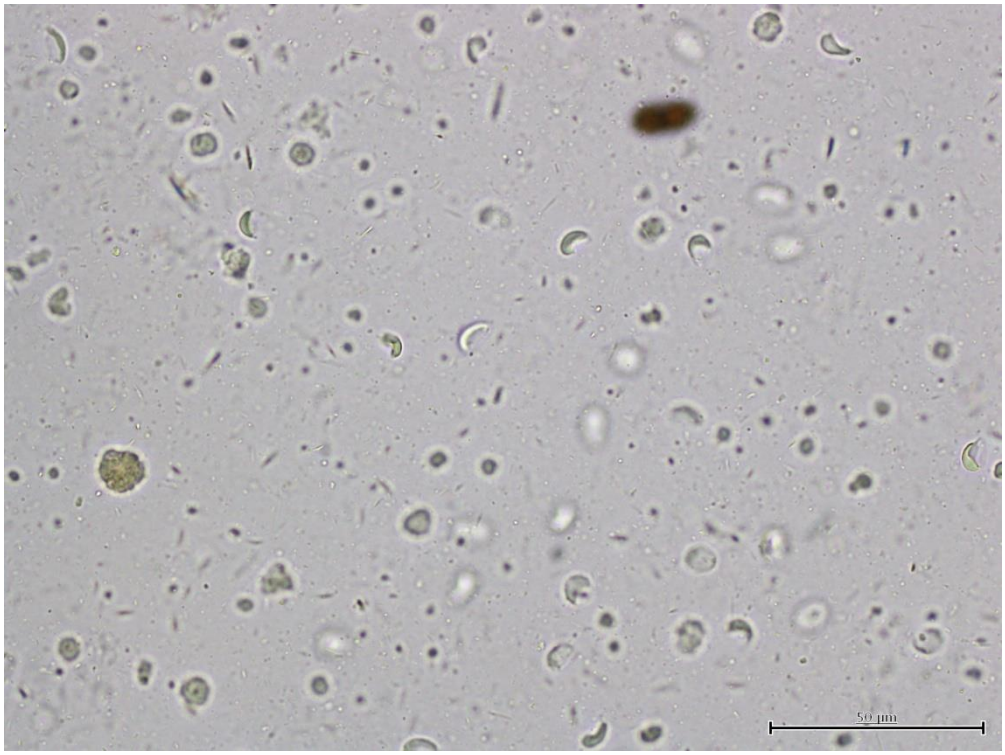
UNIVERSITY OF UTRECHT, FACULTY OF VETERINARY MEDICINE

A comparison of three diagnostic techniques for the detection of *Giardia lamblia* in pet dogs



Author: L.G.A.Verhoeven
Student number: 3575047
Date: 26-02-2015

Supervisors:
Drs. E. R. Nijsse
Drs. M. Uiterwijk
Department of Infectious Diseases and Immunology,
Faculty of Veterinary Medicine, University of Utrecht

Table of contents

Prefactory note

For the master program of veterinary Medicine at the Utrecht University, students must fulfill a research program over a time period of minimally three months. The research project gives the student an opportunity to act out one element of the veterinary profession and it is also aimed to stimulate scientific thinking in the student.
This particular research program on *Giardia lamblia* is carried out by L. G. A. Verhoeven and this report is the end result of the study.
The study from this report is part of a bigger research program taking place at the faculty of Veterinary Medicine at Utrecht University. In three different dog populations in the Netherlands the prevalence of *Giardia lamblia* is determined using different diagnostic tests. The populations used for the entire research program are: 1- Pet dogs; 2- Dogs from kennels; 3- Dogs with gastrointestinal problems.
In addition, humane genetic *Giardia*-assemblages that can also be found in dogs, are determined to obtain information on the zoonotic aspect of *Giardia* in these 3 populations. The study in this report focuses on the population of pet dogs.
Two students are cooperating in this study. In this report test comparison is the key aspect. The other student working in this study will focus on the prevalence of humane *Giardia*-assemblages in pet dogs and the zoonotic aspect of *Giardia*.

Introduction

Giardia lamblia (also named *Giardia intestinalis* or *Giardia duodenalis*) is a protozoan pathogen associated with gastrointestinal disease common in many different mammals, such as humans and dogs. *Giardia lamblia* is the summarizing name of all different known genetic *Giardia*-assemblages.

Prevalence

Giardia is commonly found in the faeces of dogs. In a Belgian study on the prevalence of intestinal parasites in different dog populations (1) *Giardia* was found in 9.3% of the household dogs. This value is the estimated true prevalence. The true prevalence was calculated by first calculating the apparent prevalence (positive animals/total animals) and secondly using the Rogen-Gladen formula that uses predetermined values for sensitivity and specificity for the tests. For the IFA test method relative sensitivity and specificity was extracted from an article by Geurden *et al.* (2) with a relative sensitivity of 0.90 and a specificity of 0.94. In kennel dogs the prevalence of *Giardia* was much higher with 43.9%. In dogs with gastrointestinal problems the prevalence was 18.1%. Pups in every tested population were more frequently infected than older dogs.
In a different study executed in the Netherlands (3) *Giardia* was found in 15.2% of the dog faeces in a population of 152 dogs. The population used in this study consisted of healthy household dogs in Dutch veterinary practices, with no age selection.(3)

Life Cycle and epidemiology

Giardia lamblia has a life cycle involving the formation of cysts that enables the parasite to maintain in usually a watery environment until ingested by a new host. The life cycle consists of two major stages, the cyst and trophozoite form. The beginning of the life cycle is infection of the host by ingestion of the cyst. Exposure of the cyst to the acidic environment of the stomach starts the process of excystation. This causes the cyst to release two trophozoites that were previously formed asexually inside the cyst into the small intestine. Excystation is a rapid process, being completed in ten minutes. The trophozoites form the second stage of the life cycle of *Giardia.* (4) In the small intestine *Giardia* trophozoites replicate asexually and attach themselves to the mucosa of the duodenum, jejunum and ileum. This creates a smaller epithelial surface of the intestines for absorption and can cause a malabsorption in the host and possibly also a hypersecretion, leading to gastrointestinal disease. (5)
The process of encystation is initiated by contact of trophozoites to biliary fluid, which makes the trophozoites form into cysts in the jejunum. Encystation can be divided into an early and late stage and is completed in approximately 26 h in total. The cyst can exit the host along with the faeces. (4)
The cysts can survive for months in watery environmental conditions. Mostly transmission to a new host is achieved by ingestion of contaminated water. The cysts can also be transmitted by contaminated food or by direct fecal-oral contact. (4,6)

Clinical signs

Giardiasis causes gastro-intestinal disease. However, most infections in dogs and humans with *Giardia* are asymptomatic. It is possible that the genetic assemblage of *Giardia* determines whether clinical signs can occur. (7,8) Clinical signs are also influenced by patient related factors, such as age, stress level, immune status and nutritional factors. Mainly younger dogs and dogs which are held in kennels develop clinical signs. Small bowel diarrhea is the most consistent symptom of giardiasis. The diarrhea can be either intermittent or continuous, acute or chronic and often is eventually self-limiting. (9)
Other symptoms can be abdominal discomfort to severe abdominal pain, the malabsorption can cause weight loss and occasionally vomiting occurs. (10)
The prepatent period is approximately one to two weeks. After this stage *Giardia* cysts can be detected in the dog feces. (11) The patent period can vary from weeks to months, in this period cysts are shed (intermittently) in the faeces. (12)

Detection methods

IFA

IFA stands for direct immunofluorescence assay and this method is considered the golden standard for detection of *Giardia* cysts. (13)
With this test method, *Giardia* cysts are fixated to a slide and a solution of antibodies with fluorescent material against *Giardia* cyst antigen is washed onto the slide. The antibodies with fluorescent material bind to the cyst wall. Detection reagent activates the fluorescence whereupon cysts can be made visible with a fluorescence microscope.
In a recent comparative study on the detection of *Giardia* and *Cryptosporidium* by European clinical laboratories, the detection limit of fluorescent-conjugated antibody-based microscopy was determined, detecting 172 cysts/ml of faeces. (14) In this study the samples that were used were fixated formalin fecal samples, with each sample containing 1 g of faeces. The detection limit of 172 cysts/ml can be interpreted as 172 cysts/g of faeces.
The Merifluor®*Giardia* test is a direct immunofluorescense assay that can be used for the detection of intact *Giardia* cysts. This test has been evaluated on human fecal specimens by Garcia *et al.* (15). In this study 70 positive *Giardia* samples (60 Giardia; 10 Giardia/cryptosporidium) and 105 negative samples were tested with the Merifluor detection kit. The status of the samples was predetermined by routine ova and parasite examination, a method which is also considered a very sensitive method for the detection of Giardia cysts in faeces.(16) All positive samples for *Giardia* showed fluorescent *Giardia* cysts, and all negative samples showed no fluorescent cysts. With possibly no false-negative and no false-positive results (compared to routine ova and parasite examination) a sensitivity of 100% and a specificity of equally 100% were determined. These results indicate why the IFA method can be considered the golden standard for the detection of *Giardia* cysts.
A different study by Zimmerman *et al.* (17) had similar results when comparing the Merifluor®*Giardia* test to the ProSpecT *Giardia* EZ Microplate Assay, with a sensitivity of 100 and 97% respectively and specificity for both tests of 99.8%, using 512 fecal samples.
A Bayesian evaluation by Geurden *et al.* compared three tests, the Merifluor®*Giardia* test, the speed-*Giardia* test from VIRBAC and the *Giardia*-strip® from Coris BioConcept using 421 fecal samples from calves (age 1-9 months). In this study the Merifluor®*Giardia* test on calves did not show as excellent results as in other studies with a sensitivity of 88% (60-99%) and specificity of 94% (90-99%). Sensitivity and specificity were calculated by a Bayesian evaluation using prior test information from a study on dairy calves by Geurden *et al.* (18). (19)
An evaluation of three commercial assays for detection of *Giardia* (Merifluor®*Giardia* test, the ImmunoCard STAT! Rapid assay and the ProSpecT *Giardia* EZ microplate assay) considered the Merifluor®*Giardia* test as most sensitive test when priority is placed on efficiency of the test. (20)

PCR

PCR stands for polymerase chain reaction and it is a method that can detect genetic material from a predetermined organism inside a sample. With the use of primers and DNA-polymerase a designated sequence of DNA material can be made. Cycles of heating are necessary for exponential multiplication. Real-time PCR is a better semi-quantitative method than conventional PCR and it enables to work with only small amounts of genetic material. Real-time PCR can be used to detect the presence of *Giardia* of all genotypes. (21)
Multiplex PCR enables amplifying multiple sequences in a single reaction. (22) Therefore multiplex PCR is a good choice for detecting more than one species in a fecal sample.
In a recent study performed by Elsafi *et al.* three different techniques for the detection of *Giardia lamblia* and *Cryptosporidium parvum* were compared. The three techniques used to detect *Giardia* were iodine-stained wet mount (microscopy), ImmunoCard STAT® *Cryptosporidium/Giardia* (rapid immunoassay) and real-time PCR detecting the 18S rRNA gene of *Giardia lamblia*. A total of 148 stool samples were tested with all three techniques. The results were used to determine relative

sensitivity and specificity for each test for the detection of *Giardia* compared to the true positive and negative results. The true positive and negative results were determined by minimally two positive or negative results from the three tests that had been executed. Real-time PCR relative sensitivity was determined at 100% (80.8–100) and relative specificity at 98.4% (93.9–99.7). (23)
In a study by Vanni *et al.* six different PCR assays were tested on the ability to detect *Giardia* assemblages A and B. 15 human DNA isolates (5 previously genotyped assemblage A isolates, 5 assemblage B isolates and 5 mixed assemblages A and B isolates) were tested using assemblage-specific PCR. Two PCR assays (4E1-HP and 5C1-P21) showed excellent results in sensitivity for both assemblages. By dilution of samples the detection limit for both assemblages was determined. Both assays were more sensitive on detection of assemblage B, with the ability of detecting an amount of only 0.15 cyst or 2 copies of genetic material in one sample. The assays for assemblage A detected *Giardia* in a sample that contained minimally 0.75 cyst or 12 copies of genetic material. The interpretation of results for mixed assemblage A and B samples was more difficult. Samples with variable proportions of mixed assemblage A and B were tested to determine the ability of detection. The results showed that the two assays can detect mixed assemblage A and B when the proportion of the lower amount assemblage is at least 10% of all *Giardia* DNA present in the sample. (24)

CSF

CSF stands for centrifuge-sedimentation-flotation and is a technique that can be used as both a qualitative and semi-quantitative method for the detection of cysts, oöcysts or parasite eggs in feces. A predefined amount of feces is used to make slides for microscopic examination. The slides must be examined by trained parasitology technicians. Besides *Giardia* many other parasite cysts, oöcysts and eggs can be detected with this method, for example *Toxocara canis* eggs, *Isospora spp.* oöcysts or *Trichuris vulpis* eggs. Only in very fresh feces it is sometimes possible to find viable trophozoites visible as moving pear-shaped structures. Trophozoites die quickly once leaving the host. Therefore in older faeces only *Giardia* cysts can be detected. The cysts are visible as oval structures, with a smooth rim. Mostly the flotation fluid used causes the cyst to collapse making it visible as oval structures with half-moon like structures within. The cysts must be viewed at a magnification of 400x for determination. It is advised to view minimally two lanes of the whole slide at this magnification for a proper execution of the CSF method. Sensitivity is increased when viewing the whole slide.
The detection limit of sedimentation/concentration followed by light microscopy has been determined by Manser *et al.* as between 17.2 and 172 cysts/ml of faeces. (14) This means that 1 ml of fecal sample (containing 1 g of faeces) needs minimally 18 cysts to be present in order to detect *Giardia* in that sample.
The CSF method is not the most sensitive method with a sensitivity of 0.34 (0.06-0.80) and specificity of 0.92 (0.88-0.96) found by Geurden *et al.* using a Bayesian evaluation. (2)

Literature comparison IFA to PCR

The IFA test can detect presence of *Giardia* by microscopic examination of the fluorescent cysts. PCR does not rely on the presence of intact *Giardia* cysts, in fact PCR can detect presence of *Giardia* by detecting *Giardia* genetic material. The detection limit for the IFA test is approximately 172 cysts/ml of faeces (14). The detection limit for assemblage-specific PCR was found at 0.15 cyst or 2 copies of genetic material for assemblage B, and 0.75 cyst or 12 copies of genetic material for assemblage A minimally present in the sample (24). When comparing the detection limit for IFA and PCR, it is clear the PCR method has a much lower detection limit. This means the PCR can detect much lower amounts of *Giardia* in the faeces than the IFA test method. Unfortunately it is not possible to determine which method is more sensitive based on these detection limits. PCR may seem very sensitive due to the low detection limit, however it uses very small amounts of the fecal sample and *Giardia* cysts are not homogenously divided over the sample, which makes it easier for *Giardia* to be missed. Also PCR can be influenced by inhibiting factors created by other fecal material in the sample, resulting in a lesser sensitivity by creating false negative results. (25)

When comparing relative sensitivity and specificity, IFA and PCR have an equal ability to correctly identify *Giardia* positive fecal samples (relative sensitivity of both 100%) though IFA is more able to correctly identify the *Giardia* negative fecal samples (relative specificity IFA of 99.8-100% and PCR of 98.4%). (15,17,23)
Based on the material of *Giardia* on which is being tested, it would be more practical to use the IFA test method. When the animal is infected it is likely to find intact cysts. However there is intermittent shedding. PCR can detect the infected animals also when cysts are not being shed in that particular sample, which may seem like a benefit of the method. However, PCR also detects *Giardia* genetic material when the infection may no longer be present, giving an unstable interpretation of the result.

Literature comparison IFA to CSF

Based on a Bayesian evaluation of three diagnostic tests for the detection of *Giardia* in dogs (2), IFA is considered to be more sensitive than CSF. Overall, the CSF was determined to be a specific diagnostic technique for *Giardia*, though not very sensitive. From fecal samples of 272 household dogs, 34 IFA results were positive for *Giardia*, indicating a test prevalence of 12.5%. From these 34 positive IFA outcomes CSF supported the outcomes of those samples with 10 positive results. The other 24 IFA positive outcomes were missed with CSF, considering IFA as the golden standard. On the other hand, CSF additionally had 15 positive outcomes were the IFA results of those samples were negative. In total CSF had 25 positive results, indicating a test prevalence of 9.2%. As this study from Geurden *et al.* used a Bayesian evaluation to compare the tests, it has been possible to compare tests without using a golden standard. The results from this evaluation showed an IFA sensitivity of 0.90 (0.80-0.99) and specificity of 0.94 (0.90-0.99). For the CSF method the results showed a sensitivity of 0.34 (0.06-0.80) and specificity of 0.92 (0.88-0.96). These results illustrate that IFA and CSF are comparable in specificity (0.94 IFA; 0.92 CSF) but that CSF has a much lower sensitivity than IFA (0.90 IFA; 0.34 CSF). However the detection limit determined by Manser *et al.* did not support a higher sensitivity of the IFA compared to the CSF method since the detection limit for the CSF method was determined at 10 times lower than for the IFA method. However Manser *et al.* used 16 laboratories to determine detection limit and only 2 of 16 laboratories achieved the 17.2 cysts/mL detection limit for CSF. Also only 2 of 16 laboratories used the IFA method, so the number of participating laboratories and therefore useful results were too low to draw conclusions and it may well be possible the detection limit for the IFA is in fact lower than for the CSF method. (14)
In the veterinary practice it would be preferable to use the IFA method since it has such high sensitivity compared to CSF. However if the choice was made to use the CSF method, the sensitivity can be increased by repetitive testing of three or more subsequent samples.

IFA as golden standard

Based on literature comparison it was found that the IFA test exceeded the CSF method by sensitivity as well as specificity. The IFA also exceeded the PCR method considering the specificity based on literature. However considering sensitivity, IFA and PCR are equally valuable.
The sensitivity of the test gives information on how effective the test is at identifying fecal samples with the presence of *Giardia* cysts or genetic material. The sensitivity is the proportion of true (golden-standard) positives identified by the test as positive. Therefore it gives an indication of the ability of the test to correctly identify those animals with *Giardia* in the faeces. The IFA has been used as the golden standard. This means that the IFA test results have been considered as true positives and true negatives. Based on literature the sensitivity of the IFA test is between 88-100%. This indicates that the IFA test is able to correctly identify up to 100% of the fecal samples with *Giardia*. Therefore based on the sensitivity determined by literature for the IFA test and the fact this report will compare IFA, CSF and PCR, it is fair to consider the IFA as the golden standard. The sensitivity of PCR and CSF based on literature are 100 and 34% respectively. CSF has an approximately 3 times lower sensitivity than both IFA and PCR. Therefore it can be mentioned that CSF will correctly identify only 1/3 of those animals with *Giardia* in the faeces comparing to the IFA and PCR.

The IFA specificity according to the literature is between 99.8-100%. Specificity of a test gives information on how effective the test is at identifying fecal samples without the presence of *Giardia*. The specificity is the proportion of true (golden-standard) negatives identified by the test as negative. Therefore it gives an indication of the ability of the test to correctly identify those animals without *Giardia* in the faeces. Based on literature the IFA test is able to correctly identify 99.8-100% of the fecal samples without *Giardia*. According to the specificity determined by literary research it is not 100% fair to consider the IFA as golden standard. However, the specificity still exceeds the PCR and CSF specificity and therefore the results of the IFA are closer to the true negatives and true positives than PCR of CSF. The specificity of PCR and CSF according to literature are approximately 98.4 and 92.0% respectively. CSF has the lowest specificity meaning this test of all three tests is the least able to correctly identify those animals without *Giardia* in the faeces. (15,17)
Other diagnostic methods for the detection of *Giardia* that are often used in veterinary practices are speed tests such as the SNAP *Giardia* test from IDEXX and the speed-*Giarda* test from VIRBAC. Speed tests are usually less sensitive than the IFA method.
Rishniw *et al.* compared the IDEXX snaptest to the Merifluor®*Giardia* test using 341 fecal samples from 20 clinically healthy household dogs that were send in weekly. The IDEXX snaptest was found to have a relative sensitivity of 77% (95% C.I. 61-72%). Relative specificity was found to be 92% (14-42%)(13)
Rishniw *et al.* also compared Techlab ELISA and the ZnSO4 flotation method (single and pooled) in relation to the Merifluor®*Giardia* test. For these tests a relative sensitivity was found at respectively 51% (45-56%), 49% (44-53%) and 78% (72-83%) and a relative specificity of respectively 96% (84-99%), 94% (85-98) and 65% (44-81%).
A Bayesian evaluation by Geurden et al. comparing the speed-Giardia test from VIRBAC, the Merifluor®Giardia test and the Giardia-strip® from Coris BioConcept on faeces from calves found a sensitivity of 26% (16-35%) and relative specificity of 93% (88-98%) for the The speed-Giardia test from VIRBAC, a and relative sensitivity of 28% (16–41%) and relative specificity of 92% (86–98%) for the Giardia-strip® from Coris BioConcept and a sensitivity of 88% (60-99%) and specificity of 94% (90-99%) for the Merifluor®Giardia test (19). It must be mentioned that faeces from calves was used instead of dog faeces. However this study also shows that the IFA method exceeds (in sensitivity and specificity) the other methods for the detection of *Giardia* (speed-*Giardia* test VIRBAC and *Giardia*-strip® Coris BioConcept).

Aim of the study

In this study 3 methods for the detection of *Giardia lamblia* in the faeces of dogs will be compared. These methods are direct immunofluorescence assay using the IFA Merifluor®*Giardia* test, detection and genotyping *Giardia* using the PCR technique and microscopical examination with the centrifuge-sedimentation-flotation (CSF) technique. Samples with positive outcomes from the IFA and/or CSF method are genotyped using the PCR method. The PCR detects if *Giardia* of any assemblage is present. A second PCR (multiplex) tests on assemblage A and B. These are the two assemblages that occur in both dogs and humans. Dog related assemblage C and D are not being tested.
Direct immunofluorescence assay (IFA) is considered the golden standard for the detection of *Giardia* cysts in faeces (2,13,20). This means the test prevalence of the IFA will be closest to the true prevalence in our dog population. Therefore the IFA outcome will be considered the golden standard in this study. However, this study will not focus on population prevalence but will only compare test methods and the outcomes of those tests.
Through literature research the correlation of IFA and PCR, respectively IFA and CSF will be viewed and it will be determined whether either PCR or CSF may be considered equally valuable as the IFA method. The value of the test will be defined as the ability of the test method to detect *Giardia* in faeces from dogs.
The aim of this study is to interpret the corresponding outcomes of the tests and to determine if the tests can be interpreted equally qualitatively valuable for the detection of *Giardia* cysts in faeces from dogs older than six months.

The aim of this study brings along two hypotheses:

1) H_0: There is no (demonstrable) significant correlation between the outcome of the IFA method and respectively PCR and CSF.
   H_1: There is a (demonstrable) significant correlation between the outcome of the IFA method and respectively PCR and CSF.

2) H_0: Based on different literature sources it can be stated that the outcome of IFA and PCR, respectively IFA and CSF, have equal ability to detect *Giardia* cysts in the faeces of dogs.
   H_1: Based on different literature sources it cannot be stated that the outcome of IFA and PCR, respectively IFA and CSF, have equal ability to detect *Giardia* cysts in the faeces of dogs.

Materials and Methods

Fecal samples

For this study the test population was used from a study on *Toxocara canis* at the faculty of Veterinary Medicine at Utrecht University. The used test samples have been tested in collaboration with the VMDC (Veterinair Microbiologisch Diagnostisch Centrum of the faculty of Veterinary Medicine). Over a 3 month period, send in fecal samples were examined by CSF method on the same day the samples were received. Also each sample was preserved by putting it into SAF solution and by freezing in a portion of faeces into little Eppendorf tubes. This was also done on the same day the fecal samples were received at the laboratory. Left over sample had been stored (maximally one week) in plastic boxes and kept refrigerated at 12 degrees Celsius. Preserved samples, such as samples stored in SAF-solution or frozen samples, are containable for a longer period making later use possible. The samples preserved in SAF were used for the IFA method. The frozen samples in the Eppendorf tubes were used for PCR genotyping.

Test population

Fecal samples of pet dogs were send in each month by the owners. The owners signed up earlier for the *Toxocara* project with one or more dogs and were periodically asked to fill in a questionnaire about their dog's living habits, behavior and more health related factors. They were asked to send in one fecal sample from each dog every month. Each month the laboratory received approximately between 400-600 dog faeces samples. The individual dogs were given a unique identification number (a combination of the number of the owner plus a number depending on the amount of dogs belonging to that owner).
Each sample that was received by the laboratory over the 3 month period was tested with the CSF method. For the IFA a sample from every dog was tested once. Positive outcomes from IFA and/or CSF during the test period were gathered and on these samples PCR was conducted.
Dog owners participated on voluntary basis. The dogs in the test population are older than 6 months, they are household pets or kennel held pets and they are not selected on presence or absence of gastro-intestinal disease. Therefore the dogs can differ from each other by breed and gender and by having or not having clinical signs.

IFA

For this study the IFA Merifluor®*Giardia* test has been used.
For the IFA Merifluor®*Giardia* test suitable samples for testing are fresh faeces or preserved faeces in 10% formalin or SAF (sodium acetate-acetic acid formalin). (14) The fecal samples were preserved in SAF-solution made into a suspension the same day the samples were received at the laboratory. The SAF samples were later used during the study period for the IFA. The SAF samples (one part faeces, three parts SAF) were meanwhile stored in tubes at room temperature. The tubes were labeled with patient numbers for sample identification. Each patient was only used once for the IFA test.
IFA has been performed on 359 samples in total. Slides were made according to the instructions supplied with the testkit. The precise protocol can be found in appendix no. 1. For determination of the microscopic view, a microscopic module has been used released by the U.S. Environmental Protection Agency (EPA).(26)
To screen the fluorescent antibody-wells, a magnification of 100x (low power) was sufficient. The examination time is therefore relatively short at 20 to 30 s compared to the examination time used for the CSF method. (15)

PCR

Faeces from every individual dog that was send in during the test period was stored in Eppendorf tubes and frozen at a temperature of -18 degrees Celsius. Positive outcomes of IFA and/or CSF could

later be gathered and send in as DNA isolates to the RIVM (Rijksinstituut voor Volksgezondheid en Milieu, The Netherlands) where PCR is executed. During this study a test batch has been tested, which consisted of 11 previously found positive results and 8 previously found negative results determined by IFA and/or CSF.
For DNA isolation the faeces from the Eppendorf tubes were first de-frozen and suspended in phosphate- buffered saline (PBS). The DNA was then isolated from the cysts using the high pure PCR template purification kit (Roche, Almere, The Netherlands). A total of 19 DNA isolates of dog faeces samples were amplified by PCR for 18S rDNA.(3)
The test batch PCR was a multiplex real-time PCR executed to catch all types of *Giardia.* Subsequently a multiplex real-time PCR will be executed that will test on *Giardia* assemblages A and B. However this has not been done during the test period of this study.
The multiplex real-time PCR to catch all types of *Giardia* consisted of an incubation of 10 minutes at 95 degrees Celsius. The incubation was followed by 45 cycles containing 10 seconds at 95 degrees Celsius, 20 seconds at 58 degrees Celsius and 20 seconds at 72 degrees Celsius. A final extension incubation of 7 minutes at 72 degrees Celsius concluded the multiplex real-time PCR.
5 out of the 19 samples that were used for PCR were not able to be used for comparing test methods since no IFA had been conducted on that sample thus far. Therefore a total of 14 PCR sample results were used for comparison of test methods.

CSF

For the detection of *Giardia lamblia* cysts a sucrose flotation technique was used. A total of 1065 fecal samples were examined using the CSF method. 3-5 g of faeces were suspended in 55 ml of water using mortars. Also the fecal consistence of each sample was determined using a scoring list, with a fecal consistence varying from zero to seven (0 = extremely fluid, 6 = extremely firm, 7 = crumbles when pressure is exerted).
For the CSF most samples were pooled in pairs of two to save time (suspended with 2 x 55 ml = 110 ml of water). The suspension was sieved to remove large debris. The suspension was then put into a centrifuge tube, whereupon the tubes were centrifuged for 2 minutes at 3000 rpm. The supernatant was decanted and to the sediment a few drops of sucrose solution was added (1.28 -1.30 g/cm^3). The sediment was made into a new suspension by vortexing the tube. The tubes were put back into the centrifuge and sucrose solution was added to fill the whole tube resulting in a half spherical meniscus on top of the tube. Then a cover glass was put on top. The tubes were centrifuged again for 2 minutes at 3000 rpm. After centrifuging the cover glass was taken of the tubes with a vertical movement and placed on a microscope slide. This slide was examined under a microscope. The whole slide was viewed with a 100x magnification for all types of parasite eggs, oöcysts or cysts and larvae. Then minimally two lanes of the slide were examined with a 400x magnification to detect *Giardia* cysts.

Results

Giardia positive outcomes

In total 359 IFA outcomes and 1065 CSF outcomes have been collected. Two IFA results were dismissed since there had been made a mistake during the execution of the test (too much time had been between centrifuging and decanting the sediment, causing loss of most of the sediment) and some IFA results could not be paired with a CSF result. Therefore a total remainder of 354 IFA results could be used for test comparison. In order to compare tests, the matching CSF outcomes to the IFA outcomes have been paired in a table. This allows a comparison in outcome per test.

Comparison of test outcomes

Cross-classified test results

The cross-classified test results obtained by the IFA and CSF test method are presented in Table 1. The results are divided into classes based on combining the IFA result with the CSF result. The different classes are illustrated in Figure 1. 313 samples were found to be negative in both test methods. 41 samples were positive for the IFA test method. CSF supported 10 of those samples with a positive result. However 31 samples were negative for the CSF method which were found to be positive with the IFA method. All negative sample outcomes for the IFA, were also negative for the CSF.

IFA	CSF	Number of samples
+	+	10
+	-	31
-	+	0
-	-	313
		Total: 354

Table 1 The cross-classified test results obtained by IFA and CSF.

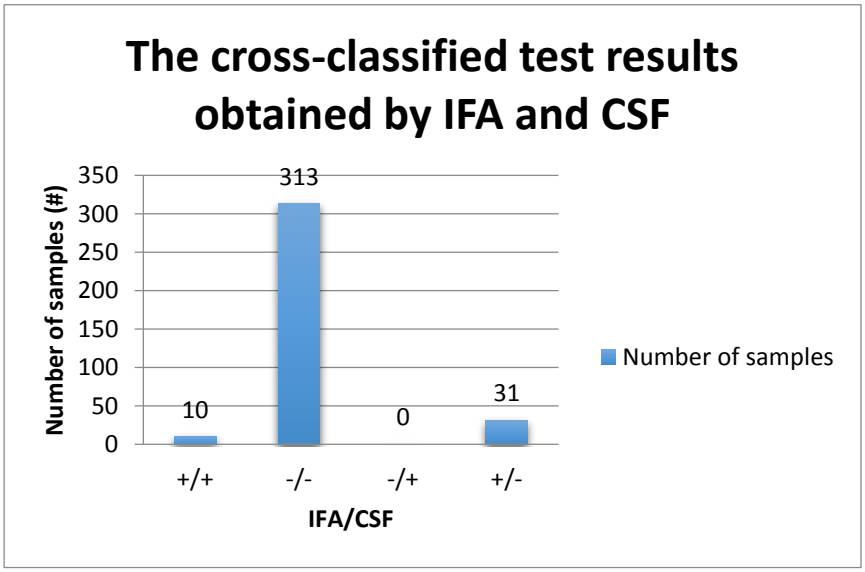


Figure 1 Overview of the cross-classified test results obtained by IFA and CSF.

Contingency table analysis

To compare the IFA and CSF test method a contingency table analysis using a golden standard reference test (IFA) has been conducted on all 354 IFA and corresponding CSF test results. The contingency table analysis is also known as the Chi-squared test. This test calculates whether the proportion of positive results for the IFA test (π_{IFA}) is equal to the proportion of positive results of the CSF method (π_{CSF}). Using Table 1 the proportions of positive results have been calculated: π_{IFA}= 41/354 = 0.1158 and π_{CSF}= 10/354 = 0.0282. The contingency table that was used can be found in Appendix no. 2, Table A.

The statistical analysis showed that the proportions of positive results of both tests were not similar (with a p-value lower than 0.001). Therefore it can be said that the IFA and CSF method are not equally sensitive for the detection of *Giardia* cysts in faeces. The execution of the statistical analysis comparing π_{IFA} and π_{CSF} can be found in Appendix no.2.

A second contingency table analysis was executed to test the correlation between the IFA and CSF test results. If there is a high correlation, sample results positive for the IFA test should also be positive for the CSF test. Vice versa, sample results negative for the IFA test should also be negative for the CSF test. The contingency table used to test the correlation can be found in Appendix no. 2, Table B.

The statistical analysis showed that there is no correlation between the IFA and CSF results (with a p-value lower than 0.001). Therefore it can be said that the IFA and CSF method are qualitatively not equally valuable for the detection of *Giardia* cysts in faeces. The execution of the statistical analysis to test the correlation can be found in Appendix no.2.

In Table 2 the outcomes of the statistical analysis that were executed are demonstrated.

Contingency table analysis	Z-value	P-value
Comparing proportions	16.35	< 0.001
Testing correlation	69.93	< 0.001

Table 2 Outcome statistical analysis for comparing proportions and testing correlation between the IFA and CSF sample results.

For testing the correlation a higher Z-value was found than for comparing the correlation.

Prevalence for each test

IFA vs. CSF

In this study the IFA method is considered the golden standard. This means a sensitivity and specificity of 100% is appointed to the IFA method. This enables the calculation of the relative sensitivity and relative specificity for the CSF method compared to the IFA method.
Table 3 is created using the IFA results as true prevalence of the sample population.

	Positive IFA result	Negative IFA result	Total
Positive CSF result	10	0	10
Negative CSF result	31	313	344
Total	41	313	354

Table 3 Observed positive results of the CSF method. The IFA results are considered the true sample prevalence.

From Table 3 it is possible to calculate relative sensitivity and relative specificity for the CSF method:
$Sensitivity_{rel./CSF} = 10/(10+31) = 10/41 = 0.24 = 24\%$
$Specificity_{rel./CSF} = 313/(0+313) = 313/313 = 1.00 = 100\%$

For these values a 95% confidence-interval (C.I.) has been determined. The 95% C.I. for the proportion of true positive sample results (considering the IFA results as the true prevalence) is $0.081<p_{true}<0.151$. This means that there is a 95% chance that the true positive samples proportion is between 0.081 and 0.151. It is noticeable that the calculated proportion of positive IFA results lies within the range of the 95% C.I. with a value of $\pi_{IFA}=0.1158$. The 95% C.I. for the proportion of CSF positive results is $0.011<p_{CSF}<0.045$, there is a 95% chance that the true proportion of positive CSF results is between 0.011 and 0.045. It was determined that $\pi_{CSF}= 0.0282$, this value lies within the range of the 95% C.I.
The confidence intervals of π_{IFA} and π_{CSF} do not overlap indicating even more that the proportions are not similar and that CSF and IFA are not equally sensitive.

IFA vs. PCR

For the PCR method a similar table has been made as for the CSF to calculate relative sensitivity and specificity, using the results from the test batch (Table 4).

	Positive IFA result	Negative IFA result	Total
Positive PCR result	4	0	4
Negative PCR result	2	8	10
Total	6	8	14

Table 4 Observed positive results of the PCR method. The IFA results are considered the true sample prevalence.

According to these results relative sensitivity and relative specificity for the PCR method are:
$Sensitivity_{rel./PCR} = 4/(4+2) = 4/8 = 0.50 =50\%$
$Specificity_{rel./PCR} = 8/(0+8) = 8/8 = 1.00 =100\%$

Discussion

Contemplating on the literature used in this report, some notifications should be made. Elsafi *et al.* found a 100% sensitivity for the real-time PCR method. Often PCR on faeces is influenced by inhibiting factors (25), therefore a 100% detection of all true positive fecal samples seems somewhat unlikely. From a total of 148 samples, two samples were determined falsely positive, possibly caused by contamination, according to the authors. The finding of false positive results indicates one should be cautious in interpreting the results from this particular study by Elsafi *et al.* (23) Additionally, true positive and negative results were determined by minimally two positive or negative results from the three different tests that were executed. This is a very unreliable manner to determine the true sample status, making the interpretation of the calculated relative sensitivity and specificity by means of this true sample status very unsettling.

A study by Geurden *et al.(19)* showed lower values of sensitivity and specificity for the IFA method than other studies. Such low values compromise the decision to consider IFA as the golden standard. However, the study used faeces from calves instead of dogs. Faeces from a different animal could have a deviating effect on the functioning of this IFA test. This may have had an effect on IFA sensitivity. Another factor that could have influenced the outcome of the IFA sensitivity is the statistical method. A Bayesian evaluation was used. This method uses a stringent prior on the specificity of the IFA test that has been considered as objective information. This might have influenced the sensitivity of the IFA method in a negative manner.

Considering test value and the comparison of tests, IFA and CSF can be called equally qualitatively valuable when both sensitivity as specificity are similar or very close to each other. It is expected that if this were true, all outcomes in Table 1 could be stored under classes +/+ and -/-. However, it is noticeable that some results are stored under the class +/- (IFA/CSF). The amount of results in the classes +/- and -/+ also greatly affect the outcome of the calculations on relative specificity and sensitivity. The incongruence in results per class has been a first indication from the results of this study that it was likely that the IFA and CSF do not have similar sensitivity and specificity and therefore are not equally qualitatively valuable.

To test if the IFA and CSF method truly are not equally qualitatively valuable, a contingency table analysis has been performed. Here it was determined that the proportion of positive results for the IFA was not similar to the proportion of positive results for the CSF method. From this outcome it can be deduced that IFA and CSF are not equally qualitatively valuable. The IFA test significantly detected more *Giardia* positive samples.

Using the IFA as the golden standard, the relative sensitivity and specificity for the CSF method are calculated. Relative sensitivity for the CSF was calculated as 24%. In literature CSF sensitivity was 34%. The 34% found in literature correspond with the confidence interval calculated from the results in this study. So the results from this study circumstantiate the result found in literature. As in literature, this study indicates that CSF is not as able as the IFA to correctly identify faeces from animals that contain Giardia cysts.

The relative specificity for CSF according to the results from this study is 100%. This indicates that CSF is compared to the IFA equally able to identify faeces that does not contain *Giardia* cysts. Based on literature a specificity of 92% was found. The result from this study differs from the results in previous literature. However the difference is not extreme. Perhaps the difference could be explained by a different team of microscopists, which was more careful in identifying samples as positive based on the microscopic view. Also the team on this study viewed less lanes of the microscopic slide, possibly causing a lower a lower chance of accidentally appointing false positive results.

From the 14 sample results obtained with the PCR method, relative sensitivity and specificity was calculated as well. Relative sensitivity for PCR according to the results from this study is 50%. This is a much lower value than found in literature, at 100%. Relative specificity according to the results from this study is 100%. This value also diverges from the value according to literature, at 98.4%. This difference however is not very remarkable. From a scientific perspective, it is not right to make calculations and assumptions upon relative sensitivity and specificity for the PCR method with such low sample size. Therefore no conclusion should be drawn from the calculated relative sensitivity and specificity for PCR in this study.

For testing the correlation between IFA and CSF, a higher Z-value was found than for comparing the proportions of positive results. This means the correlation has a lower P-value than the P-value for comparing proportions. The P-value is the probability of obtaining the observed value of the test statistic (Z-value) if the null hypothesis is true. From this it can be extracted that the IFA and CSF do not only differ in (positive) results (low p-value testing proportions), they are also even less correlated (lowest p-value testing correlation). This is eligible considering the first contingency table analysis only compares positive results, and the second contingency table analysis takes both positive and negative results into consideration, giving a more detailed comparison between the two tests.
A few notifications on execution of this study should be made. First, the execution of the Merifluor®*Giardia* test in this study is somewhat disputable. The test originally contains an extra concentration step that has not been executed. In a study by Garcia *et al.*(15) a sensitivity for the Merifluor®*Giardia* test was determined at 100%, including the extra concentration step. A concentration step enables a higher sensitivity of the test by lowering the detection limit. The amount of *Giardia* in the sample is accumulated and brought onto the examination slide. In case of a positive sample, more cysts will be on the slide than an execution of the Merifluor®*Giardia* test without the concentration step. This increases the sensitivity of the test. Therefore, the Merifluor®*Giardia* test executed in the study from this report possibly has a lower sensitivity than 100%. Since the IFA method has been used as golden standard and sensitivity has been considered as 100%, it would have been better to have included the extra concentration step of the Merifluor®*Giardia* test. Unfortunately this was not obtained due to financial reasons. If the IFA sensitivity in this study is actually lower than 100% due to the missing execution step, than the other two test methods calculated relative sensitivity should be interpreted with a larger confidence interval than given in this report.
Secondly, due to an administrative error, instead of just one sample two consecutive samples for a few dogs were put into SAF-solution. This error lead to double IFA results from samples from two dogs in the population. These double IFA results could still be paired with a CSF result and therefore they did not have to be eliminated for use in test comparison.
For only 19 samples PCR was executed of which just 14 sample results could be used. Originally the aim was to execute PCR on all IFA and/or CSF positive results. Perhaps this will still be achieved in the future. However, for the 3 month period this study has endured, it has not been possible to achieve the aim. The results that have been obtained so far were part of a 'test batch'. This test batch was used to determine whether the PCR method was effective on the samples provided. When the results are determined to be legit, the idea is that the remaining samples of this study can be tested with the PCR method consequently.
The test batch did not only consist of samples with IFA and/or CSF positive results (not enough preserved faeces from IFA and/or CSF positive samples were available at the time PCR was conducted). This has made it possible to implement a calculation of relative sensitivity and specificity for the PCR. However, the total number of PCR results is too low to draw conclusions from these calculations. In order to determine a reliable relative sensitivity and specificity a much higher number of PCR test results should be obtained. Due to the low sample size for PCR results, it was considered not to be useful to calculate a confidence interval for the PCR relative sensitivity and specificity.
In the future only IFA and/or CSF positive results will be tested with the PCR method, therefore unfortunately a relative sensitivity and specificity will not be possible to determine in the future in this particular study.
This study relied on dog fecal submissions send in by the owners. The owners were asked to send in the sample after collecting the sample from their dog. All samples send in should be equally fresh. However, it was often seen that the dog faeces was covered in fungus, indicating the sample was no longer to be considered fresh. Also the peak moment of samples received at the laboratory on a week basis was mostly on a Tuesday. The suspicion has arisen this has to do with the mail delivery system in the local area and this would create a possibility that samples send in during the weekend did not arrive sooner than Tuesday. That means samples send in by the owners during the weekend might be older than other samples. Even so, the age of samples could interfere with the observable prevalence of *Giardia* in the test population, this is not obstructive to this study. Population prevalence is not evaluated, only test prevalence. It still may be possible sample age interferes with our test prevalence, since it might lower the concentration of cysts in the sample making them more difficult to detect. For

this detection limit is very important. Unfortunately not much is known about detection limits of the three test methods used in this study for *Giardia* apart from the detection limits that were mentioned before.
Fresh samples were stored in SAF-solution. The samples send in from each day have been put into solution that same day. Since IFA is considered more sensitive than CSF, it is expected that some negative sample results with the CSF method would be positive results for the IFA. However, if fresh samples would have been kept cooled for more than one day and then put into SAF-solution, it is possible that *Giardia* cysts would have collapsed and disappeared from the sample. In such a case it could be expected that positive CSF results would come out negative in the IFA. For this reason all samples have been stored in SAF-solution the same day they were send in.
For the test comparison of the IFA and CSF method it would have been better if sample size was larger. The idea was to examine faeces from each dog in the test population once using the IFA test. This was not entirely achieved. 359 samples were tested using the IFA method. Had all the faeces from the dogs in the test population been tested, a total of 400-600 results could have been achieved. The reason it was not possible to obtain all 400-600 results is the time limit of the study and the time occupying aspect of the IFA test. At the same time the people who were using the microscopes were learned a new technique during the test period to be able to view and detect *Giardia* and *Cryptosporidium* cysts using the IFA and fluorescence microscope. If the microscopists would have been previously trained staff, the efficiency of the IFA execution could have been better from the start. However, the microscopists did achieve a high improvement of efficiency towards the end of the study period.
Since the microscopists were learned a new skill during this study and did not have years of experience, it raises the question whether they are as competent as well experienced laboratory workers that have working with these test methods for years building on their microscopic examination skills. The microscopists in this study were trained by experienced microbiologists. Viewed slides were checked by the microbiologists the first two weeks of the study. Hereafter examination had become the responsibility of the learning microscopists (often microscopic findings were still validated by the experienced staff, with the necessity of it judged by the learning microscopists themselves). It is possible the little experience of the microscopists had an effect on the results. However, it was decided by the specialists in the team that the microscopists are competent in detecting *Giardia* cysts with the guidance they were given.
In the future it would be an idea to compare tests on different parameters besides relative sensitivity and specificity, such as user friendliness and time consuming aspect and to ground this with test data. Test results on user ability could subsequently be translated for use in the veterinary practice. Extracted from this study design, those parameters can be carefully discussed. Each of the tests that were executed in this study have a certain preparation time and user friendliness. It is interesting to know which test, apart from reliability, is the most easy and efficient to use. It is also interesting which test would be preferable to use in the veterinary practice.
The Merifluor®*Giardia* test preparation time is depending on multiple factors. In case of testing one sample, it takes approximately 1.5h to prepare the slide. A slide consists of three wells, a negative and positive control and the actual sample. 1h of the preparation time is waiting time, thus not very efficient. However, waiting time can be efficiently used by performing other tasks to prevent unnecessary time loss.
In case of testing one sample with the CSF method, it takes approximately 1h to prepare the slide. With the IFA method, multiple slide can be made at once. In this study each preparation round produced 4 slides. Preparation time for 4 slides was 1.5h. Two wells were used for the control and 14 wells were used for sample. The available material was the limiting factor, therefore 4 slides was the maximum count made at once.
With the CSF method also multiple slides can be made at once. In this study maximally 8 pooled samples were made in one preparation round, consisting of 16 samples. *Giardia* positive CSF outcomes that had been pooled, needed to be repeated with CSF singular to determine which of the two samples (or both) was positive for *Giardia*. Preparation time for 8 slides was 1.5h
Looking at preparation time and samples produced, IFA and CSF are compatible.

Microscopic examination for one IFA well is less than 5 min. Examination time of one slide (three wells) was 5-10 min. Examination time decreased when many wells are *Giardia* positive (finding one cyst is already considered positive). Microscopic examination of one CSF slide was approximately 10-15 min. (for either pooled or singular slides). To examine a whole batch of IFA slides (4 slides) approximately 30 min. were needed. Examining a whole batch of CSF (8 slides) was roughly 100 min. Looking at examination time the IFA test is more efficient.
For both IFA and CSF trained microscopic examiners are needed. Especially for CSF it is important the examiner routinely examines slides, since the microscopic skill is very delicate. Examining the IFA slide on the other hand is less delicate. The fluorescent cysts are visible more clearly than the cysts in the CSF slide. Also CSF shows other parasitic eggs, cysts and oöcysts that make examination more difficult. The Merifluor®*Giardia* test principally only shows *Giardia* and *Cryptosporidium* cysts. Slides from both techniques are bothered with fecal debris making examination more difficult. The IFA slides are easier to examine than the CSF slides if the examiner is moderately skilled.
Based on the information obtained in this study on user qualities for the IFA and CSF test method, one can make a choice on which method is more suitable for the veterinary practice. Overall, the IFA method is more efficient and qualitatively better than the CSF method. It is fast with little examination time. Preparation time is similar for both methods. However IFA preparation contains waiting time in contrast to the CSF method, which can efficiently be used for other activities and therefore indirectly shortens preparation time for this technique. Both techniques require examination skills, however IFA examination is easier to learn and therefore faster to learn. The downside to the IFA is that it principally only provides information on two parasites (*Giardia* and *Cryptosporidium),* while CSF can detect many more parasites. And the IFA test method requires a very specific and expensive microscope, which requires a very large investment by the veterinary practice. Concluding, IFA is more practical and efficient. However if a practice is willing to invest time and effort into training staff with up to date examination skills and to obtain those skills over time, the CSF method would be preferred for it provides more information about a patient than IFA is able to.
PCR is usually not executed in veterinary practices. The laboratory technique requires experience and skill and many special equipment is needed. When practitioners would like a PCR executed on a fecal sample, the sample can easily be send in to a certified laboratory. For the IFA test a fluorescent microscope is needed which can be very expensive. Veterinary practitioners should determine if acquiring a fluorescent microscope is beneficial based on the expected use (in most cases the use would be too little to earn back the investment). For the CSF equipment is needed as well, such as a centrifuge and preferably a vortexing machine. However, acquiring this equipment comes with a lower cost than a fluorescent microscope. Therefore, based on costs, a CSF is more accessible for most veterinary practices. (20)
It is interesting to consider how this study design could be altered to get more accurate results. For this study the IFA method was used as a golden standard based on literature. For this reason sensitivity and specificity were calculated in relation to the IFA results, resulting in a relative sensitivity and specificity. In an ideal situation the true sensitivity and specificity could be calculated exactly, in contrary to the relative sensitivity and specificity. Only when true sensitivity and specificity for the different tests are known, it is possible to determine which test truly exceeds above the others. In order to determine true sensitivity and specificity, true positive and true negative samples would have to be known with certainty upfront. This could possibly be achieved using two dog populations consisting of SPF animals (specific pathogen free). Half of that group could be intentionally infected with *Giardia lamblia*. By observation of clinical signs infection of the SPF animals with *Giardia* should be confirmed. The other half of the group should be *Giardia* free. This way it could be made possible to have certainty about the sample status of all samples used. (27) However, such a study design would be very difficult, time-consuming and expensive.
Another option to calculate sensitivity and specificity is by using a Bayesian evaluation as done by Geurden *et al.*(2) The Bayesian statistical method is more complicated and therefore was not chosen for the study in this report.

Conclusion

In this study it has become clear that the IFA method can be used as a golden standard. Based on literature and sensitivity and specificity of tests, the PCR and CSF method are not equally qualitatively valuable as the IFA test method. Based on the results of the study from this report, the CSF method is less reliable than the IFA method for the detection of *Giardia* cysts in faeces with lowest sensitivity and specificity. To decide if PCR is as reliable as the IFA test, more research should be performed. Each veterinary practice should determine individually if any of these tests is suitable for the practice. The IFA method is more practical an efficient but requires expensive equipment. CSF is more difficult to learn, less reliable, but gives more information on a patient than IFA and is more accessible due to lower equipment costs.

Acknowledgement

First of all I would like to thank the management of the department of Infectious Diseases and Immunology, for giving me the opportunity to participate in this project. It has been a very educational study design and the facilities and surrounding gave great opportunity to learn about working in a department together with colleagues and developing new skills.
Secondly I would like to thank W. J. Scholten, for being such a good lab partner. Working together with you was very pleasant and together we have made a huge effort to make this project succeed.
Also I would like to thank Drs. H. Ploeger for helping me with the statistics performed in the study and for the laughs.
I would like to thank the employees of the VMDC, for showing me around in the laboratories and helping me out when needed.
I also would like to thank my mother, for being an amazing moral support.
Finally last but definitely not least, I would like to thank my supervisors Drs. E. R. Nijsse and Drs. M. Uiterwijk. You have given me great guidance with this project. You have learned me a lot. Thanks for the many feedback, in my report as well as in my personal development. It was hard work but incredibly educational and rewarding.

References

(1) Claerebout E, Casaert S, Dalemans A-, De Wilde N, Levecke B, Vercruysse J, et al. Giardia and other intestinal parasites in different dog populations in Northern Belgium. Vet Parasitol 2009;161(1-2):41-46.

(2) Geurden T, Berkvens D, Casaert S, Vercruysse J, Claerebout E. A Bayesian evaluation of three diagnostic assays for the detection of Giardia duodenalis in symptomatic and asymptomatic dogs. Vet Parasitol 2008;157(1-2):14-20.

(3) Overgaauw PAM, van Zutphen L, Hoek D, Yaya FO, Roelfsema J, Pinelli E, et al. Zoonotic parasites in fecal samples and fur from dogs and cats in The Netherlands. Vet Parasitol 2009 7/7;163(1–2):115-122.

(4) Adam RD. Biology of Giardia lamblia. Clin Microbiol Rev 2001;14(3):447-475.

(5) Buret AG. Mechanisms of epithelial dysfunction in giardiasis. Gut 2007;56(3):316-317.

(6) Esch KJ, Petersen CA. Transmission and epidemiology of zoonotic protozoal diseases of companion animals. Clin Microbiol Rev 2013;26(1):58-85.

(7) Xu F, Jerlstrom-Hultqvist J, Andersson JO. Genome-wide analyses of recombination suggest that Giardia intestinalis assemblages represent different species. Mol Biol Evol 2012 Oct;29(10):2895-2898.

(8) Uehlinger FD, Greenwood SJ, McClure JT, Conboy G, O'Handley R, Barkema HW. Zoonotic potential of Giardia duodenalis and Cryptosporidium spp. and prevalence of intestinal parasites in young dogs from different populations on Prince Edward Island, Canada. Vet Parasitol 2013 9/23;196(3–4):509-514.

(9) Thompson RCA, Palmer CS, O'Handley R. The public health and clinical significance of Giardia and Cryptosporidium in domestic animals. Veterinary Journal 2008;177(1):18-25.

(10) Overview of Giardiasis. September, 2013; Available at: http://www.merckmanuals.com/vet/digestive_system/giardiasis/overview_of_giardiasis.html?qt=giardia%20lamblia&alt=sh.

(11) Payne PA, Artzer M. The Biology and Control of Giardia spp and Tritrichomonas foetus. Vet Clin N Am : Small Anim Pract 2009 11;39(6):993-1007.

(12) The Center for Food Security and Public Health. Giardiasis. December, 2012; Available at: http://www.cfsph.iastate.edu/Factsheets/pdfs/giardiasis.pdf, 2013.

(13) Rishniw M, Liotta J, Bellosa M, Bowman D, Simpson KW. Comparison of 4 Giardia Diagnostic Tests in Diagnosis of Naturally Acquired Canine Chronic Subclinical Giardiasis. Journal of Veterinary Internal Medicine 2010;24(2):293-297.

(14) Manser M, Granlund M, Edwards H, Saez A, Petersen E, Evengard B, et al. Detection of Cryptosporidium and Giardia in clinical laboratories in Europe-a comparative study. Clinical Microbiology and Infection 2013.

(15) Garcia LS, Shum AC, Bruckner DA. Evaluation of a new monoclonal antibody combination reagent for direct fluorescence detection of Giardia cysts and Cryptosporidium oocysts in human fecal specimens. J Clin Microbiol 1992;30(12):3255-3257.

(16) Dryden MW, Payne PA, Smith V. Accurate diagnosis of Giardia spp and proper fecal examination procedures. Vet Ther 2006 Spring;7(1):4-14.

(17) Zimmerman SK, Needham CA. Comparison of conventional stool concentration and preserved-smear methods with merifluor Cryptosporidium/Giardia Direct Immunofluorescence Assay and ProSpecT Giardia EZ microplate assay for detection of Giardia lamblia. J Clin Microbiol 1995;33(7):1942-1943.

(18) Geurden T, Claerebout E, Vercruysse J, Berkvens D. Estimation of diagnostic test characteristics and prevalence of Giardia duodenalis in dairy calves in Belgium using a Bayesian approach. Int J Parasitol 2004;34(10):1121-1127.

(19) Geurden T, Levecke B, Pohle H, De Wilde N, Vercruysse J, Claerebout E. A Bayesian evaluation of two dip-stick assays for the on-site diagnosis of infection in calves suspected of clinical giardiasis. Vet Parasitol 2010 Sep 20;172(3-4):337-340.

(20) Johnston SP, Ballard MM, Beach MJ, Causer L, Wilkins PP. Evaluation of three commercial assays for detection of Giardia and Cryptosporidium organisms in fecal specimens. J Clin Microbiol 2003;41(2):623-626.

(21) Hunt M. Real Time PCR. July, 2010; Available at: http://pathmicro.med.sc.edu/pcr/realtime-home.htm, 2013.

(22) Markoulatos P, Siafakas N, Moncany M. Multiplex polymerase chain reaction: A practical approach. J Clin Lab Anal 2002;16(1):47-51.

(23) Elsafi SH, Al-Maqati TN, Hussein MI, Adam AA, Hassan MMA, Al Zahrani EM. Comparison of microscopy, rapid immunoassay, and molecular techniques for the detection of Giardia lamblia and Cryptosporidium parvum. Parasitol Res 2013;112(4):1641-1646.

(24) Vanni I, Caccio SM, van Lith L, Lebbad M, Svard SG, Pozio E, et al. Detection of Giardia duodenalis assemblages A and B in human feces by simple, assemblage-specific PCR assays. PLoS Negl Trop Dis 2012;6(8):e1776.

(25) Oikarinen S, Tauriainen S, Viskari H, Simell O, Knip M, Virtanen S, et al. PCR inhibition in stool samples in relation to age of infants. J Clin Virol 2009 Mar;44(3):211-214.

(26) Method 1622/1623 Microscopy module on *Giardia* and *Cryptosporidium by The U.S. Environmental Protection Agency (EPA)*. Available at: http://216.54.19.111/~corp2002/epa/sb/crypto/gandcrypto/index.html. Accessed september, 2013.

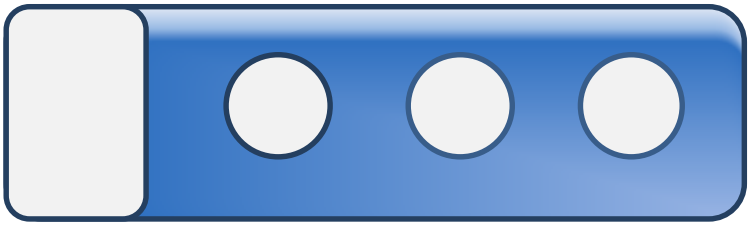
(27) Deblanc C, Gorin S, Queguiner S, Gautier-Bouchardon AV, Ferre S, Amenna N, et al. Pre-infection of pigs with Mycoplasma hyopneumoniae modifies outcomes of infection with European swine influenza virus of H1N1, but not H1N2, subtype. Vet Microbiol 2012 May 25;157(1-2):96-105.

Appendix no. 1 IFA Merifluor *Giardia/Cryptosporidium* protocol (Dutch)

1 deel feces op minstens 3 delen SAF
wasbuffer maken: 5 ml 20x wasbuffer (uit kit) + 95 ml aquadest (in afzuigkast) → in glazen flesje met groene draaidop

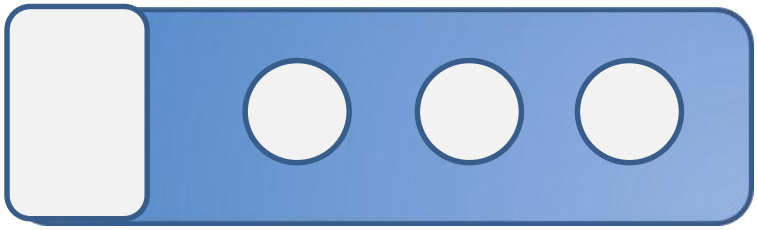
SAF:

- vortexen
- door zeef (theezeef; kleinst mogelijke maaswijdte), opvangen in bakje
- overgieten in centrifugeerbuis, evt. aanvullen met aquadest tot gelijke hoogten
- centrifugeren, 3000 toeren, **5** min.
- supernatant afgieten (SAF in afvalcontainer!)
- aanvullen tot 1 ml met aquadest
- vortexen
- Merifluor test procedure volgens bijsluiter: m.b.v. 10µl *transfer loop* op IFA glaasje aanbrengen en verspreiden binnen cirkel (niet krassen!). Goede administratie van welk monster waar aangebracht.



datum monster A B C

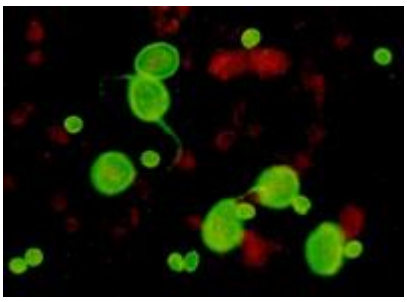
slidenummer



\+ -

- per ronde ook 1 positieve en 1 negatieve controle
- laten drogen aan de lucht, kamertemperatuur. Ongeveer half uur.
- één druppel Detection reagent in elke cirkel (blauwe dop)
- één druppel Counterstain erbij (groene dop)
- voorzichtig mengen (niet krassen!)
- half uur incuberen in humidified chamber bij kamertemperatuur (stoof met bakje water erin), in donker
- afwassen met 1x wasbuffer (glazen flesje, groene dop) m.b.v. plastic pasteurse pipet; overmaat detection reagent & counterstain verwijderen. Niet onderdompelen. Vermijd kruiscontaminatie
- overmaat buffer verwijderen door zachtjes op de kant op een tissue te tikken

- Glaasje niet laten indrogen!
- één druppel Mounting medium per cirkel (grijze dop); dekglaasje erop
- Bekijke de preparaten onder de fluorescentie-microscoop
- de microscoop moet een kwartier opwarmen. Let op: niet het fluorescentie licht te lang op de lens laten schijnen → schuifje dichtdoen
- de Giardia cysten en Cryptosporidium oöcysten (stuk kleiner dan Giardia) lichten appelgroen op. In de cysten zijn de structuren i.h.a. goed zichtbaar. Ook de 'kapotte' (niet meer mooi intacte) cysten zijn aangekleurd



Bron:
Geurden, T. *et al.*, 2008, A Bayesian evaluation of three diagnostic assays for the detection of *Giardia duodenalis* in symptomatic and asymptomatic dogs

Appendix no. 2: The contingency table analysis for comparing the proportion positive test results for the IFA and CSF method.

	IFA	CSF
+ Outcome	41	10
- Outcome	313	344
Total	354 (=n)	354 (=n)

Table A: The contingency table used for the Chi-squared test comparing the proportions of positive results for IFA (π_{IFA}) and CSF (π_{CSF}).

π_{IFA}= 41/354 = 0.1158
π_{CSF}= 10/354 = 0.0282

H_0: π_{IFA}= π_{CSF}
H_1: $\pi_{IFA} \neq \pi_{CSF}$

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

O = the frequencies observed
E = the frequencies expected
$\sum$ *= the 'sum of'*

Figure A: The formula of the statistical Chi-squared test.

Outcome statistical analysis:

$Test_8$ = 16.35 P < 0.001
→ P < 0.05 therefore H_0 is rejected, $\pi_{IFA} \neq \pi_{CSF}$

Conclusion: the IFA and CSF method are not equally sensitive for the detection of *Giardia* cysts in faeces.

	IFA +	IFA –	Total
CSF +	10	0	10
CSF -	31	313	344
Total	41	313	354 (=n)

Table B: The contingency table used for the Chi-squared test to test the correlation between IFA and CSF outcomes.

Outcome statistical analysis:

$Test_8$ = 69.93 P < 0.001
→ P < 0.05 therefore H_0 is rejected.

Conclusion: the IFA and CSF method are qualitatively not equally valuable.