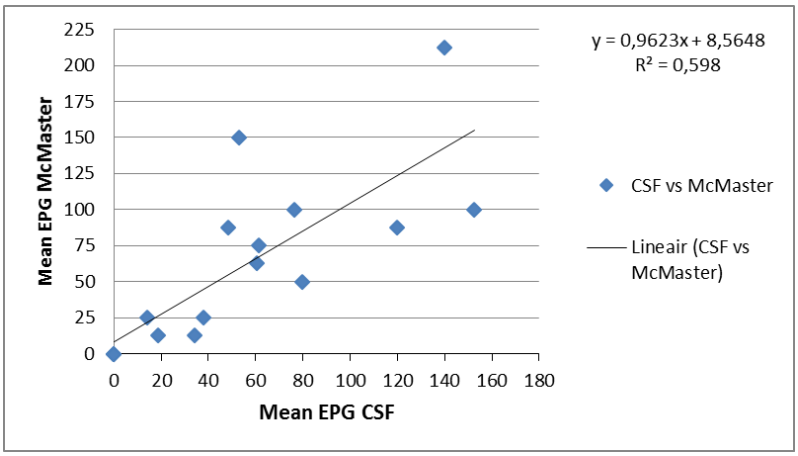

Figure 4. Mean *T. canis* EPG CSF in relation to the mean EPG of the McMaster

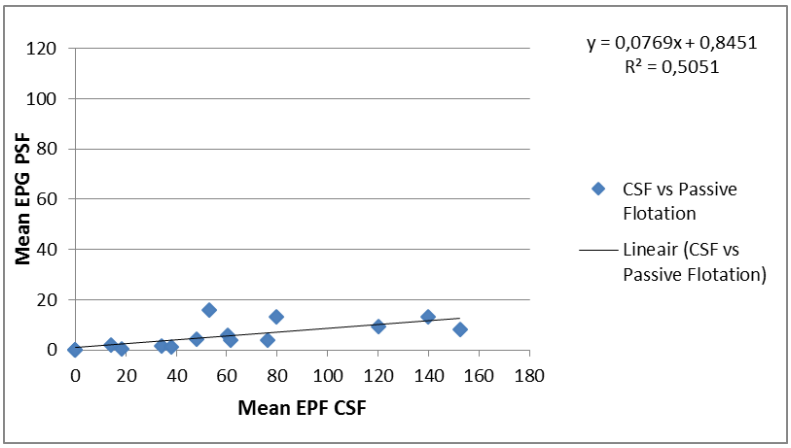

Figure 5. Mean *T. canis* EPG CSF in relation to the mean EPG of the passive flotation

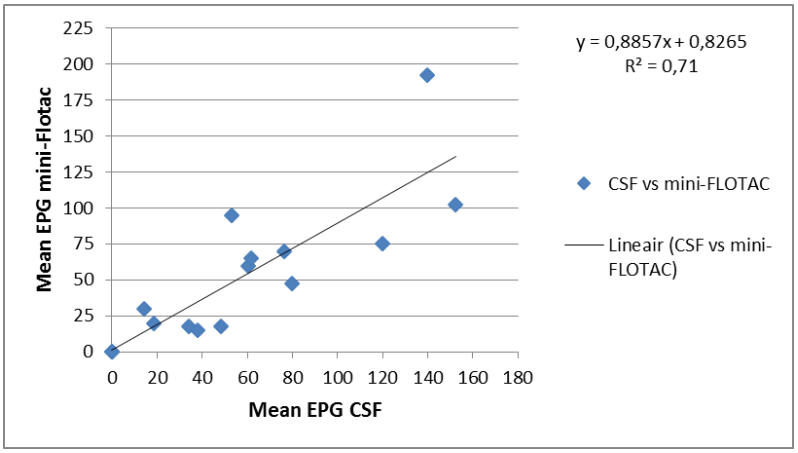

Figure 6. Mean *T. canis* EPG CSF in relation to the mean EPG of the mini-FLOTAC

The repeatability of the 4 different techniques is showed in figure 7 – 10, with every technique the means of the observations are related to the individual observations regarding every fecal sample examined. The R^2 value is an indication for the acceptance of the repeatability of the technique. As the different techniques are quite rough, a very high R^2 (e.g. around 1,0) is not achievable. When the R^2 is above 0,81 the repeatability is acceptable. Only the CSF (R^2= 0,88) and mini-FLOTAC technique (R^2= 0,87) have an acceptable repeatability. The repeatability of the Passive flotation (R^2= 0,76) and the McMaster technique (R^2= 0,64) are not acceptable.

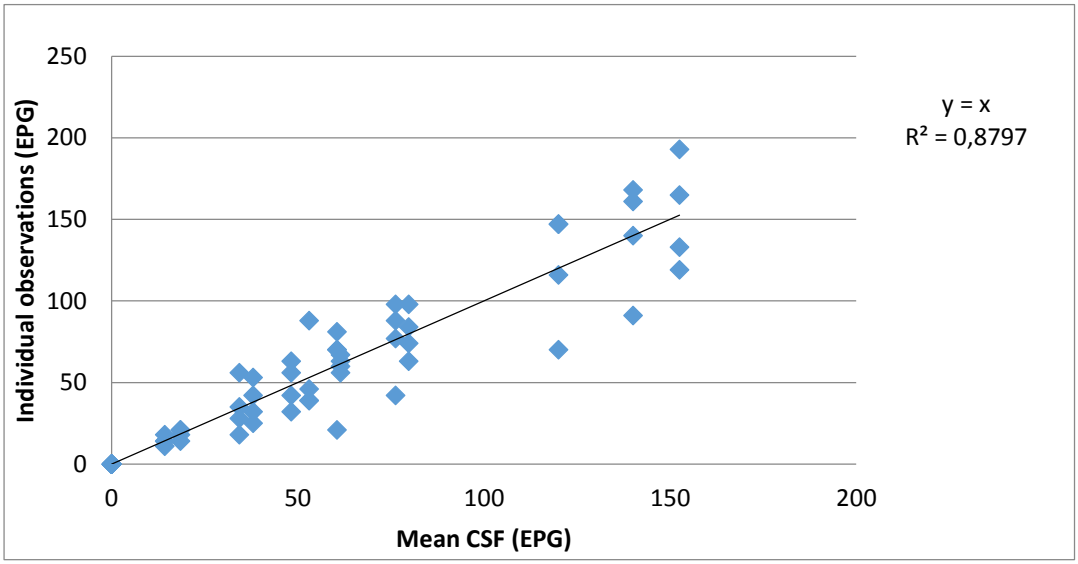

Figure 7. The average of the observations in relation to the individual observations with the CSF technique.

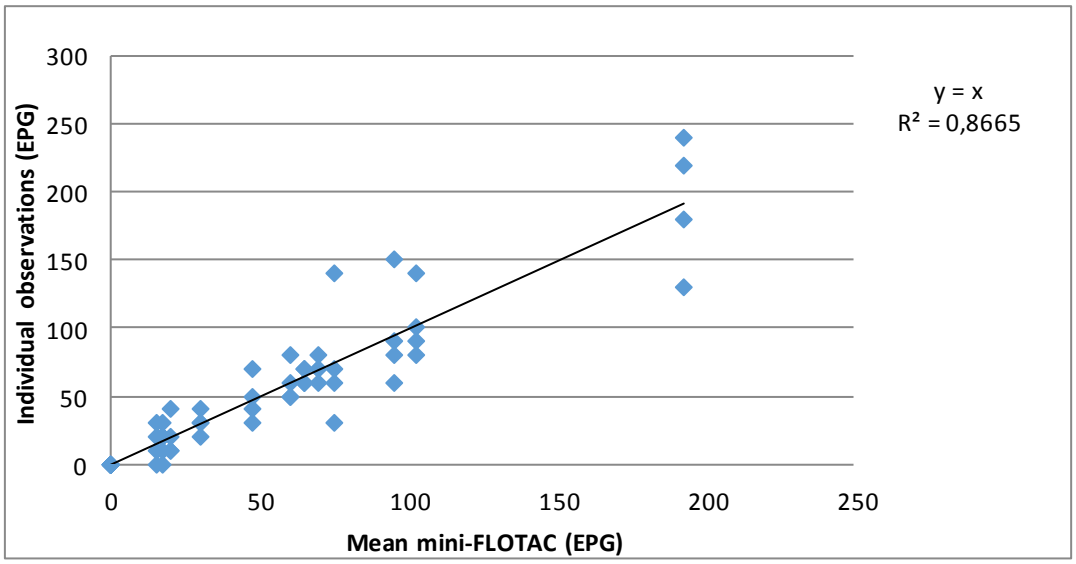

Figure 8. The average of the observations in relation to the individual observations with the mini-FLOTAC technique.

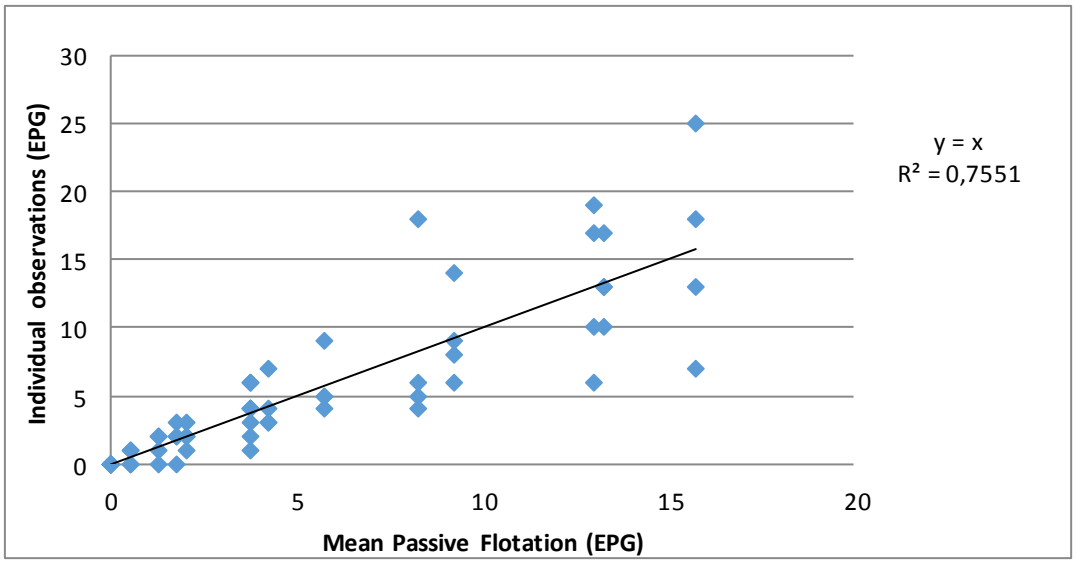

Figure 9. The average of the observations in relation to the individual observations with the PF technique.

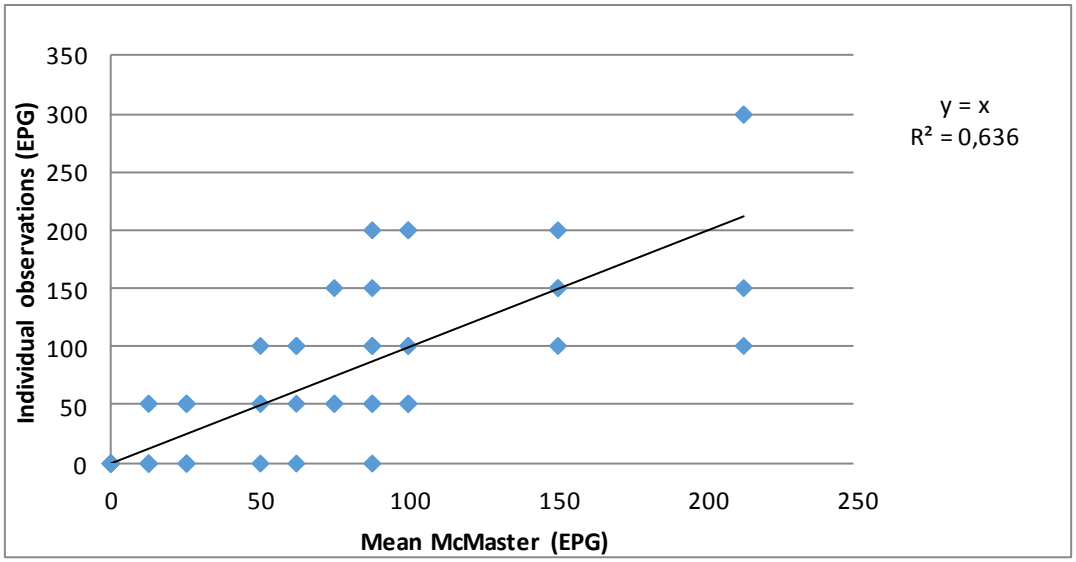

Figure 10. The average of the observations in relation to the individual observations with the McMaster technique.

In table 4 the results of the mean EPG values of *Toxocara canis* of the different techniques are compared with each other with each individual fecal example that had been examined. This figure shows that the McMaster technique (46,1%) rendered the highest mean EPG values followed by the CSF technique (38,5%). The mini-FLOTAC technique never has the highest mean EPG value, but usually is only a small percentage below the highest mean EPG value. The passive flotation is the technique that constantly rendered the lowest mean EPG value.

Mean EPG	**CSF**	**McMaster**	**Passive flotation**	**Mini-FLOTAC**
Sample 1	0	0	0	0
Sample 2	0	0	0	0
Sample 3	0	0	0	0
Sample 4	**34**	13	2	18
Sample 5	76	**100**	4	70
Sample 6	53	**150**	16	95
Sample 7	61	**63**	6	60
Sample 8	**80**	50	13	48
Sample 9	14	25	2	**30**
Sample 10	**120**	88	9	75
Sample 11	62	**75**	4	65
Sample 12	19	13	1	**20**
Sample 13	48	**88**	4	18
Sample 14	**38**	25	1	15
Sample 15	**152**	100	8	103
Sample 16	140	**213**	13	193

Table 4. The means of the EPGs of the different techniques for every fecal sample examined for *Toxocara canis*

5. Discussion

In this study the CSF technique was selected as a golden standard to compare with the other coproscopical techniques. As mentioned earlier, the CSF technique (as well as all other coproscopical techniques) can only detect a gastro-intestinal infection when egg shedding occurs. Besides that, results in this study show that not all the eggs were collected every time when the CSF technique is applied, which suggests that extreme light egg shedding could be missed by the CSF. This combined is an indication that the results from the CSF technique are not 100% reliable to make the diagnosis of a gastro-intestinal infection. For the reason that probably not all eggs that were shed were calculated, all conclusions about the different techniques made in this study are made in relation to the data gathered with the CSF technique. As the experiment where the CSF technique negative entitled fecal samples were examined by all of the techniques resulted negative, it makes it more plausible that the CSF is sensitive enough to be titled the golden standard in this study. In a study by Alarcon et al. the CSF was compared to the Kato-Katz technique, the latter is the advised technique in human coproscopical examination. This study revealed that the CSF had such good results that that technique could be used in human coproscopical examination. (34) Undoubtedly idea liter, spiked fecal samples were used for all of the techniques to make the best comparison, but because of the high work load it was not accomplishable in this study. Therefore all the assumptions made regarding the sensitivity are in relation to the EPG of the CSF. Another point of interest is the fact that in this study no definite difference was made between *Toxocara canis* and *Toxocara cati* eggs. This could have resulted in *T. cati* eggs that were assumed to be *T. canis* eggs. That made it more difficult to make any assumptions regarding the specifity of the different tests.

The CSF technique is a mainly used qualitative technique to examine feces, not a quantitative technique. To make it possible to compare the results from all the techniques, an EPG calculation was needed for all techniques. The EPG calculation of the CSF technique as described above (3.2.1.) assumed that all eggs of the homogenous fecal suspension in the test tube floated to the surface and that all eggs of the 0,28 gram of feces were counted. This approach to calculate the EPG does not include the fact that not all eggs are examined because some of the eggs did not stick to the coverslip because they were not high enough in the test tube to get in contact with the coverslip or did not stick tight enough to the coverslip during the removal from the test tube. This was displayed in the experiment performed to discover how many eggs did not get onto the coverslip. The examination of the second coverslip revealed that sometimes not all eggs were examined the first time, but that it are only a few eggs that remain in the test tube. Therefor it is assumable that the EPG calculation of the CSF gives a good enough representation of the real EPG value of the feces.

The McMaster technique is used as a quantitative technique to examine feces. The EPG calculation as described above (3.2.2.) did not include the eggs and oöcysts that did not float to the surface in the 10 minutes of waiting time. It is possible that some larger particles prevented them to float quickly enough to the surface. But if we compare the EPG of the McMaster with the EPG of the CSF technique, results are quite similar and therefor it is assumable that the calculation is close to reality. The low repeatability of the McMaster is probably explicable by the fact that this technique needed the large extrapolation of 50x to calculate the EPG value.

The passive flotation technique is also a qualitative technique to examine feces and an EPG calculation had to be created to be able to compare the four different techniques with each

other. This calculation as described above (3.2.3.) does not include the fact that eggs and oöcysts could get stuck underneath the sieve or that they stick to particles. The sieve prevents large particles to float through it, but could then also form a barrier of particles impermeable for the eggs and oöcysts. Regarding the low passive flotation technique EPG results compared with the other three higher EPG's, it is quite evident that many eggs and oöcysts did not float or stick to the coverslip. Another possible imperfection of this technique is the likelihood of spilling some of the flotation solution including the eggs when the container is filled until a slight meniscus is formed or when the coverslip was taken from the container. Therefor the EPG for the passive flotation gives an underestimation of the real EPG and could result in false negative results for gastro-intestinal infections.

The mini-FLOTAC EPG calculation as described above (3.2.4.) is based on the assumption that all eggs in the apparatus float to the surface in 10 minutes. A result from an experiment with examinations after different waiting times is that there are more eggs found after 12 minutes compared to a waiting time of 10 minutes. This shows that the calculation might be a small underestimation of the real EPG value. Furthermore, the re-fill of the mini-FLOTAC might have an influence on the EPG because of the loss of fluid en thereby possibly also eggs from the counting chambers into the small space between the upper and bottom part. To minimize this effect on the EPG value, it was made sure that the counting chambers were completely filled at the moment of turning the apparatus. Still the refill could result in a little overestimation of the EPG, but this is difference would be so diminutive that this is not processed in the EPG calculation for the mini-FLOTAC. But in comparison to the EPG of the CSF technique, it still seems that the used EPG calculation gave a good reflection of the real EPG value.

The fecal samples used in this experiment were send by mail to the Univerity of Utrecht, wrapped in a plastic bag which was put in a plastic container in a seal bag. During the transport to the University of Utrecht the feces stayed a couple of days on room temperature, which could have had an influence on the eggs, oöcysts and parasites that were possibly present in the feces. Once the mail was delivered to the University of Utrecht, the feces was stored in a fridge after the first examination for the ongoing experiment. Depending on the number of fecal samples that contained a gastro-intestinal parasitical infection of interest, some of the feces was used immediately and some was stored for a couple of days before being used in this experiment. Giardia can stick to different particles which could made it more difficult to diagnose. The influence of the room temperature on the feces during the process of transport to the University could result in the further development or hatching of nematode eggs. When hookworm eggs are about 24 hours in humid and warm conditions, they can develop in larvae. This could make it more difficult to identify and count the exact amount of nematode and hookworm eggs present in the fecal sample.(8)

The fecal samples used for this experiment were taken directly from the feces presented in the plastic bag. No kneading of the feces preceded, therefor it must be taken in regard that it is possible that some of the eggs, like *Taenia* spp., did not have a homogenous concentration in the feces. Especially the eggs and oöcysts that were passed into the feces through coprophagy have a high chance of having very different concentrations in the feces. Therefor it cannot be excluded that slightly varying results in EPG by the four techniques result from a non-homogenous spread of eggs and oöcysts. Besides that, the mini-FLOTAC had regularly smaller or bigger air-bubbles in the counting chambers, which could have resulted in lower EPG values compared to the others. Despite this flaw, the apparatus still gave quite similar EPG values in comparison with the CSF and the McMaster technique.

In this study only a certain assembly of eggs and oocysts of gastro-intestinal parasites was used to compare the different techniques with each other. We know from these eggs and oocysts that they would be detected by the CSF technique. Furthermore, mainly Toxocara canis in this study was used because of the high zoonotic behavior, but to make a more accurate comparison to the veterinary practice, a broader range of canine gastrointestinal parasites should be used. It is important to recognize that there are many other gastro-intestinal parasites that can infect dogs and that there are also other procedures for the diagnosing of the different gastro-intestinal parasites. *Taenia* spp. and *Echinococcus* spp. are for example 2 important and wide-spread gastro-intestinal parasites that also have a zoonotic character that cannot be diagnosed with the 4 techniques used in this study. (35, 36) For that reason, when there is a suspicion of a certain gastro-intestinal parasitic infection, the most appropriate diagnostic test should be used.

In this study different flotation solutions and different coproscopical examination techniques were used to make the most accurate comparison to both the veterinary practice and to the laboratory. The result of these differences is that not all of the gastro-intestinal parasites can always be detected. For example, *Trichuris* spp. and *Capillaria* spp. need a higher specific gravity than the saturated salt solution with a specific gravity of 1.18 - 1.20 g/cm³ that was used with the McMaster technique or the sodium nitrate solution with a specific gravity of 1.20 g/cm³ used with the passive flotation. Another example is *Giardia intestinalis* that was only detected by the CSF technique. This was probably due to the fact that the centrifugation with the CSF technique made it possible to separate the sticky protozoa from particles in the test tube, but also the possibility to have a clear view of it with the 400x magnification. With some microscopes it is possible to examine the mini-FLOTAC with the 400x magnification, but because of the less transparent base part of the apparatus the view is a bit vague and therefor the smaller particles, like *Giardia intestinali*s, are less detectable. It is practical impossible to examine the McMaster with the 400x amplification which also results into a smaller assortment of gastro-intestinal parasites that can be detected. The device of the passive flotation is probably the most hindering problem of that technique, as it is possible to examine the slide exactly like the CSF technique slide. The sieve of the passive flotation technique impedes many of the eggs and oocysts to float to the surface where it could stick to the coverslip, because the s.g. of sodium nitrate solution of 1.20 g/cm³ was high enough to render most of the eggs and oocysts, obviously not for *Trichuris* spp. and *Capillaria* eggs. Besides that it is worth noting that the McMaster and the mini-FLOTAC technique both did not have any centrifuging but still rendered about the same EPG's in comparison with the CSF technique.

The study by Barda et al. in 2013 has similar results for the mini-FLOTAC compared to this study. In both studies the technique has a good results for diagnosing gastro-intestinal parasites and poorer results with diagnosing protozoa, for the same reason, namely the blurred view with the 400x amplification. The durability of the apparatus was respectable in both studies and therefor the technique is not very expensive. The suggestion in the study by Silva et al. in 2013 that the mini-FLOTAC technique could be a good alternative for the McMaster is justified in this study as the same gastro-intestinal parasites were rendered, it has a higher egg detection limit, high user-friendliness and the mini-FLOTAC is a quick and cheap technique.

The study by Rinaldi et al. in 2010 had similar results for the passive flotation which gave very low and variable egg counts. In the same study the McMaster was thought to be less

precise then the methods that did not need extrapolation, but in this study that was not very obvious as the EPG did not differ much from the EPG of the CSF and mini-FLOTAC technique. This difference might be due to the fact that every technique was performed 4 times and the average of the 4 results was used. Although the EPG of the McMaster did not differ much from the other 2, it is clear that with a detection limit of 50 EPG a bigger chance of a false negative diagnosis occurs. As many studies about the FLOTAC compared with other coproscopical examination techniques had very positive results for the FLOTAC, the mini-FLOTAC also succeeded on many aspects of coproscopical examination.

As there is no research published where dog feces is examined with the 4 different coproscopical examination techniques, further research is needed. It is advisable that a more diverse assortment of gastro-intestinal parasites are examined and that for each experiment to examine a specific parasite more fecal samples are used. The aim of this study was to examine different gastro-intestinal parasites, but because we were completely dependent on the fecal samples that were send to us, it was not possible to do so. The fecal samples we examined with for instance the strongyle type of eggs did show similar results like the experiments on *Toxocara canis*. As in this study the mini-FLOTAC was only used with a sugar-solution, it is recommended to try different flotation solutions to examine whether this makes a big difference in the results. Recommendations regarding the mini-FLOTAC include the alteration of the base of the apparatus. As this base is not completely see through, it hinders the examination at the 400x amplification. Possibly when the base is transparent the amplification is better to use and could result in the likelihood to diagnose *Giardia intestinalis*. Another recommendation concerns the quantitative property of the mini-FLOTAC. In pet animals a zero tolerance policy is used, whereby it does not matter if a dog sheds enormous or just little amounts of eggs or oocysts. But possibly in the future the FECRT that is used in livestock could also become important in pet animals as anthelmintic resistance can occur in every field veterinary medicine. Still, at this moment the quantitative property is probably more appreciated in livestock fecal examination where the amount of egg shedding is far more important. In this study the EPG values were quite low compared to the values that can be found in e.g. ovine feces. A cost-benefit analysis for the different techniques might also be interesting. For that reason more research should be performed to compare the mini-FLOTAC with the commonly used McMaster technique.

6. Conclusion

The CSF technique had the highest sensitivity and specificity because it had the clearest view with the 400x amplification in which all of the parasites could be identified with a detection limit of only 3,5 EPG. With the CSF there were no false negative diagnoses made and this technique had the highest repeatability. The downside of the CSF technique is that is needs much time to be performed and there are many steps in the process where mistakes could be made like contemning of the surroundings. Furthermore a centrifuge is needed, an expensive apparatus which also needs lifelong maintenance.

The mini-FLOTAC showed a lower sensitivity and specificity compared with the CSF technique for it could not detect all of the gastro-intestinal parasites that the CSF technique did and had a detection limit of 10 EPG. But the detection limit from the mini-FLOTAC illustrated no significant difference with the detection limit of the CSF technique. The mini-FLOTAC followed the CSF with the amount of false negative diagnoses, with 3,3%. The repeatability of the mini-FLOTAC was almost exactly the same as for the CSF technique. Beneficially, this technique is very quick in usage, user-friendly and is inexpensive. Although the 400x amplification is not completely clear, with the alteration mentioned in chapter 5. it could become clearer. Unfortunately *Giardia intestinalis* is not detectable with this technique, but this could be resolved by using the SNAP *Giardia* test for the diagnosis of the protozoa, but with this technique there is no indication of the amount of *Giardia* present. The latter is also advisable for that the protozoa sheds intermittently whereby the chance of false negative results are more likely for all the coproscopical examination techniques used. The down side of the mini-FLOTAC is that when the mini-FLOTAC upper part is turned too quickly, small underestimations of the EPG could be made, but the results don't show a big difference between the 5 minute or 12 minute waiting time. This essential step could form a weakness for the usage in the veterinary practice when they don't respect the 5 minutes waiting time. The result is that when a veterinary practitioner decides to work with the apparatus, it should respect this characteristic.

The McMaster technique renders similar results compared to the mini-FLOTAC regarding the different gastro-intestinal parasites it can detect, the user-friendliness and the costs.
The detection limit from the McMaster illustrated no significant difference with the detection limit of the CSF technique. Disadvantages of the McMaster technique are that it is not possible to examine it with the 400x amplification and that the detection limit is 50 EPG, which resulted in the highest percentage of false negative diagnoses, namely 18,3%. This technique also had the lowest (unacceptable) repeatability value compared to the other techniques. Therefore the sensitivity and specificity of the McMaster are lower compared to the mini-FLOTAC and consequently also the CSF.

The passive flotation technique has the lowest results for almost all of the criteria. The detection limit of the technique should be 1 EPG but this study expressed something completely different. The detection limit for the passive flotation technique is significant lower compared to the CSF technique and this criteria should be very careful considered whenever a veterinary practitioner applies this technique. Although it is a cheap and quick technique whereby the slide can be examined with the 400x amplification, there is a chance of 6,7% that a false negative diagnosis is made. The repeatability of this technique was like the McMaster technique very low. Furthermore there was a high chance of spilling contaminated fluid in the surroundings with this technique. Despite the amplification possibilities it is also not possible to detect *Giardia intestinalis*. The passive flotation technique should not be

readily advised to be used in practice. Whenever a veterinary practitioner chooses to use this technique, awareness of the flaws should be needed to make the best decisions regarding gastro-intestinal parasitical infections.

Regarding the hypotheses in this study;

Hypothese$_1$:
The mini-FLOTAC has the highest EPG values compared to the CSF, passive flotation and the McMaster technique

Hypothese$_2$:
The mini-FLOTAC has the highest repeatability compared to the CSF, passive flotation and the McMaster technique

Both of the hypotheses have to be rejected, but it is worth noting that the mean EPG values of the mini-FLOTAC do not significantly differ from that of the CSF technique and that the repeatability of the mini-FLOTAC only differed 0,01 from that of the CSF technique. As the CSF technique was chosen in this study as the "golden standard" it is quite a big achievement for the mini-FLOTAC to render such results. The central question of this study was "Is the mini-FLOTAC a suitable alternative to use in laboratories or veterinary practices for the coproscopical examination of dog feces?" The answer is that the mini-FLOTAC could indeed be a suitable alternative to in the veterinary practice, as it performed far better in comparison with the nowadays usually used passive flotation technique. As regards to the laboratory, the CSF technique is probably the best method to use if the costs for a centrifuge can be made and the laboratory technicians have enough time to spend per fecal sample. Concerning a low budget laboratory with an under manned staff, the mini-FLOTAC could be a very good alternative for the CSF technique. When a laboratory of veterinary practitioner selects the mini-FLOTAC technique to work with, it should always be aware of the waiting time before turning the top from the bottom and also the fact that this technique can't diagnose *Giardia intestinalis* compared to the CSF technique.

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9. Attachement

Below the preparation of the different flotation solutions are presented. After the preparation of these flotation solutions they should always be checked on their specific gravidity before usage.

Flotation Solutions

Saturated salt solution (s.g. 1,18- 1,20 g/cm³)
350 grams NaCL
1,000 ml tap water

Sugar solution (s.g. 1,27 – 1,30 g/cm³)
1,000 grams sugar
640 ml tap water

Sodium nitrate solution (s.g. 1,20 g/cm³)
338 grams $NaNO_3$
1,000 ml tap water