Tritrichomonas foetus in young cats with chronic diarrhoea
Comparison of the different diagnostic methods

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Contents

Abstract

Tritrichomonas foetus has recently been identified as a cause of chronic diarrhoea in young cats. Up until recently, there was no effective treatment for *T. foetus* infection in cats. Recently, they have found that ronidazole (30-50 mg/kg q24h) for 14 days results in longterm elimination of *T. foetus* infection. Because of the serious adverse effects of ronidazole, a successfully diagnostic method is very important. The specific aims of the present study were to compare the different diagnostic methods (PCR at feces, fecal culture in the In Pouch TF® medium and PCR at In Pouch TF® medium), to determine the detection limits of our PCR and to determine the shedding pattern of the parasite through longitudinal follow-up study of infected cats.

Based on the results of our study, we can conclude that PCR at In Pouch TF® medium is the most sensitive diagnostic method for detection of a *T. foetus* infection. Based on a comparison of the different diagnostic methods, we concluded a relative efficacy for the detection of *T. foetus* organisms to be as follows: PCR at feces < fecal culture in In Pouch TF® medium < PCR at In Pouch TF® medium.

Results of our longitudinal follow-up study have shown that we can not detect *T. foetus* by PCR assay in all collected fecal samples and that we can speak of intermittent shedding of the parasite in measurable levels by PCR at feces.

The absolute detection limit of our real-time PCR was 10 organisms per 200 µl, the practical detection limit in feces we can not determine in our study, while the practical detection limit in In Pouch TF® medium sediment was 10 organisms per 200 µl.

Introduction

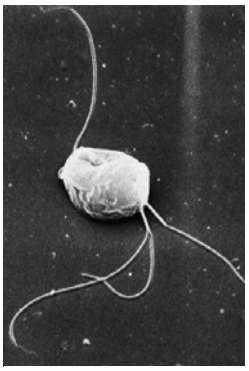

Figure 1. Electron micrograph of *Tritrichomonas foetus*. Pear shape with three anterior flagella and a single posterior flagellum [43].

Tritrichomonas foetus is a flagellated protozoan parasite that has recently been identified as a cause of chronic diarrhoea in cats [11][15].
Trichomonads are characterized morphologically by a spindle to pear shape, by three anterior flagella and a single posteriorly directed flagellum that arises at the anterior end (figure 1) [15]. These flagellum courses along the body attached to the characteristic undulating membrane, which is visible because of its wave-like motion [15][39]. Another characteristic feature of *T. foetus* is the axostyle, a rigid, rod-shaped organelle, which runs through the parasite and protrudes from the posterior end [15].
Trichomonads don't have the ability to synthesize many essential macromolecules, so they are dependent on host secretions or on phagocytosis of host bacterial flora [16]. Trichomonads don't have mitochondria needed for aerobic metabolism either. Instead, trichomonads have hydrogenosomes, which are specialized double-membane-bound organelles [16][33]. These organelles are unusual anaerobic energy-producing organelles, in which the production of adenosine triphosphate (ATP) takes place by a process that involves the generation of hydrogen; they are found in some eukaryotic microbes that inhabit oxygen-deficient environments [28][33].
T. foetus exists only in a trophozoites stage; trichomonads do not form environmentally stable cysts [9][15][17]. Trophozoites reproduce by binary fission [9][15][17].

T. foetus is best known as a venereal pathogen of cattle [16]. Bovine trichomoniasis is well known since the middle of the 20th century and this venereal disease results in infertility, abortion, pyometra, endometritis, vaginitis and cervicitis in infected cows [41][36]. *T. foetus* in cows is transmitted from the prepuce of the bull to the vagina and uterus of the cow [40]. The disease has been detected in all major cattle producing countries [29]. Artificial insemination has reduced or eliminated bovine trichomoniasis in many countries, but *T. foetus* is still of great economic importance in naturally bred cattle in many areas of the world [5][29].

Trichomonads are occasionally observed in the feces of dogs with diarrhoea [14]. Based on morphological appearance these infections have been attributed to opportunistic overgrowth of the commensal *Pentatrichomonas hominis* [14]. *P. hominis*, unlike *T. foetus*, has five anterior flagella and inhabits the large intestine of a large number of mammalian hosts, such as cats, dogs and people, and is considered to be a commensal [14][15][18].
The study of Gookin et al. (2005) was performed to determine, by means of rRNA gene sequence analysis, the identity of trichomonads observed in feces from dogs with diarrhoea. In this study one dog has been diagnosed *T. foetus* infection by PCR, so canine trichomoniasis may also be caused by infection with *T. foetus*. The origin of *T. foetus* infection in this dog of this study remains unknown. The dog was born into a closed research colony and had no contact with cats or cattle [14].
Trichomonads in pigs, i.e. species *Trichomonas suis*, are an inhabitant of the porcine gastrointestinal and nasal mucosa, in which their pathogenicity is uncertain [5][40]. *Tritrichomonas foetus* and *Tritrichomonas suis* are considered to be strains of the same species based on their morphology and sequence identity of rRNA [13][40]. *T. suis* has been shown to cause vaginal pathology similar to that caused by bovine isolates of *T. foetus* when transferred to ruminants [26].

So, all hosts for *T. foetus* are mammalian, and the site of infection is the lumen of either the gastrointestinal or reproductive tract [36]. Probably, *T. foetus* recovered from cats may have adapted to the new host environment [36]. The reproductive tract and large intestine are both luminal environments, which require the parasite to develop a means of adherence to epithelial cells, a common characteristic of *T. foetus* in both cows and cats [36].
Otherwise, the natural history of *T. foetus* in the feline host and any relationship to bovine or swine trichomoniasis needs further evaluation [15].

Since 1996, *T. foetus* has become increasingly recognized as an important enteric pathogen of cats [5][15][40]. Before 1996 only one published report of a cat with intestinal trichomonosis had been submitted, perhaps because of the assumption that trichomonads are nonpathogenic commensal fauna and only flourish when there is a coexisting enteric disease [10][15].
Nowadays *T. foetus* is recognized as an emerging parasite of felines.

Nowadays, the prevalence of feline trichomoniasis has been estimated in different countries. First of all, Gookin et al. (2004) examined prevalence for feline *T. foetus* infection in the USA. They gathered data for 117 cats from 89 catteries at an international cat show and they found a prevalence of *T. foetus* 31% among cats (36/117) and catteries (28/89) included in their study. In the UK, Gunn-Moore et al. (2007) found that sixteen (14.4%) samples of fecal samples from 111 cats with diarrhoea positive by polymerase chain reaction (PCR).
In France, Brigui et al. sampled 141 cats from 19 purebred breeding catteries in the Ile-de-France region (around Paris). In this study 15 (10.2%) and 9 catteries (47%) were found infected by *T. foetus*. The mean prevalence inside the positive catteries was 21%. Holliday et al. (2009) examined fecal samples from 74 cats with chronic large bowel diarrhoea that were living in a rescue colony in Italy. Twenty-four (32%) of the cats in this rescue colony were found to be infected with *T. foetus*. Frey et al. (2009) investigated 45 cats of Switzerland suffering from chronic diarrhoea and found that about 25% of the cats were positive. Other countries, where they found *T. foetus* in their cat population are among others Norway [5], the Netherlands [6], Australia [1][2], Germany and Austria [35].

Observations of the study of Gookin et al. 2001 have fulfilled Koch's postulates [10][40]. First of all, resulted orogastric inoculation with axenic cultures of a *T. foetus* isolate of SPF cats in infection of the ileum and colon [10]. Subsequently, *T. foetus* with 100% sequence identity to the infecting isolate was recultured from 1 of the experimentally inoculated cats [10].

T. foetus causes histologic changes, like a mild-to-moderate lymphoplasmatic and neutrophilic colitis with crypt epithelial cell hypertrophy, hyperplasia and increased mitotic activity, loss of goblet cells, crypt microabscesses, and attenuation of the superficial colonic mucosa [40]. Trichomonads were seen in close proximity to the mucosal surface and less frequently in the lumen of colonic crypts [40]. In a study of Gookin et al. 2001 a *T. foetus*-specific monoclonal antibody was used and it was discovered that trichomonads were found in close association with the surface epithelia and mucus of the caecal and colonic mucosa. The positive immunolabeling of enterocytes seen in proximity to overlying *T. foetus* in the experimentally infected cats in their study had been observed in preputial epithelium of naturally infected bulls as well [10].
These histologic changes of the caecum and colon result in clinical signs, such as diarrhoea with mucus, or even with blood, tenesmus, flatulence, increased frequency of defecation, the passage of semi-formed to liquid often foul-smelling feces, which in some cases may wax and wane [13][15][21][23][24][37]. Sometimes severe diarrhoea may cause the anus to become

inflamed and to appear edematous, erythematous and painful, and fecal incontinence or rectal prolapse may be seen [15][21]. In general however, most affected cats maintain good health and body condition [13][15][21][23].
Feline *T. foetus* infection is typically characterized by chronic large bowel diarrhoea signs. However, Dahlgren et al. (2007) described the first and to the authors' knowledge only report of a natural feline uterine infection with *T. foetus*. The infection in this cat was associated with signs of an endometritis/pyometra. Large numbers of live trophozoites were seen in the uterine contents, suggesting that the feline uterus might be a favorable environment for *T. foetus*, like the reproductive tract of cows [5]. How *T. foetus* might have caused the uterine infection in this cat is not clear. There are some speculations. First of all, spreading of an intestinal *T. foetus* infection might have caused the uterine infection, but the cat had no history of diarrhoea and *T. foetus* was not detected in the feces by direct fecal smear examination and PCR. Otherwise an ascending infection through the cervix without intestinal involvement is possible. At least three other cats in the same cattery shed *T. foetus* in their feces, so the infection can be from other sources [5].

Feline trichomoniasis is mainly seen in young cats [11][21][23][38][40]. In several studies the infected cats were 1 year or younger, with in one study of Yaeger et al. (2005) a range of 10 weeks to 12 months. However, Holliday et al. (2009) found that the infected cats in a rescue centre in Italy predominantly were over a year of age (67% of the infected cats). The majority of these infected cats were adults of over 18 months of age [23]. A probable explanation of this discrepancy is that the infection might have entered this cat population for the first time, so a lack of acquired immune protection allowed older cats as well as younger cats to develop clinical signs [23].

Feline trichomoniasis is spread via the fecal-oral route and is primarily found in purebred cats living in densely populated housing, such as catteries or animal shelters [12][16][24]. It is unknown whether *T. foetus* infection is seen more often in pedigree cats because of genetic predisposition or because these cats lived in large multi-cat households, which predispose them to this infection [21][23]. The latter is consistent with the outcome of the study of Gookin et al. (2004), in which high housing density (low number of square feet of facility area per cat) was identified as a likely risk factor for *T. foetus*, because it predisposes to high levels of fecal-oral spread [13][21][23]. The method(s) of transmission of *T. foetus* infection in cats remain unknown. It is unclear whether it is transmitted through shared litter boxes or between queens and kittens [38].

In the study of Foster et al. (2004), they determined the longterm outcome of cats infected with *T. foetus*. Among the cats of their study, chronic *T. foetus* infection without clinical signs after resolution of diarrhoea was common. These cats kept shedding *T. foetus* for many months after resolution of diarrhoea [7][38].
Several other studies [5][13][16][38] have showed the existence of carriers in the cat population too. The exact role of these carriers in the transmission route and epidemiology is not clear yet.

Several studies [1][10][13][38] suggest that co-infection with *Giardia* is common. In a study of Gookin et al. (2004) co-infection with *T. foetus* and *Giardia* sp. was diagnosed in 12% of cats, but an association between *T. foetus* and *Giardia* sp. infection was not significant. However, Steiner et al. (2007) found that most infected cats of their study were often positive for fecal *Giardia* antigens as well, Bisset et al. (2008) reports a percentage of 66%.

A co-infection with coccidia was frequently reported too [1][10][13][38]. Gookin et al. (2001) experimentally infected (with a feline isolate of *T. foetus*) eight cats of which four cats were already infected with *Cryptosporidium* spp. and the other four cats were specific pathogen free cats (SPF). Following inoculation with *T. foetus*, cats infected with *Cryptosporidium* spp. developed diarrhoea earlier and with greater severity than SPF cats of this study [10][38]. The cats infected with *Cryptosporidium* spp. had an increased number of trichomonads on direct fecal examination compared with the SPF cats [10]. However, there was no detectable change in the number of oocysts shed after inoculation with *T. foetus* [10]. These results suggested that coexisting enteric infection with *Cryptosporidium* spp. was associated with proliferation of *T. foetus*, more than suggesting natural worsening of cryptosporidial infection [10]. In the study of Stockdale et al. (2009) additional infections in *T. foetus* infected cats included *Isospora* spp., *Toxascaris leonina*, *Giardia* sp. and *Cryptosporidium* spp. So these different studies [1][10][13][38] have shown that other infections were observed when *T. foetus* is present. There is a possibility that one infection may exacerbate the other, as suggested in the study of Gookin et al. (2001) in cats with a *Cryptosporidium* spp. infection and a *T. foetus* infection, or common risk factors might be involved, as *T. foetus* and *Giardia* sp. do [1]. Common risk factors for trichomoniasis and giardiasis include diarrhoea, high-density housing and young age [1][13]. However, the question still remains whether *T. foetus* is the single cause of the diarrhoea problems in infected cats or if a concurrent infection leaves young cats more vulnerable to *T. foetus* infection [38]. Otherwise, maybe an impaired or immature immune system plays a role in the pathogenesis of this infection [38].

Feline *T. foetus* infection can be diagnosed by direct fecal smear examination looking for motile trophozoites, cultivation of feces using the specific 'In Pouch TF' culture system (BioMed Diagnostics, Oregon, USA) or Diamonds medium, or by extraction of DNA from feces and amplification of *T. foetus* rRNA via a polymerase chain reaction (PCR) technique involving species-specific primers [7][8][21][23][40]. In the study of Gookin et al. (2004), they found different sensitivities: direct smear examination of feces was positive in 5/36 of cases, culture of feces in modified Diamond's medium was positive in 9/36 of cases, culture of feces with 'In Pouch TF' culture medium was positive in 20/36 of cases and PCR was positive in 34/36 of cases.

Direct smear examinations are simple, quick and economical to perform, but the test sensitivity is really poor for detecting *Tritrichomonas foetus* (≈14%) [1][22]. Direct smear examination is done by observation of motile trophozoites in freshly voided feces diluted with physiologic saline solution (0,9% NaCl) and examined with a light microscope at 200 to 400x magnification [12][15]. The sensitivity of this technique is low, because of dependence on the presence of high number of viable organisms within fecal samples [22]. Further, the examiner must be able to differentiate between trophozoites from *T. foetus* and trophozoites from similar protozoa such as *Giardia* and *Pentatrichomonas hominis* [12][15][22][32]. The trophozoites are similar in size (≈ 10-20 µm in length), but there are some morphological differences between the different trophozoites [1][22]. As described before, *T. foetus* trophozoites are pear-shaped, have three anterior flagella and one posterior flagellum, 1 nucleus and an undulating membrane along the entire length of the body [1][32]. On the other hand, *Giardia* trophozoites are spindle-shaped, have two large nuclei and do not possess an undulating membrane [1][32]. The motion of *Tritrichomonas foetus* is characteristically forwardly progressive, while *Giardia* has a 'falling leaf' pattern of motility [1][32]. *Pentatrichomonas hominis* and *Tritrichomonas foetus* differ in appearance only by the number of anterior flagella, respectively five versus three anterior flagella [15].

For increasing the sensitivity of fecal smear examination it is important to collect fresh feces by use of a plastic loop in stead of using voided samples. Use of fresh feces collected by use of a plastic loop yielded more positive results than did voided samples, probably because *T. foetus* survives better in fresh feces [10]. Furthermore, survival of trophozoites of *T. foetus* in feces can be extended from 0 to 4 days by removal of adherent litter and dilution of the sample with saline solution to avoid desiccation (3 ml 0,9% saline per 2 g feces) [10][15].

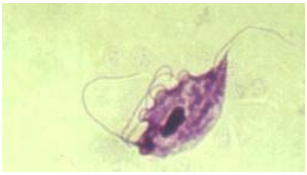

Figure 2. Light micrograph of *Tritrichomonas foetus* [44].

Use of a cultivation method for detection of a *T. foetus* infection is technically simple, cost-effective and has a reported relative sensitivity of approximately 55% for detecting *T. foetus* in cats [1][13]. There are two different cultivation methods, namely the In Pouch TF® feline medium (BioMed Diagnostics White City Oregon, USA) (figure 2) and the commercially unavailable Diamond's medium. For the fecal culture voided feces is used or a specimen obtained by a rectal swab or fecal loop. It is then incubated in a growth medium. The difference between the growth medium Diamond's medium and the In Pouch TF® medium is that culture in Diamond's medium feces were first diluted 100-fold (to prevent bacterial overgrowth) and then transported overnight to a laboratory. In the laboratory sterile medium must be prepared and maintained and this medium requires 37°C incubation [12][15], whereas the In Pouch TF® medium is inoculated directly with ≤ 0,1 g of feces and can be cultured at 25°C. In Pouch TF® medium doesn't require dilution or shipment of feces, there is no requirement for preparation of sterile media or facilities for incubation of cultures at 37°C [12]. So use of the In Pouch TF® medium is more practical for use in clinical settings than use of the Diamond's medium [12].
The In Pouch TF® medium has to be examined every 48 hours for motile trophozoites at 200 to 400x magnification with a light microscope during 12 days [12][15]. After 12 days there is no multiplication of *T. foetus* possible, so the In Pouch TF® medium can be discarded if they continue to yield negative results after 12 days [12]. If *T. foetus* trophozoites are present, they will multiply in the culture over time, increasing the likelihood of their detection [19][32]. The detection limit of the In Pouch TF® medium in absence of cat feces is 1 *T. foetus* organism, while supplemented with 0,05 g of feces, the In Pouch TF medium yielded positive results after inoculation with ≥ 1000 *T. foetus* organisms [12]. Inclusion of cat feces reduced the sensitivity, presumably because feces contain large numbers of competitive microorganisms [12]. In the study of Gookin et al. (2003) all culture system isolates were identified as *T. foetus* by PCR assay. This assumed that neither *Giardia* or *P. hominis* organisms survived in the In Pouch culture system [12]. Addition of physiologic saline solution to the fecal sample (approximately 3 ml of saline solution/2 g feces) is recommended if the fecal sample has to be transported or stored prior to culture; further the fecal sample should be free of cat litter, because this may desiccate the sample and kill the organisms [12].

Figure 3. In Pouch TF® medium is one of the diagnostic methods for detection a *T. foetus* infection [Biomed Diagnostics].

Because the diagnostic methods described above depend on the demonstration of live organisms, PCR remains the best method of detection of *T. foetus*, because it can detect both live and dead organisms [11][32]. PCR testing has the highest sensitivity, in a study of Gookin et al. (2004) a sensitivity of 94,4% (34 out of 36) was determined, but PCR testing may be cost-prohibitive for many cat owners [1][13]. The most used kind of PCR is the specific single-tube nested PCR based on amplification of a conserved portion of the *T. foetus*

internal transcribed spacer region (ITS1 and ITS2) and 5.8S rRNA gene from feline feces [11][15]. In this single-tube nested PCR two pairs of primers are used, namely the primer pair TFR3 and TFR4 and the other primer pair TFITS-F and TFITS-R [11]. Several studies showed that the rRNA gene unit sequence is highly conserved among *T. foetus* isolates and is reliably different than from related trichomonads [11]. For that reason primers TFR3 and TFR4 were designed to specifically amplify a 347bp fragment of *T. foetus* DNA by PCR [11]. The second primer pair (TFITS-F and TFITS-R) amplify a 208bp fragment of the rRNA gene unit that lies internal to the sequence amplified by the other primer pair, described above [12]. Use of the combination of these primer pairs (TFR3 and TFR4 with TFITS-F and TFITS-R) resulted in a detection limit of 50 organisms per g of feces 90% of the time and 500 organisms per g of feces 100% of the time [11]. The study of Gookin et al. (2002) have showed that these single-tube nested PCR did not amplify genomic DNA from either a feline isolate of *G. lamblia* or *P. hominis*. The difficulty of PCR testing in fecal samples is that feces is considered to be one of the most complex biological samples, because of the presence of inherent PCR inhibitors [11][16][17][22]. These PCR inhibitors could include heme, bilirubin, bile salts and complex carbohydrates, which are often coextracted along with pathogen DNA [12][16]. The type and quantity of PCR inhibitors present vary with composition of the feces, which is largely influenced by species, diet and concurrent disease [24]. A DNA sample can be tested on the presence of PCR inhibitors by a separate reaction in which bacterial 16S rRNA genes are amplified. If bacterial 16S rRNA genes can not be amplified, PCR inhibitors are present in the fecal sample [16][17]. The presence of PCR inhibitors makes optimal extraction of DNA from fecal samples very important [11][16]. Several previous studies [11][24] have focused on the optimization of DNA extraction; Gookin et al. (2002) found that improvement in reproducibility and sensitivity was obtained by optimizing the duration and temperature of proteinase K digestion and by adding an additional wash step prior to DNA elution.
The sensitivities of the diagnostic methods described above may be further reduced by intermittent shedding [23]. Several studies have suggested possible intermittent shedding of *T. foetus* [17][22][37][42]. For instance, Capital Diagnostics SAC Veterinary Services at Penicuik have analysed fecal samples collected every three hours over a 12-hour period from one infected cat and identified *T. foetus* by PCR in the first and last sample, but not in the 2nd, 3rd and 4th samples. They have repeated collecting samples from several cats, where *T. foetus* was not detected in the first, but was detected in samples taken a week or so later [45]. In the study of Gookin et al. (2007) *T. foetus* was identified in feces from all untreated cats in this study by PCR assay or culture, but only intermittently. So, shedding intensity of *T. foetus* in infected cats is likely to vary over the course of infection [22].

Several medications (such as fenbendazole and metronidazole) are used as treatment for *T. foetus* infection in cat, but they are poorly effective [10][23][32]. However, fecal consistency may improve during treatment with these medications, likely because of a possible role of the colonic microflora in the pathogenesis of *T. foetus* related diarrhoea [10][13][15]. Because of the alteration in colonic microflora there is a temporal improvement in diarrhoea, but diarrhoea returned soon after discontinuation of treatment with these medications [13][32]. Mostly, it results in prolongation of time to resolution of diarrhoea [7].
In a study of Gookin et al. (2006) they found that ronidazole at 30 to 50 mg/kg bodyweight PO q12h for 14 days results in longterm elimination of *T. foetus* infection and diarrhoea in the cat. Ronidazole is a nitroimidazole (similar to metronidole) and the primary mechanism of action of most nitroimidazoles (including ronidazole) is thought to be related to production of nitro anion radicals, leading to destabilization of deoxyribonucleic acid [33]. The disadvantage of use of ronidazole are the adverse effects, in the form of neurologic signs,

attributed to ronidazole in cats. Adverse effects included decreased appetite, altered mentation, trembling, weakness in the pelvic limbs, ataxia, hyperesthesia and occasionally seizures [23][32][33]. In a recent study of LeVine et al. (2008) the pharmacokinetics of ronidazole in cats were examined. Ronidazole is rapidly and completely absorbed in the proximal gastrointestinal tract and persists in plasma over 48 hours [25]. This may explain why neurotoxicosis can occur with twice a day dosing and the most recent recommendation for treatment of *T. foetus* is 30 mg/kg body weight, q24h (instead of q12h) for 14 days [25][32].
In a study of Gookin et al. (2007) they determined the efficacy of tinidazole for treatment of cats with experimentally induced *T. foetus* infection. Susceptibility of feline *T. foetus* to tinidazole has been detected in vitro, but in this study tinidazole suppressed *T. foetus* infection in only 2 of the 4 cats during 33-week period after completion of treatment and is less effective than ronidazole [18].

Despite of possible effective treatment of *T. foetus* infection with ronidazole, spontaneous resolution of feline trichomonosis is possible [7][15][21][23]. However, Foster et al. (2004) determined the long-term outcome of cats infected with *T. foetus*, and found that this can take a long time, ranging from 2 months to 3 years (with a median of 9 months) [7][21]. Most of the infected cats continue to shed low levels of the organism for many years after resolution of diarrhoea [21][23].

The role of *T. foetus* in the field of public health is unknown. There is one case report of Okamoto et al. (1998) of *T. foetus* meningoencephalitis occurring in a severely immunosuppressed patient following alloPBSCT. This is the first and only single case of *T. foetus* infection in humans [15][30].

The specific aims of the present study were to compare the different diagnostic methods for detection of *T. foetus* infection in young cats with chronic diarrhoea, to determine the detection limits of the RT-PCR, used at the Veterinary Microbiological Diagnostic Centre (VMDC) of the Faculty of Veterinary Medicine, Utrecht University and to determine the shedding pattern of the parasite through longitudinal follow up of infected cats. Because of the severe adverse effects of effective therapy of a *T. foetus* infection, setting up blind therapy is not acceptable and therefore a reliable diagnostic method is important. In order to say more about the sensitivities of the different diagnostic methods, it is important to determine the detection limits.
As previously described, several studies have suggested possible intermittent shedding of *T. foetus* [17][22][37][45]. Through longitudinal follow up of infected cats, it is possible to say something more about the shedding pattern of *T. foetus*.

Materials and Methods

Sample population
Veterinarians, interested in feline medicine, were selected for this research project and, by means of telephone contact, were asked if they were interested in participating in this research project.
Participating veterinarians were asked to select cats aged between 2 to 18 months with chronic diarrhoea (between loose stool and real diarrhoea), that didn't stop after therapy for a *Giardia* infection. Participating veterinarians had to send fresh fecal samples, taken directly from the litter box and an In Pouch TF® medium, inoculated with voided fecal sample or a specimen (approximately 0,05 g, peppercorn sized amount of feces) obtained by a rectal swab, from each selected cat.
The following information was obtained from each cat when possible: Name cat, age, gender, breed, number cats in household, way of housing, kind of and duration of diarrhoea, established therapy and effect of this therapy.

Comparison of the different diagnostic methods
Immediately upon arrival at the Veterinary Microbiological Diagnostic Centre of the Faculty of Veterinary Medicine, Utrecht University, 1 gram of the fecal sample was parasitologically examined by sedimentation-flotation technique to evaluate presence of ova, cysts and oöcysts. This parasitological examination was to make it more attractive for the veterinarians to send samples to us and to exclude other parasitological causes of chronic diarrhoea. Beside, the In Pouch TF® medium was examined by light microscopy at 160x magnification and was cultured at 37°C. Finally, 200 mg of the fecal sample was frozen and stored at -20°C for PCR assay at feces.

(i)DNA isolation procedure
Total DNA was isolated from this 200 mg fecal sample using a commercial kit (QIAamp DNA stool mini kit; Qiagen, Benelux B.V. Venlo, the Netherlands) in accordance with manufacturer instructions and with a modification to optimize the DNA isolation. The modification involved 15 minutes incubation with the ASL buffer at 95°C.
The principles and procedures of this DNA isolation kit are lysis of stool samples in Buffer ASL, adsorption of inpurities to InhibitEX matrix and purification of DNA on QIAamp Mini spin columns.
The first step of the procedure of the DNA isolation is adding 1,4 ml Buffer ASL to each stool sample (200 mg or if the sample is liquid 200 µl). This should be vortexed until the stool sample is thoroughly homogenized. The suspension should be heated for 15 minutes at 95°C (this is a modification to the protocol of the manufacturer, step 3). This heating step helps to lyse *T. foetus* and through this total DNA concentration in the lysate will be increased 3- to 5-fold. The samples should be vortexed for 15 seconds and centrifuged 14.000 rpm for 1 minute to pellet stool particles. Hereafter 1,2 ml of the supernatant is pipetted into a new 2 ml microcentrifuge tube. To each sample 1 InhibitEX tablet should be added and this mixture should be vortexed continuously until the tablet is completely suspended. This suspension should be incubated for 1 minute at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. DNA-damaging substances and PCR inhibitors present in the stool sample are absorbed at this way to InhibitEX matrix. After inhibitors and DNA-degrading substances have been absorbed to InhibitEX matrix, the InhibitEX matrix is pelleted by centrifugation at 14.000 rpm for 3 minutes to pellet inhibitors bound to InhibitEX matrix. After this step, the supernatant should be pipetted into a new 1,5 ml microcentrifuge tube and the sample should be centrifuged at 14.000 rpm for 3 minutes again. Into a new 1,5 ml microcentrifuge tube 15

µl proteinase K should be pipetted, followed by 200 µl supernatant and finally 200 µl Buffer AL. This entire suspension should be vortexed for 15 seconds to form a homogeneous solution. This solution should be incubated at 70°C for 10 minutes. Proteins are digested and degraded under denaturing conditions during a 70°C incubation with proteinase K. After the incubation 200 µl ethanol (96-100%) should be added to the lysate and this should be vortexed. The complete lysate should be applied to the QIAamp spin column and should be centrifuged at 14.000 rpm for 1 minute. After the centrifugation, the QIAamp spin column should be placed in a new 2 ml collection tube. 500 µl Buffer AW1 is added to the QIAamp spin column and this has to be centrifuged at 14.000 rpm for 1 minute. Hereafter, the QIAamp spin column should be placed in a new 2 ml collection tube and 500 µl Buffer AW2 should be added. This should be centrifuged at 14.000 rpm for 3 minutes. The QIAamp spin column should be transferred into a new 1,5 ml microcentrifuge tube and then should be added 200 µl Buffer AE. This should be incubated for 1 minute at room temperature. After the incubation, it should be centrifuged at 14.000 rpm for 1 minute to elute DNA. In the previous steps DNA is absorbed onto the QIAamp silica membrane and in the last step, after adding Buffer AE, DNA passes through the membrane and the QIAamp spin column can be removed. Concentrated purified DNA is obtained in a volume of 200 µl in the 1,5 ml microcentrifuge tube. According to the manufacturer of the DNA isolation kit, the obtained DNA concentration is about 75-300 ng/µl. The obtained DNA is stored at a temperature of 4°C. To avoid false positive results, fecal samples of negative tested cats (non purebred cats with no diarrhoeal complaints) were included in every DNA isolation procedure.

(ii) PCR amplification of T. foetus rRNA genes

The obtained DNA of the fecal samples was tested on *T. foetus* by amplification of *T. foetus* rRNA via Real-time PCR using Lightcycler 1.5 (Roche). For this Real-time PCR used a 15 µl volume of PCR mix containing 10 µl TaKaRa SYBR® *Premix Ex Taq*™, 2,4 µl $MgCl_2$ (25mM), 1,0 µl TFITS R primer (10 µM), 1,0 µl TFITS F primer (10 µM), and 0,6 MiliQ. TaKaRa SYBR® *Premix Ex Taq*™ is a premixed reagent, which includes Taq-polymerase, SYBR® Green I (which binds to double-stranded DNA and emits green light), dNTP's, Mg^{2+} and buffer. The primers used in this RT-PCR were TFITS R primer with a sequence of 5'-GCA ATG TGC ATT CAA AGA TCG-3' (Biolegio) and TFITS F with a sequence of 5'-CTG CCG GTT GGA TCA GTT TCG-3' (Biolegio). The primers are provided by Biolegio in a concentration of 100µM and the working dilution is 4 times (25 µM).

In short, a cycle of PCR consists of three steps, namely a denaturation step, an annealing step and an elongation step. The denaturation is the first step and consist of heating the reaction to 95°C and causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary basepares, yielding single strands of DNA. In the annealing step the reaction temperature is lowered to 57°C allowing annealing of the primers to the single-stranded DNA template. In the elongation step the reaction temperature is 72°C (the optimal temperature for Taq DNA polymerase) and the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand. Specific to real-time PCR is that through using a DNA-binding dye, in this case SYBR® Green I, which binds to all double-stranded DNA, a fluorescence signal arises. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity which is measured at each cycle by a photometer. This leads to the typical amplification curve. After that, by raising the temperature from 70°C to 95°C, a melting curve is generated and the change in fluorescence is measured. The double-stranded DNA melts and falls apart, as a result of which the fluorescence decreases. At the melting point, the two strands of DNA will separate and the fluorescence will rapidly decrease. At this point there is an exponential decrease in fluorescence.

To reduce possible contamination, different working spaces were used. In advance the UV lamp was turned on for 15 minutes in the working space, where the PCR mix and DNA templates were pipetted in the LightCycler Capillaries (Roche). In each LightCycler Capillary were pipetted 15 µl PCR mix. After that, 5 µl of DNA template were added. The capillaries are closed with the associated caps through the Lightcycler Capping Tool (Roche). In the other working space the capillaries are placed in the Lightcycler Carousel, which is placed in the Lightcycler Carousel Centrifuge 2.0 (Roche), where the capillaries are centrifuged. After centrifugation, the Lightcycler Carousel is placed in the Lightcycler and the program selective for *Tritrichomonas foetus* is run. This program consists of the following phases:
- Hotstart, 1 cycle: 10 seconds 95°C.
- Denaturation, annealing, elongation, 50 cycles: 10 seconds 95°C, 5 seconds 57°C, 10 seconds 72°C.
- Melting curve, 1 cycle: 15 seconds from 70°C to 90°C.
In each PCR we took control DNA samples along, namely negative control DNA samples (of negative fecal sample, sediment and supernatant of a negative In Pouch TF® medium), a positive control DNA sample and Miliq as an another negative control sample.
After the program of the PCR the capillaries were removed in another working space through the Lightcycler Capillary Releaser (Roche).
For the interpretation of the results of the RT-PCR the amplification curve (which tells after how many cycles the signal came) was examined, as well as the peak of the melting temperature and further the course of the melting curve. For specific products, the amplification curve rises at around 20-25 cycles. Furthermore, DNA of *T. foetus* has a peak of the melting curve around 81°C. Around this temperature there is an obvious exponential decrease in fluorescence seen. Using these specific features, the results of the RT-PCR were interpreted.

(iii) In Pouch TF® medium
The In Pouch TF® media were incubated vertically at 37°C in the dark for 12 days and microscopically examined by light microscopy at 160x magnification for motile trophozoites every day. Before microscopical examination the fluid of the In Pouch TF® was moved up and down 3-4 times. Because *T. foetus* gravitates to the edges of the In Pouch TF® medium, first the edges were examined with the light microscope. After this the liquid of the In Pouch TF® medium were examined microscopically. This procedure was repeated daily during 12 days.

(iv) PCR In Pouch TF® medium
After these 12 days the fluid of the In Pouch TF® medium was centrifuged at 3000 x g for 1 minute. For the DNA isolation procedure we used 200 µl supernatant and 200 µl sediment (each in its own 2,0 ml microcentrifuge tube). These samples were frozen and stored at 20°C for PCR assay. For the DNA isolation of supernatant and sediment of the In Pouch TF® medium fluid and the PCR assay the same protocols were used as described above.

Longitudinal follow-up study:
For the longitudinal follow-up study two infected cats were followed up for 4 weeks. The owner of these cats got the instructions to collect fecal samples the same for 3 consecutive days per week. Once every week the owner sent us the fecal samples and in the meantime the owner stored the fecal samples in the freezer. The owner had to fill out a scoring form regarding the clinical course of the cat of each day, when the fecal sample of the corresponding cat was collected. This scoring form contained questions regarding the overall

condition of the cat, the aspect of the diarrhoea and whether the cat is treated with regard to diarrhoea symptoms. 200 mg of each fecal sample was frozen and stored at -20°C for DNA isolation and the PCR assay.

Pilot study: Time before incubation In Pouch TF® medium
Due to the fact that sometimes the post was delayed and thus the In Pouch TF® medium was cultured at 37°C later than desirable, it was decided to do a pilot study aimed at the time before incubation the in pouches.
We have used an live positive In Pouch TF® culture medium and with this medium we have inoculated six different new In Pouch TF® media with 0,5ml. At different time points (day 0, 1, 2, 3, 4 and 5) after inoculation we have incubated these pouches at 37°C and have examined these pouches for motile trophozoites every day with a light microscope at 160x magnification.
We also instructed an owner with an infected cat to inoculate 5 different In Pouch TF® culture media with 5 different sterile cotton swabs. For inoculation of these pouches an amount smaller than a peppercorn was obtained of just voided feces with the sterile cotton swab. The intention was to incubate these pouches at different time points (day 1, 2, 3, 4 and 5 after inoculation) at 37°C and to examine these pouches for motile trophozoites every day with a light microscope at 160x magnification.

Determination detection limits real-time PCR:
(i) Absolute detection limit:
In Pouch TF® culture medium with cultivated *T. foetus* organisms of an infected cat of our study in the logarithmic phase of growth was first centrifuged for 1 minute at 3000 x g and the pellet were washed 3 times by centrifugation for 5 minutes at 1500 x g in sterile PBS solution, just as described in the study of Gookin et al. (2002; 2007). The organisms were counted on a haemocytometer (Bürker-Türk) by use of a light microscope. Organisms were serially diluted in PBS, and genomic DNA was extracted from 200 µl aliquots containing 0,01; 0,1; 1; 10; 100; 1000 or 10.000 organisms (n=2 each) by using a QIAamp DNA minikit, just like as previously described.
(ii) Detection limit in feline feces:
Twenty-microliter aliquots containing 0,01; 0,1; 1; 10; 100; 1000 or 8000 organisms (n=2 each) were added to 180 mg samples of feline feces obtained from a cat tested negative for *T. foetus* by PCR at feces, culture in In Pouch TF® medium and PCR at In Pouch TF® culture medium fluid. DNA was extracted from spiked fecal samples by using a QIAamp DNA minikit, just like as previously described.
(iii) Detection limit in sediment of In Pouch TF® medium:
Twenty-microliter aliquots containing 0,01; 0,1; 1; 10; 100; 1000 or 8000 organisms (n=2 each) were added to 180 µl samples of an for 12 days incubated (at 37°C) In Pouch TF® medium inoculated with feline feces obtained from a cat tested negative for *T. foetus* by PCR at feces, culture in In Pouch TF® medium and PCR at In Pouch TF® culture medium fluid. DNA was extracted from spiked fecal samples by using a QIAamp DNA minikit, just like as previously described.

Statistical analyses
All statistical analyses were performed with the use of a statistical software SPSS16 package. Statistical test results with a P value less than 0.05 were reported as significant.
The Chi-squared test and McNemar test were used to determine if there was a significant difference in outcome of the different diagnostic methods. The Wilcoxon rank sum test was

used to detect differences between the outcome positive or negative for PCR at feces for scores for fecal consistency in the longitudinal follow-up study.

Results

Comparison of the different diagnostic methods

Fifty-seven fecal samples and sixty-two inoculated In Pouch TF® media were obtained from veterinarians throughout the Netherlands. Samples were from 48 pure bred cats and 7 non purebred cats (6 domestic shorthair cats and 1 mixed breed), age ranging from 6 weeks to 10 years old. Twenty-four cats of the fifty-five sampled cats (43,6%) were tested positive for *T. foetus*. Diagnosis of *T. foetus* infection was made on the basis of results of the PCR at the feces (12 out of 24), microscopic examination of culture of feces with In Pouch TF® medium (11 out of 24) and/or PCR at the In Pouch TF® fluid medium (22 out of 24). Positive cats were between the ages of 6 weeks and 10 years, with a mean age of 20,8 months. Fifteen out of the twenty-four positive cats were ≤ 1 year old (62,5%). Most of the positive cats were reported to have chronic diarrhoea, often watery with blood or mucus and malodorant. The positive cats were often reported to respond not well to the established therapy, like metronidazole, fenbendazole and Finidiar®. Four of the positive cats were reported to have a concurrent infection with *Giardia* sp.. All of the positive cats were pure bred and included Maine Coon (6), Turkish Angora (3), Tonkinese (4), Persian (1), Sphynx (1), Burmese (3), Bengal (3), Devon Rex (1) and Abyssinian (2). Eight positive cats were intact female, eight were intact male, four were spayed female and four were neutered male.
Table 1 shows the summarized results of the outcome of the different diagnostic methods, used in this study. For complete testresults is referred to table 9 in the attachments.
Some cats were sampled repeated in our study and that is the reason this table shows results of 62 cats instead of the 55 sampled cats. Not from every cat in this study was feces available for microscopic examination and PCR.

Table 1. Summary data of the samples with the different diagnostic methods.

Outcome	PCR feces	In Pouch TF® medium	PCR In Pouch TF® culture medium
Positive	12	12	23
Negative	45	50	39
Total	57	62	62

Because we are interested in determining whether the outcomes described above in table 1 are related in some way, we test the null hypothesis of no association by calculating a Chi-squared test statistic. Table 2 shows the observed frequencies of positive and negative outcome with the 3 different diagnostic methods. The expected frequencies (rounded numbers) are shown in the table in brackets.

Table 2. Observed frequencies of positive and negative outcome with PCR at feces, In Pouch TF® medium and PCR at In Pouch TF® medium (expected frequencies are shown in brackets).

	PCR feces	In Pouch TF®	PCR In Pouch TF®	Total
+	12 (15,7)	12 (15,7)	23 (15,7)	47
-	45 (41,3)	45 (41,3)	34 (41,3)	124
	57	57	57	171

The test statistic gives ≈ 7,09. Referring this value to the Chi-squared distribution with 2 degrees of freedom, we obtain 0,025 < P < 0,050.
So we have evidence to reject the null hypothesis that the positive rates are the same for the three different methods. Thus, there is some evidence that the three different diagnostic methods show different rates of success.
Table 3, 4 and 5 show the observed frequencies of positive and negative outcome of two different diagnostic methods, so we can compare the diagnostic methods themselves. The expected frequencies (rounded numbers) are shown in the table in brackets.

Table 3. Observed frequencies of positive and negative outcome using methods PCR at feces and In Pouch TF® medium (expected frequencies are shown in brackets).

		In Pouch TF® medium		
		+	**-**	**Total**
PCR feces	**+**	8 (2,5)	4 (9,5)	12
	-	4 (9,5)	41 (32,4)	45
		12	45	57

The test statistic gives ≈ 19,37. Referring this value to the Chi-squared distribution with 1 degree of freedom, we obtain P < 0,001 (a computer analysis gives P=0,000).
The 95% confidence interval for the true difference is 0,298 to 0,857.
So we have evidence to reject the null hypothesis that the positive and negative rates are the same for the methods PCR at feces and In Pouch TF® medium. Thus, there is some evidence that the PCR at feces and the method In Pouch TF® medium show different rates in outcomes and that there is an association between these methods.

Table 4. Observed frequencies of positive and negative outcome using methods In Pouch TF® medium and PCR In Pouch TF® culture medium (expected frequencies are shown in brackets).

		PCR In Pouch TF® culture medium		
		+	**-**	**Total**
In Pouch TF® medium	**+**	12 (4,5)	0 (7,5)	12
	-	11 (18,5)	39 (31,4)	50
		23	39	62

The test statistic gives ≈ 24,81. Referring this value to the Chi-squared distribution with 1 degree of freedom, we obtain P < 0,001 (a computer analysis gives P=0,000).
The 95% confidence interval for the true difference is 0,318 to 0,726.
So we have evidence to reject the null hypothesis that the positive and negative rates are the same for the methods In Pouch TF® medium and PCR at In Pouch TF® medium. Thus there is some evidence that the In Pouch TF® medium and the method PCR at the In Pouch TF® medium show different rates of outcomes and that there is an association between these methods.

Table 5. Observed frequencies of positive and negative outcome using methods PCR feces and PCR In Pouch TF® culture medium (expected frequencies are shown in brackets).

		PCR In Pouch TF® culture medium		
		+	**-**	**Total**
PCR feces	**+**	10 (4,8)	2 (7,2)	12
	-	13 (18,2)	32 (26,8)	45
		23	34	57

The test statistic gives ≈ 11,52. Referring this value to the Chi-squared distribution with 1 degree of freedom, we obtain P < 0,001 (a computer analysis gives P=0,001).
The 95% confidence interval for the true difference is 0,158 to 0,593.
So we have evidence to reject the null hypothesis that the positive and negative rates are the same for the methods PCR at feces and PCR at In Pouch TF® medium. Thus there is any evidence that the PCR at feces and the method PCR at the In Pouch TF® medium show different rates of outcomes and that there is an association between these methods.

Officially, the Chi-square test can not be used to test correlated data, like paired observation, by which each observation in one group is paired or individually matched with an observation in the other group. With a Chi-square test the observations are always assumed to be independent of each other. Because we are dealing with paired observations, we used McNemar's test, which approximates the Chi-squared distribution. This test used to determine whether the null hypothesis, that the true proportions of successes using the two different diagnostic methods are equal, is true.

The McNemar test with the comparison of the methods PCR at the feces and the method In Pouch TF® medium gives ≈ 0,13. Referring this value to the Chi-squared distribution with 1 degree of freedom, we obtain P >0,5 (a computer analysis gives P=1,000).
So we don't have any evidence to reject the null hypothesis and the true proportions detected are the same using PCR at feces and the In Pouch TF® medium.

The McNemar test with the comparison of the methods culture of feces in In Pouch TF® medium and PCR at In Pouch TF® medium gives ≈ 9,09. Referring this value to the Chi-squared distribution with 1 degree of freedom, we obtain 0,010 < P > 0,001 (a computer

analysis gives P=0,001). The 95% confidence interval for the true difference in the proportions is 0,082 to 0,273.
So we have evidence to reject the null hypothesis and there is a difference in true proportions of successes, in this case PCR at In Pouch TF® medium detected more positive cats than the method In Pouch TF® medium.

The McNemar test with the comparison of the methods PCR at feces and PCR at In Pouch TF® medium gives ≈ 6,67. Referring this value to the Chi-squared distribution with 1 degree of freedom, we obtain 0,010 < P > 0,001 (a computer analysis gives P=0,007). The 95% confidence interval for the true difference in the proportions is 0,070 to 0,316.
So we have evidence to reject the null hypothesis and there is a different in true proportions of successes, in this case PCR at In Pouch TF® medium detected more positive cats than the method PCR at feces.

In order to say more about the sensitivity, specificity and predictive values of the different diagnostic values, we have established the PCR at the In Pouch TF® culture medium as the gold standard. In our experience this diagnostic method is the most sensitive diagnostic method in our situation (detects 22 out of 24 positive cats in our study).
Table 5 shows the comparison of the outcome of the methods PCR at feces versus PCR at the In Pouch TF® culture medium. We established with making a rough assumption the PCR at the In Pouch TF® culture medium as the gold standard, which means it is the same as the true diagnosis with outcome positive or negative.
Calculations show that the PCR at feces has:
a sensitivity of 10/ (10+13) ≈ 43,5%;
a specificity of 32/(32+2) ≈ 94,4%;
a positive predictive value (PPV) of 10/(10+2) ≈ 83,3%;
a negative predictive value (NPV) of 32/(13+32) ≈ 71,1%

Calculations show that the In Pouch TF® medium has:
a sensitivity of 12/(12+11) ≈ 52,2%;
a specificity of 39/(39+0) = 100%;
a positive predictive value (PPV) of 12/(12+0)= 100%
a negative predictive value (NPV) of 39/(39+11) ≈ 78%

For the DNA isolation procedure were 200 µl supernatant and 200 µl sediment of the fluid of the In Pouch TF® medium used. Table 6 shows the observed frequencies of positive and negative outcome comparing PCR at sediment of In Pouch TF® medium with PCR at supernatant of In Pouch TF® medium.
The McNemar test with the comparison of PCR at sediment of In Pouch TF® medium and PCR at supernatant of In Pouch TF® medium gives ≈ 0,17. Referring this value to the Chi-squared distribution with 1 degree of freedom, we obtain P >0,5 (a computer analysis gives P= 0,687).
So we don't have any evidence to reject the null hypothesis and the true proportions detected are the same using PCR at sediment of In Pouch TF® medium and PCR at supernatant of In Pouch TF® medium.

Table 6. Observed frequencies of positive and negative outcome with PCR In Pouch TF® sediment versus PCR In Pouch TF® supernatant.

		PCR In Pouch TF® sediment		
		+	-	Total
PCR In Pouch TF® super-natant	+	9	2	11
	-	4	28	32
		13	30	43

Longitudinal follow-up study:
Table 7 shows the results of the longitudinal follow-up study of two infected cats followed up for 4 weeks. In the table are the dates of the collected fecal samples described with the outcome of PCR assay at feces. We identified *T. foetus* by PCR at feces in 8 fecal samples and not in 16 of the 24 collected fecal samples of these two infected cats.

Table 7. Results of the longitudinal follow-up study of two infected cats followed up for 4 weeks. In the table stood dates of the collected fecal samples with the outcome of PCR assay at feces (neg.= negative; pos.= positive).

	Week 1			Week 2			Week 3			Week 4		
Cat 1	30/7: neg.	31/7: pos.	1/8: neg.	6/8: neg.	7/8: neg.	8/8: neg.	17/8: neg.	19/8: neg.	20/8: neg.	25/8: neg.	26/8: neg.	27/8: pos.
Cat 2	24/7: neg.	30/7: neg.	31/7: neg.	6/8: neg.	8/8: pos.	9/8: pos.	17/8: neg.	18/8: pos.	19/8: neg.	25/8: pos.	26/8: pos.	27/8: neg.

On the basis of the completed scoring forms, we have looked for any association between the outcome of the PCR assay at feces and fecal consistency. The different scores for fecal consistency were:
Stone-hard feces: 0
Normal feces : 1
Semiformed feces: 2
Gruel-like feces: 3
Liquid feces: 4
Number of observations of fecal consistency scores with outcome positive or negative with PCR at feces are shown in table 8. The Wilcoxon rank sum test was used to detect differences between the outcome positive or negative for PCR at feces for scores for fecal consistency in the longitudinal follow-up study. Table 11 in the attachments shows results of the outcome of the PCR assay at feces and fecal consistency, where the observations of fecal consistency were ranked together.

Table 8. Number of observations of fecal consistency scores with PCR at feces positive or negative.

	Faecal consistency scores				
PCR assay feces	**0**	**1**	**2**	**3**	**4**
+	0	1	4	3	0
-	0	7	4	5	0

Using the Wilcoxon rank sum test with these results, a computer analysis gives P=0,299. Thus the data are consistent with the null hypothesis that there is no difference between the outcome positive or negative for PCR at feces for different scores for fecal consistency.

Pilot study: Time before incubation In Pouch TF® medium
The pouches incubated at 37°C at different time points (day 0, 1, 2, 3, 4 and 5 after inoculation) were all positive for motile trophozoites.
The 5 different pouches inoculated with voided feces of an infected cat arrived at 4 days after inoculation. Because the overgrowth of fecal flora impaired the observation of 2 In Pouch TF® pouches, we have inoculated 2 new pouches with medium fluid of each In Pouch TF® medium. Only these pouches were positive for motile trophozoites, namely since 10 and 11 days after inoculation.

Determination detection limits real-time PCR:
(i) Absolute detection limit:
The absolute detection limit of our real-time PCR was 10 organisms per 200 µl. The PCR was able to detect 10 organisms 66,7% of the time (4 of 6 reactions) and 100 organisms 100% of the time (6 of 6 reactions).
(ii) Detection limit in feline feces:
T. foetus was not detected by RT-PCR in our spiked fecal samples, thus the detection limit is higher than 800 organisms per 200 mg of feces.
(iii) Detection limit in sediment of In Pouch TF® medium:
The practical detection limit in 180 µl samples of sediment of In Pouch TF® medium spiked with 20 µl aliquots serially diluted *T. foetus* was 10 organisms per 200 µl. The PCR was able to detect 10 organisms 66,7% of the time (4 of 6 reactions) and 100 organisms 100% of the time (6 of 6 reactions).

Conclusion

In this study diagnosis of *T. foetus* infection was made on results of the PCR at feces, culture of feces with In Pouch TF® medium and/or PCR at In Pouch TF® fluid medium. In the present study *T. foetus* infection was found in cats between ages of 6 weeks and 10 years, with a mean age of 20,8 months. The majority of the positive cats (62,5%) were ≤ 1 year old. All of the positive cats were pure bred cats and most of the cats were reported to have chronic diarrhoea, often water-thin, with blood or mucus and malodorant.
Based on the results of present study, *T. foetus* infection was found with PCR at feces in 12 of the 24 positive cats, with the In Pouch TF® medium in 11 of the 24 positive cats and with PCR at In Pouch TF® fluid medium in 22 of the 24 positive cats. Comparison between the three different diagnostic methods shows a statistic significant difference based on the Chi-squared test.
The Chi-squared test shows there is a difference between the outcomes of the methods PCR at feces and the In Pouch TF® medium. However, based on the McNemar test we can conclude there is no difference between the true proportions of successes using these two methods. In comparison with our established gold standard the PCR at In Pouch TF® culture medium we found the PCR at feces has a sensitivity of 43,5%, a specificity of 94,4%, the positive predictive value is 83,3% and the negative predictive value is 71,1%, while the In Pouch TF® medium has a sensitivity of 52,2%, a specificity of 100%, the positive predictive values is 100% and the negative predictive value is 78%.
Based on these results we can conclude that the In Pouch TF® medium is a more sensitive method and furthermore gives no false positive results in comparison with the method PCR at feces. The usefulness of positive outcome with In Pouch TF® medium is 100% and higher than that from the PCR at feces. In other words, the probability that the patient really has a *T. foetus* infection in case of a positive test is higher (and 100%) with the method In Pouch TF® medium than with the method PCR feces (94,4%). The negative predictive value is also higher with the method In Pouch TF® medium in comparison with the method PCR at feces. Thus, the probability that in case of a negative test result, the patient has really not a *T. foetus* infection is higher with use the In Pouch TF® medium than with use of PCR at feces.
When comparing the methods In Pouch TF® medium with the method PCR at In Pouch TF® medium the Chi-square test shows there is a difference between the outcomes of these methods. Moreover, the McNemar test shows a statistic significant difference in true proportions of successes. The PCR at In Pouch TF® medium detected more positive cats and is a more sensitive method than the In Pouch TF® medium.
In the comparison of the PCR at feces with the PCR at In Pouch TF® medium, the Chi-square test shows there is difference between the outcomes of these methods. The McNemar test shows further a difference in true proportions of successes between these methods. The PCR at In Pouch TF® medium detected more positive cats than the PCR at feces and therefore the PCR at In Pouch TF® medium is a more sensitive method than the PCR at feces.
For the PCR at In Pouch TF® medium supernatant and sediment of fluid of this medium is used. The McNemar test shows no statistic significant difference between the positive outcome of using sediment or supernatant in the PCR at In Pouch TF® medium.
Based on the results of present study, we can conclude that PCR at In Pouch TF® fluid medium is the most sensitive diagnostic method for detection of a *T. foetus* infection. Furthermore, there is a minimum difference between sensitivity of PCR at feces versus fecal culture in In Pouch TF® medium. Based on a comparison of the different diagnostic methods, we concluded a relative efficacy for the detection of *T. foetus* organisms to be as follows: PCR at feces < fecal culture in In Pouch TF® medium < PCR at In Pouch TF® medium.

Furthermore, our results have not shown any difference in true proportions of successes between use of sediment or supernatant in the PCR at In Pouch TF® medium.

Results of our longitudinal follow-up study of two infected cats have shown that we can not detect *T. foetus* by PCR in all of the collected fecal samples of these cats. From this we can conclude that we can speak of intermittent shedding of the parasite.
Furthermore, we can conclude, based on the result of the Wilcoxon rank sum test, that there are no differences between the outcome positive or negative for PCR feces for different scores for fecal consistency.

Based on the results of our pilot study aimed at time before incubation of an In Pouch TF® medium, we can conclude that an inoculated In Pouch TF® medium can be succesfully incubated 5 days after inoculation. Five days after inoculation, without incubation at 37°C, the pouches were still positive for motile trophozoites.

In the current study the detection limits of our real-time PCR were determined. The absolute detection limit of our real-time PCR was 10 organisms per 200 µl. In the field of reproducibility, the PCR was able to detect 10 organisms 66,7% of the time (4 of 6 reactions) and 100 organisms 100% of the time (6 of 6 reactions). *T. foetus* was not detected by RT-PCR in our spiked fecal samples and for that reason we can only conclude that the detection limit is anyhow higher than 800 organisms per 200 mg of feces. The practical detection limit in 180 µl samples of sediment of In Pouch TF® medium spiked with 20 µl aliquots serially diluted *T. foetus* was 10 organisms per 200 µl. This is similar to the detection limit of the absolute detection limit. The PCR was able to detect 10 organisms 66,7% of the time (4 of 6 reactions) and 100 organisms 100% of the time (6 of 6 reactions). The reproducibility is also the same as that of the absolute detection limit. Thus, we can conclude that sediment of In Pouch TF® medium has no effect on the detection limit of the PCR.

Discussion

In the present study *T. foetus* infection was found in young cats (62,5% were ≤ 1 year old), in pure bred cats and most cats had a medical history of chronic diarrhoea. This is consistent with results of previous studies [11][16][21][23][24][37][38].
This study was aimed at the comparison of the PCR at feces, fecal culture in In Pouch TF® medium and PCR at In Pouch TF® medium. In this study it was attempted to compare the different diagnostic methods as fair as possible. Important is that the samplings have taken place at the same moment (at least on the same day), because of the possible intermittent shedding of *T. foetus*. However, this was not always practically feasible.
Based on the results of our study, we concluded a relative efficacy for the detection of *T. foetus* organisms to be as follows: PCR at feces < fecal culture in In Pouch TF® medium < PCR at In Pouch TF® medium. In the present study the PCR at feces is the least sensitive method and has a low relative sensitivity of 43,5%. Thus, the PCR at feces gives a lot of false-negative results, probably because of the presence of inherent PCR inhibitors, such as heme, bilirubins, bile salts, and complex carbohydrates, which are often coextracted along with pathogen DNA [11]. On the other hand, the PCR at feces has rather a high specificity of 94,4% and a positive predictive value of 83,3%, thus in case of a positive test result it is quite certain that we can speak of a *T. foetus* infection. Taking into account this is based on determined prevalence.
In our study *T. foetus* was not detected by RT-PCR in our spiked fecal samples. Further research should be done with a higher concentration of organisms in the dilution series. In a previous research project the detection limit in spiked fecal samples could be determined. The detection limit was 1000 organisms per 200 mg of feces [6]. This is in contrast with the detection limit found in the study of Gookin et al. (2002). They found a detection limit of 10 organisms per 200 mg feces. However, Gookin et al. (2002) use a single-tube nested PCR instead of our used real-time PCR. Based on a comparison of diagnostic methods, Gookin et al. (2004) found a sensitivity of 94,4% (34 out of 36). This is in contrast with the sensitivity found in our study. Probably this has something to do with difference in type of PCR and the refinement of their used technique. In the use of our PCR assay, refinement of our technique is certainly possible. Our used PCR runs a programme with 50 cycles. However, for positive samples, the amplification curve rises at around 16-32 cycles and for negative samples at around 34-37 cycles. Thus, the number of PCR cycles can be reduced, so less non-specific products may amplify. Reducing the number of PCR cycles, can provide optimization of our technique. It is important that more focused research in this field is done.
Because of possible presence of inherent PCR inhibitors in fecal samples, the DNA isolation procedure can be optimized. Isolating DNA from feces is one of the most challenging aspects of molecular diagnostics [22]. For example, considerable improvement in sensitivity can be obtained by optimizing the duration and temperature of proteinase K digestion and by adding an additional wash step prior to DNA elution [11].
DNA samples can be tested for PCR inhibitors by a separate reaction in which bacterial 16S rRNA genes were amplified. If bacterial 16S rRNA genes can not be amplified, PCR inhibitors are present in the fecal sample [16][17]. To minimize the possibility of false negative results caused by the presence of PCR inhibitors, DNA samples could be tested for PCR inhibitors in the future. Probably this is not practically feasible and is more common in a experimental setting.
Sometimes the signal, seen in the melting curve, in the real-time PCR is unclear, making it difficult to determine whether a sample is positive or negative. Probably the number of organisms in the sample is below the detection limit of the PCR. Possibly, it will help to get higher concentration of higher DNA elute during the DNA isolation procedure. However,

according the QIAamp DNA Stool Handbook of the QIAamp DNA Stool Mini kit one must use the minimum amount of DNA eluate possible in PCR. The volume of eluate used as template should not exceed 10% of the final volume of the PCR mixture. In this area it is likely to achieve minimal optimization of the PCR assay.
In our study we found a minimal difference in the sensitivities of the methods PCR at feces and the In Pouch TF® medium. In comparison with our gold standard, the In Pouch TF® medium has a sensitivity of 52,2%, which is higher than the sensitivity of the PCR at feces. The specificity of the In Pouch TF® medium is 100%, which means there are no false positives with this method. The manufacturer of the In Pouch TF® medium also guarantees based on the study of Gookin et al. (2003) that the In Pouch TF® medium is selective and *P. hominis* and *Giardia* sp. do not survive in the In Pouch TF® medium. However, the negative predictive value of the In Pouch TF® medium is 78%. The diagnostic method depend on the demonstration of live organism, thus use of fresh voided feces for the inoculation of the In Pouch TF® medium is very important. Hale et al. (2009) found recently that the environmental resilience of *T. foetus* is greater than previously anticipated. They recommend that cat feces has to be inoculated into culture within a 6 hour period from voiding. Inoculation of the In Pouch TF® medium with too much feces reduced the sensitivity, presumably because feces contains large number of competitive microorganisms [12]. To minimize this it is, in our experience, important to inoculate an In Pouch TF® medium with feces collected by a rectal swab and not from voided feces. It is too difficult to obtain a small amount of feces, when specimen for inoculation is collected of feces that has been voided. If the overgrowth of fecal flora impaired the observation, we inoculate a new In Pouch TF® medium with fluid of the In Pouch TF® medium with overgrowth of fecal flora. However, Hale et al. (2009) found that overgrowth of fecal flora within the In Pouch TF® medium could be tolerated by adopting the following protocol for pouch examination: (1) best results were achieved by examining the In Pouch without mixing of the contents prior to visualisation due to the concentration of *T. foetus* amongst the sediment at the bottom seam of the pouch and otherwise 2) gently pulling the pouch against a bench top prior to re-examination of the pouch contents followed by focusing around the remaining sediment at the bottom of the lower chamber.
In comparison of the different diagnostic methods, the PCR at In Pouch TF® medium is the most sensitive method (detects 22 out of 24 positive cats). The detection limit in 180 µl samples of sediment of In Pouch TF® medium spiked with 20 µl aliquots serially diluted *T. foetus* was 10 organisms per 200 µl. This is similar to the detection limit of the absolute detection limit. The reproducibility is also the same as that of the absolute detection limit. Probably, presence of inherent PCR inhibitors plays no role in the sediment of an In Pouch TF® medium. It is plausible that this is because of the smaller amount of specimen inoculated in the In Pouch TF® medium compared to the 200 mg fecal sample used in the DNA isolation procedure for the PCR assay. This possibly is the reason why in this study in 2 cats the infection is not diagnosed by PCR at In Pouch TF® medium, but only by PCR at feces. A smaller amount of fecal sample can reduce the chance of detecting the parasite. Another possibility is that DNA is destructed during inoculation.
Possibly, the PCR at In Pouch TF® medium is more sensitive than the PCR at feces, because multiplication of *T. foetus* has occurred in the In Pouch TF® medium, although the parasite was not seen by microscopic examination. In this way there is also multiplication of DNA of the parasite, making it easier to be detected by the PCR.
Our study shows no statistic significant difference between the positive outcome of using sediment or supernatant in the PCR at In Pouch TF® medium. Because of the weight of the trophozoites, it would be most logical that the probability of detection of *T. foetus* is greatest in the sediment. In our study we could not prove that statistically, probably because we had

too few samples. To show this difference in the use of sediment or supernatant in the PCR at In Pouch TF® medium, more research should be done. However, in some cats the infection is only detected in the supernatant by PCR at In Pouch TF® medium. One reason could be that sublethal trophozoites were in the In Pouch TF® medium, which have fallen apart during the 12 days incubation. DNA of these trophozoites is released into solution, whereby it can be detected only in the supernatant. The question remains what the clinical value of such a outcome is. To be able to say more about this, it is important that more research is done to the shedding patron of infected cats with clinical signs and aimed at how much organisms shedding by an infected cat is infective for another cat.

Based on the fact we can not detect *T. foetus* by PCR in all of the collected fecal samples of two infected cats in our longitudinal follow-up study, we can possibly speak of intermittent shedding of the parasite. This is consistent with several studies, which have suggested possible intermittent shedding of *T. foetus* [17][22][37][42]. Capital Diagnostics SAC Veterinary Services at Penicuik have analysed fecal samples collected every three hours over a 12-hour period from one infected cat and identified *T. foetus* by PCR in the first and last sample, but not in the 2nd, 3rd and 4th samples [45]. However, the problem with documenting intermittent shedding is that although the PCR assay is highly sensitive, negatives will be seen, which might be caused by low numbers of organisms, intermittent shedding or sample storage/carriage conditions. Whilst developing the PCR assay technicians of Capital Diagnostics SAC Veterinary Services at Penicuik had the opinion that bacterial DNAases in feces were affecting the amount of *T. foetus* DNA available for extraction, and the longer they left a sample at room temperature the more difficult is was to extract TF DNA [45].
In our study no differences were found between the outcome positive or negative for PCR at feces for different scores for fecal consistency. Our expectation was that if a cat is shedding more *T. foetus*, there is more damage to the colon, leading to reduction of fecal consistency. Shedding more of the parasite, ensures a greater chance of detection of the parasite. However, as previously described, more things are important in the field of the detection of *T. foetus* by PCR assay at feces, such as sample storage and carriage conditions. Moreover, there are probably too few samples in this survey to demonstrate a statistically significant difference.

Results of our pilot study aimed at time before incubation an In Pouch TF® medium show that an inoculated In Pouch TF® medium can be incubated 5 days after inoculation. Five days after inoculation, without incubation at 37°C, the pouches were still positive for motile trophozoites. Nevertheless, this is of course dependent on the specimen collection technique for inoculation of the In Pouch TF® medium, because of the risk of overgrowth of fecal flora and how much organisms the sampled cat is shedding.

Gookin have described that overgrowth of fecal flora developed rapidly in cultures incubated at 37°C as compared to cultures incubated at 25°C [12]. Our experience is that survival of trophozoites in In Pouches TF® incubated at 25°C is longer than In Pouches TF® incubated at 37°C. In pouches incubated at 37°C also show trophozoites in a more rounded shape. Such changes in morphology have been described in several reports as signs of stress caused by variations in temperature or other physical influences or as effects of treatments with various drugs [8]. These conditions induce internalisation of flagella. It is a reversible formation of pseudocysts, which is probably a responsive survival mechanism [20].

At last, based on previously described conclusions, we advise the use of the In Pouch TF® medium and the PCR at In Pouch TF® medium as diagnostic methods for detection of a *T. foetus* infection. However, if motile trophozoites are seen by microscopial examination PCR

at In Pouch TF® medium is not necessary because the manufacturer of the In Pouch TF® medium guarantees based on the study of Gookin that the In Pouch TF® medium is selective and *P. hominis* and *Giardia* sp. do not survive in the In Pouch TF® medium [12]. When using the In Pouch TF® medium as diagnostic method, it is important that the In Pouch TF® medium is not inoculated by an excessive amount of feces. The best way to obtain a fecal sample for the In Pouch TF® medium is with the rectal swab. If overgrowth of fecal flora develops anyhow, we advise to inoculate a new In Pouch TF® medium with minimal amount of fluid of the old In Pouch TF® medium. Otherwise, the protocol compiled by Hale et al. (2009) can be followed, but we do not have experience with this protocol. Although we have demonstrated that pouches are still positive for motile trophozoites when they are incubated 5 days after inoculation without inoculation at 37°C, it remains important to incubate an inoculated In Pouch TF® medium as soon as possible to avoid risk of false negatives.
Furthermore, we advise use of sediment of the medium fluid with PCR at In Pouch TF® medium. Theoretically, use of the sediment in the PCR at In Pouch TF® medium has a greater sensitivity for detection of a *T. foetus* infection. Based on the results of this study, some infected cats can not be detected with only use of sediment in the PCR at In Pouch TF® medium, but the question still remains what the clinical value is of such case.
First of all further research should be done on the shedding pattern of cats, especially cats which are only positive in the PCR at the In Pouch TF® medium using supernatant of the culture fluid. Use the sensitive diagnostic method the PCR at In Pouch TF® medium may be important for the detection of carriers in the population. Whether these carriers are shedding enough to infect other cats, needs further research.

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Attachments

Table 9. The testresults of the samples with the different diagnostic methods.

	PCR feces	In Pouch TF® medium	PCR In Pouch TF® culture medium
Cat 1	Negative	Negative	Positive
Cat 2	Positive	Positive	Positive
Cat 3	Negative	Negative	Negative
Cat 4	Positive	Negative	Positive
Cat 5	Positive	Negative	Positive
Cat 6	Negative	Negative	Negative
Cat 7	Negative	Negative	Positive
Cat 8	Negative	Negative	Positive
Cat 9	Negative	Negative	Negative
Cat 10	Negative	Negative	Negative
Cat 11	N/A	Negative	Negative
Cat 12	Negative	Positive	Positive
Cat 13	N/A	Negative	Negative
Cat 14	N/A	Negative	Negative
Cat 15	Negative	Negative	Negative
Cat 16	Negative	Negative	Negative
Cat 17	Negative	Negative	Negative
Cat 18	Negative	Negative	Negative
Cat 19	Negative	Negative	Negative
Cat 20	Negative	Negative	Negative
Cat 21	Negative	Negative	Negative
Cat 22	Negative	Negative	Negative
Cat 23	Negative	Negative	Negative
Cat 24	Negative	Negative	Negative
Cat 25	Negative	Negative	Negative
Cat 26	Negative	Negative	Negative
Cat 27	Negative	Negative	Negative
Cat 28	Negative	Negative	Negative
Cat 29	Negative	Negative	Negative
Cat 30	N/A	Negative	Negative
Cat 31	Negative	Negative	Positive
Cat 32	Negative	Positive	Positive
Cat 33	Negative	Negative	Positive
Cat 34	Negative	Negative	Positive
Cat 35	Negative	Negative	Positive
Cat 36	Negative	Negative	Positive
Cat 37	Negative	Negative	Positive
Cat 38	Negative	Negative	Negative
Cat 39	Negative	Positive	Positive
Cat 40	Negative	Negative	Negative
Cat 41	Negative	Negative	Negative
Cat 42	Negative	Negative	Negative
Cat 43	Positive	Negative	Negative
Cat 44	Positive	Positive	Positive

Cat 45	Positive	Positive	Positive
Cat 46	Negative	Negative	Negative
Cat 47	Negative	Positive	Positive
Cat 48	Positive	Negative	Negative
Cat 49	Positive	Positive	Positive
Cat 50	N/A	Negative	Negative
Cat 51	Positive	Positive	Positive
Cat 52	Negative	Negative	Negative
Cat 53	Negative	Negative	Negative
Cat 54	Negative	Negative	Negative
Cat 55	Positive	Positive	Positive
Cat 56	Positive	Positive	Positive
Cat 57	Positive	Positive	Positive
Cat 58	Negative	Negative	Negative
Cat 59	Negative	Negative	Negative
Cat 60	Negative	Negative	Negative
Cat 61	Negative	Negative	Negative
Cat 62	Negative	Negative	Negative

Table 10. The testresults of the samples with PCR In Pouch TF® sediment versus PCR In Pouch TF® supernatant.

	PCR In Pouch TF® sediment	**PCR In Pouch TF® supernatant**
Cat 1	Positive	Positive
Cat 2	Positive	Positive
Cat 3	N/A	Negative
Cat 4	N/A	Positive
Cat 5	N/A	Positive
Cat 6	N/A	Negative
Cat 7	N/A	Positive
Cat 8	N/A	Positive
Cat 9	N/A	Negative
Cat 10	N/A	Negative
Cat 11	N/A	Negative
Cat 12	N/A	Positive
Cat 13	N/A	Negative
Cat 14	N/A	Negative
Cat 15	N/A	Negative
Cat 16	Negative	Negative
Cat 17	Negative	Negative
Cat 18	Negative	Negative
Cat 19	Negative	Negative
Cat 20	Negative	Negative
Cat 21	Negative	Negative
Cat 22	Negative	Negative
Cat 23	Negative	Negative
Cat 24	Negative	Negative
Cat 25	Negative	Negative
Cat 26	Negative	Negative
Cat 27	Negative	Negative

Cat 28	Negative	Negative
Cat 29	Negative	Negative
Cat 30	Negative	Negative
Cat 31	Negative	Positive
Cat 32	Positive	Positive
Cat 33	Negative	Positive
Cat 34	Positive	Positive
Cat 35	Positive	Positive
Cat 36	Positive	Positive
Cat 37	Positive	Negative
Cat 38	Negative	Negative
Cat 39	Positive	Positive
Cat 40	Negative	Negative
Cat 41	Negative	Negative
Cat 42	Negative	Negative
Cat 43	Negative	Negative
Cat 44	Positive	Positive
Cat 45	Positive	Positive
Cat 46	Negative	Negative
Cat 47	Positive	Negative
Cat 48	Negative	Negative
Cat 49	Positive	Negative
Cat 50	Negative	Negative
Cat 51	Positive	Negative
Cat 52	Negative	Negative
Cat 53	N/A	Negative
Cat 54	N/A	Negative
Cat 55	N/A	Positive
Cat 56	N/A	Positive
Cat 57	N/A	Positive
Cat 58	N/A	Negative
Cat 59	Negative	Negative
Cat 60	Negative	Negative
Cat 61	Negative	Negative
Cat 62	Negative	Negative

Table 11. Results of the observations of fecal consistency with PCR at feces positive or negative ranked together.

PCR assay feces positive	PCR assay feces negative	Rank
1		4,5
	1	4,5
	1	4,5
	1	4,5
	1	4,5
	1	4,5
	1	4,5
	1	4,5

2		12,5
2		12,5
2		12,5
2		12,5
	2	12,5
	2	12,5
	2	12,5
	2	12,5
3		20,5
3		20,5
3		20,5
	3	20,5
	3	20,5
	3	20,5
	3	20,5
	3	20,5

Calculation dilution series for determination detection limits real-time PCR:

Through using a haemocytometer (Bürker-Türk) 40 x 10^4 trophozoietes per millilitre were counted. This represents 8 x 10^4 trophozoietes per 200 microlitre. Trophozoietes were 1:8 diluted in PBS and the 200 µl aliquots containing 10.000 organisms were obtained. This is serially diluted 1:10 and 200 µl aliquots containing 1.000; 100; 10; 1; 0,1; 0,01 were obtained. The 20 µl aliquots containing 1000 organisms were obtained by a 1:10 dilution of the 200 µl aliquot containing 10.000 organisms. This is serially diluted 1:10 and 20 µl aliquots containing 100; 10; 1; 0,1; 0,01 were obtained. The 20 µl aliquots containing 8.000 organisms were obtained by a 1:10 dilution of the 200 µl aliquot containing 8 x 10^4 trophozoietes.

Brief voor de dierenarts met gedetailleerde informatie met betrekking tot het onderzoek

Geachte dierenarts (naam),

Naar aanleiding van ons telefonisch contact op (datum) stuur ik u deze brief.
Eerst zal ik mij even voorstellen. Ik ben Bianca Vermeulen en ik ben 5e jaars student diergeneeskunde. Ik doe op dit moment een onderzoeksstage bij de afdeling Klinische Infectiologie/VMDC van het Departement Infectieziekten en Immunologie van de Faculteit Diergeneeskunde. Mijn onderzoek betreft de diagnostiek van *Tritrichomonas foetus* bij de kat.

T. foetus is een flagellaire darmprotozo die chronische diarreeklachten bij voornamelijk jonge katten kan veroorzaken. In Nederland is de infectie sporadisch en wordt op dit moment met name gediagnosticeerd bij raskatten. *T. foetus* lijkt in Nederland bij de kat pas recent te zijn geïntroduceerd en er is weinig bekend over de prevalentie en de epidemiologie. Het frustrerende probleem van chronische diarree bij jonge katten, die niet op de ingezette therapieën reageren, zou eventueel te wijten kunnen zijn aan deze parasiet.
De infectie is weliswaar te behandelen met ronidazol (2dd gedurende 14 dagen!), maar dit geeft soms ernstige bijwerkingen. Naar alle waarschijnlijkheid is de infectie uiteindelijk zelflimiterend; de meeste katten 'groeien er overheen'.

Trichomoniase bij de kat kan op verschillende manieren gediagnosticeerd worden:
1. Door microscopisch onderzoek van een warm natief fecespreparaat. Onder praktijkomstandigheden is hierbij de parasiet (beweeglijkheid) te detecteren, dit vereist echter de nodige ervaring.
2. Daarnaast kan *T. foetus* in een geschikt medium, In Pouch TF®medium, gekweekt worden. Dit medium kan microscopisch onderzocht worden. Hierbij is het gebruik van verse, warme feces eveneens vereist.
3. Tenslotte maakt het VMDC gebruik van een PCR. Hiervoor hoeft het fecesmonster niet vers (warm) te zijn, aangezien er voor de detectie van het DNA geen levende parasieten aanwezig hoeven te zijn. Het is nog niet bekend hoe gevoelig deze test is, m.a.w. wat de diagnostische waarde van een negatieve uitslag is.

Voorts zou er sprake kunnen zijn van intermitterende uitscheiding, wat uitsluiting bemoeilijkt. Door middel van een longitudinaal onderzoek, waarin door *T. foetus* geïnfecteerde katten gevolgd worden in de tijd, kunnen we meer zeggen over het uitscheidingspatroon van *T. foetus*. De op deze manier verkregen informatie is voor de praktijk van groot belang voor de interpretatie van toekomstige negatieve uitslagen.

Van de door u opgestuurde monsters wordt er ten eerste een volledig parasitologisch onderzoek gedaan op wormeieren, cysten en oöcysten.
Daarna wordt er ten behoeve van het onderzoek naar *T. foetus* een PCR gedaan op het directe fecesmonster. Tevens wordt het met verse feces beënte In Pouch TF® medium gedurende 12 dagen bebroed en microscopisch onderzocht. Tenslotte wordt er een PCR uitgevoerd op het sediment van de beënte In Pouch. Deze verschillende diagnostische methodieken worden met elkaar vergeleken.
Herhaald fecesonderzoek van een positief bevonden kat gedurende een bepaalde periode kan voor de interpretatie van de uitslagen veel extra informatie geven. Bij een positieve uitslag neem ik dan ook contact met u op om nadere afspraken te maken voor wat betreft het mogelijk longitudinaal vervolgen van de patiënt.

U krijgt via het VMDC de uitslag van het parasitologisch onderzoek (binnen 1 werkdag) en het onderzoek specifiek op *T. foetus* op de gebruikelijke wijze (in dit geval binnen 14 werkdagen).
Voor dit onderzoek ben ik op zoek naar fecesmonsters van jonge (ras)katten met chronische diarree. Insluitcriteria hierbij zijn:
- Jonge katten in de leeftijd vanaf 2 maanden tot 18 maanden .
- Chronische diarree, die aanhoudt ondanks de gebruikelijk ingestelde therapie.
- Diarree: van brijige feces tot aan duidelijke diarree.
- Beschikbaarheid van verse -warme- feces: niet ouder dan een half uur.

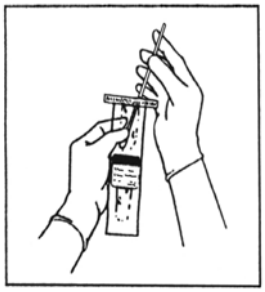
Bij deze brief zijn drie fecesmonsterpotjes, drie In Pouch TF® kweekmedia, drie swabs, een gebruiksaanwijzing en enveloppen voor het retourneren geleverd.
Voor het fecesmonster voor de PCR en het parasitologisch onderzoek heb ik ongeveer 5 gram feces nodig en de In Pouch dient beënt te worden met een swabje verse, nog warme, liefst rectaal genomen feces (iets kleiner dan een peperkorrel). De benodigde patiëntinformatie (naam kat, geslacht, leeftijd, ras, datum monstername, gegevens DAP, naam eigenaar, aantal katten en de huisvesting, aard en duur van de diarree, ingestelde behandeling en het effect hiervan) kan op het bijgeleverde aanvraagformulier worden ingevuld.
Monsters kunnen tot eind augustus 2009 ingestuurd worden.
Hopelijk heb ik u hiermee voldoende geïnformeerd. Indien u nog vragen heeft, kunt u contact opnemen met ondergetekende.

Met vriendelijke groeten,

Bianca Vermeulen
Tel: -
e-mail: B.D.Vermeulen@students.uu.nl

Gebruiksaanwijzing voor het bemonsteren van jonge katten met chronische diarree:

Ongeveer 5 gram feces is voldoende voor het parasitologisch onderzoek en de PCR, dit kan in het meegestuurde buisje verstuurd worden. Indien dit niet binnen enkele uren verstuurd kan worden dient het in de koelkast te worden bewaard. Voor het In Pouch TF® medium is het de bedoeling dat u dit beënt met een rectaal genomen fecesmonster. Gebruik hiervoor de bijgeleverde steriele swab. De swab hoeft alleen maar bedekt te zijn met feces, een kleine hoeveelheid voor het beënten is namelijk al voldoende. Mocht het rectaal afnemen met een swab niet mogelijk zijn, dan kan voor het beënten van het medium ook een swab van vers gedeponeerde warme ontlasting van ongeveer 0,05 gram (dit is iets kleiner dan een peperkorrel) genomen worden, waarmee het medium kan worden beënt.
Voor het beënten van het medium wordt eerst grotendeels de vloeistof, die in de bovenste kamer zit naar beneden gemasseerd om lekken vanuit de bovenkant te voorkomen. De bedoeling is dat er ongeveer 1 ml in de bovenste kamer achterblijft voor het beënten.



Hierna kan de bovenkant opengeknipt worden, waarna het zakje geopend kan worden door te trekken aan de witte stripjes, die aan beide kanten zitten. Dan kan de swab in het medium in de bovenste kamer gebracht worden en kan het monster door middel van het rollen van de swab tegen de wand aan in het medium terecht komen. Als laatste wordt het In Pouch TF® medium zakje gesloten door de bovenkant dicht te drukken en het verder voor 2-3 keer op te rollen. Het is gewenst om op het etiket van de In Pouch (gelieve niet op de rest van het mediumzakje) en het potje de volgende informatie te noteren: Naam kat, geslacht, leeftijd kat, ras, datum monstername, gegevens DAP en naam eigenaar.
Op het bijgeleverde aanvraagformulier kan de uitgebreide patiëntgerelateerde informatie, namelijk naam kat, geslacht, leeftijd, ras, datum monstername, gegevens DAP, naam eigenaar, aantal katten en de huisvesting, aard en duur van de diarree, ingestelde behandeling en het effect hiervan en eventuele andere informatie die u van belang acht, genoteerd worden. Het monster dezelfde dag opsturen in de bijgeleverde envelop. Dit kan via de reguliere post of via de VMDC-ophaalservice als u hier al gebruik van maakt.

Bij voorbaat dank. Hopend u hiermee voldoende geïnformeerd te hebben. Bij vragen kunt u contact opnemen met ondergetekende.

Mocht u meer monstermateriaal nodig hebben, dan kunt u ook contact opnemen met ondergetekende.

Met vriendelijke groeten,

Bianca Vermeulen
Tel: -
e-mail: B.D.Vermeulen@students.uu.nl

Naam kat:…….
Naam eigenaar:……
……...

Scoring formulier klinisch verloop voor longitudinaal vervolg

- Datum monstername: ……
- De kat is vandaag: levendig / sloom / anders*, namelijk……
- Vragen met betrekking tot het aspect van de ontlasting op dag van monstername*:

Slijm bij de ontlasting	Wel	niet

Bloed bij de ontlasting	Wel	niet

Consistentie ontlasting	Keiharde ontlasting	harde ontlasting	wat slap	brijachtig	waterdun	anders,nl.:... .....

Kleur	Bruin	zwart	lichtbruin	geel	grijs	anders,nl.:... .....

Persen bij het poepen	Wel	niet	onbekend

Winderigheid	Wel	niet	onbekend

- Vragen met betrekking tot mogelijke behandeling van de kat:

De kat wordt op dit moment WEL / NIET* behandeld voor de diarreeklachten, namelijk met het middel:…….(naam middel en dosering).

Op dit moment is de kat op dag*:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	...

van de behandeling.

* Omcirkelen wat van toepassing is.

Informatie voor eigenaar over een *Tritrichomonas foetus* infectie bij de kat

Sinds korte tijd is bekend dat de parasiet *Tritrichomonas foetus* bij de (jonge) kat voorkomt en chronische diarreeklachten kan veroorzaken. Verder is er nog vrij weinig bekend over de infectie bij de kat en is verder onderzoek van groot belang.

T. foetus kan buiten de kat slecht overleven. Men zegt dat de parasiet buiten de kat binnen een half uur sterft.[1] Om een andere kat dus te kunnen infecteren, moeten katten intensief contact met elkaar hebben. De kat infecteert zichzelf door hele verse ontlasting van een geïnfecteerde kat op te likken.

Uit verschillende onderzoeken blijkt dat de gemiddelde leeftijd van een geïnfecteerde kat rond de 7 à 9 maanden ligt. Infectie bij katten ouder dan één jaar wordt nauwelijks gezien.
Over het algemeen zijn de geïnfecteerde katten niet ziek, maar wordt er alleen diarree gezien. Soms zit er slijm of zelfs bloed bij de diarree. In Nederland wordt de infectie nog maar zelden gezien.

Er zijn verschillende manieren om de infectie bij een kat te diagnosticeren; microscopisch onderzoek, kweken van de parasiet en het aantonen van het DNA van de parasiet.
Geen enkele van deze onderzoeksmethoden kan echt op ieder moment de parasiet aantonen.

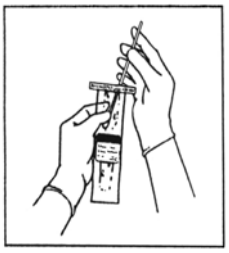
Een *T. foetus* infectie kan behandeld worden met het middel ronidazol. Dit moet gedurende 14 dagen gegeven worden en kan nare bijwerkingen hebben in de vorm van neurologische verschijnselen. U moet dan denken aan evenwichtsstoornissen, bewustzijnsverandering, trillen, zwakte en verminderde eetlust.
Uit verschillende onderzoeken blijkt dat de infectie uiteindelijk zelflimiterend is en dat de meeste katten er dus uiteindelijk wel ‘overheen groeien’. Afhankelijk van de situatie is het niet behandelen van de kat dus wel een degelijk een optie.

1. Nieuwe inzichten hierin geven aan dat *Tritrichomonas foetus* langer buiten de kat kan overleven [22].

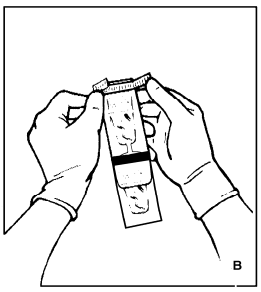
Extra brief wat betreft monstername voor de In Pouch® kweekmediazakjes

Deze extra monstername is van belang om meer informatie te verkrijgen over hoelang de parasiet in het kweekmediumzakje kan overleven zonder dat het gelijk in een warme stoof gelegd wordt. Dit is belangrijk om te weten aangezien de post soms vertraagd is en het diagnostisch laboratorium daardoor de zakjes later krijgt aangeleverd. We zien dan geen parasiet in het kweekzakje, maar weten niet of de kat de parasiet dan echt niet bij zich draagt of dat het mogelijk komt doordat het zakje te lang te koud heeft gelegen.

Deze monstername geldt alleen voor de kat **Rhumba** (van deze kat hebben we namelijk eerder de parasiet kunnen kweken):
Voor de monstername heeft u ontlasting nodig, dat net 'geproduceerd' is, dus vers en warm is. Van tevoren is het handig om eerst al het monstermateriaal (5x swabs en 5x In Pouch® kweekmedia) vast klaar te leggen. Eerst dient u de vloeistof, die in het bovenste gedeelte van de In Pouch® kweekmedium zit naar beneden te masseren om lekken vanuit de bovenkant te voorkomen. De bedoeling is dat nog een klein beetje vloeistof bovenin blijft.
Hierna kan de bovenkant opengeknipt worden, waarna het zakje geopend kan worden door te trekken aan de witte stripjes, die aan beide kanten zitten. Het handigste is dat u de 5 kweekmediumzakjes ergens tegenaan op een rij kunt zetten, zodat de vloeistof er niet uit kan lopen.



De swab kunt u dan door de verse ontlastinghoop halen. Er hoeft maar echt een klein beetje aan de swab te zitten (hoeveelheid iets kleiner dan een pepperkorrel). De swab kan dan in het kweekmediumzakje in het bovenste gedeelte gebracht worden en het monster kan dan door middel van rollen van de swab tegen de wand aan (tussen duim en wijsvinger) in het medium terecht komen.



Als laatste wordt het In Pouch TF® medium zakje gesloten door de bovenkant dicht te drukken en de bovenkant verder voor 2-3 keer op te rollen. Hierna kunnen de crèmekleurige lipjes omgevouwen worden en is het zakje dicht.
Op het etiket van het In Pouch® medium zakje kunt u de naam van de kat, datum van de monstername en uw naam neerzetten.

Deze hierboven beschreven handeling dient voor ieder kweekmediumzakje met een andere swab gedaan te worden.

De 5 zakjes gelieve gelijk op de dag zelf opsturen (in tussentijd gewoon op kamertemperatuur bewaren). De 5 zakjes kunnen weer in het plastic doosje gestopt worden waar ze in verstuurd zijn. Dit plastic doosje kan dan in een Laboratorium Sealbag® gestopt worden. Deze sealbag kan dan in de envelop gestopt worden, die u niet hoeft te frankeren, omdat deze naar een antwoordnummer verstuurd wordt.

Alvast heel erg bedankt voor de moeite.

Mochten er nog bepaalde onduidelijkheden zijn, dan kunt u contact opnemen met ondergetekende.

Met vriendelijke groeten,

Bianca Vermeulen

Werkformulier DNA isolatie feces

Datum:
Uitgevoerd door:
Lot nr:

	Omschrijving	Tijd	OK
1	Monsters controleren		
2	180-200 mg feces in 2,0 ml epje +1,4 ml ASL buffer. Vortex 1 min 15 minuten incuberen bij 95 ºC		
3	15 seconde vortexen Centrifugeer 1 minuut bij 14000 rpm.		
4	1,2 ml supernatant in nieuw 2,0 ml epje. Voeg hieraan 1 InhibitEX tablet toe, 1 min vortexen, 1 min incuberen bij kamertemperatuur.		
5	Centrifugeer 3 minuut bij 14000 rpm		
6	Pipetteer al het supernatant in een 1,5 ml epje. Centrifugeer 3 minuut bij 14000 rpm.		
7	15 µl proteinase K in een 1,5 ml epje + 200 µl supernatant + 200 µl AL-buffer , vortex 15 seconden. 10 minuten incuberen bij 70ºC in hitteblok		
8	200 µl Ethanol toevoegen en goed vortexen Breng alles over in een QIAamp -spin kolom. Centrifugeer 1 minuut bij 14000 rpm.		
9	500µl AW1- buffer Centrifugeer 1 minuut bij 14000 rpm.		
10	500µl AW2- buffer Centrifugeer 3 minuut bij 14000 rpm		
11	200 µl AE buffer toe Incubeer 1 minuut bij KT		
12	Centrifugeer 1 minuut bij 14000 rpm		
13	bewaar DNA bij 4ºC in koelkast 4.		

	naam	tijd
Geconfirmeerd door		
Werkformulier gegeven aan microbioloog		
Geautoriseerd door		

Werkvoorschrift real-time PCR Tritrichomonas

Doel:
Aantonen van Tritrichomonas uit katten feces.

Inleiding:

Instrumentarium:

- Lightcycler 1.5 (Roche)	W345
- LC Carousel Centrifuge 2.0 (Roche)	W345
- Lightcycler Capillary Releaser (Roche)	W327B
- Lightcycler Capping tool	W327C
- Computer (HP)	W345
- Pipetten (Biohit en Gilson)	W345,W327C
- Fluorescentie lamp	W327C
- Centrifuge voor epjes	W345
- Hitteblok	W345
- Vortex	W345,W327C

Disposables

- Filtertips (steriel) (Omnilabo of IKS) bovenkast 0,5-10.0 µl cat. nr. 817200 1,0-100,0 µl cat. nr. 817210 1,0-230,0 µl cat. nr. 817220	W345,
- LightCycler Capillaries (Roche) cat nr:11909339001	W345, bovenkast
- Handschoenen ongepoederd (Vermeulen Poultry sciense) large cat nr: 724049 medium cat. nr. 724039	ladeblok
- Steriele epjes (1,5 ml en 2.0 ml)) (FO)	W345,kast 10
- Pipetpuntjes (FO 6^{e} etage)	W344A, lade?

Chemicalien, reagentia

- QIAamp® DNA Stool Mini kit (Qiagen) cat.no. 51504 (Bij nieuw ingebruikname kit ehtanol toevoegen aan AW buffer)	W345. Kast 10
- Ethanol (96-100%)	bij kit
- TaKaRa SYBR®*Premix Ex Taq*™ 200Reacties (x 50 µl PCR) cat.nr. RR041A	vriezer , la 4
- TFITS R primer 100µM (Biolegio) Tm= 60.0 ºC 5'-GCA ATG TGC ATT CAA AGA TCG -3' werkverdunning 4x (is 25µM)	vriezer , la 3
- TFITS F primer 100µM (Biolegio) Tm= 66.0 ºC 5'-CTG CCG GTT GGA TCA GTT TCG -3' werkverdunning 4x (is 25µM)	vriezer , la 3

- MgCl2 (Roche) koelkast 4
- MilliQ, uitgevuld in groene epjes van 500 µl W345, vriezer
- RNA-away (VWR) kast 5 voorraad
  cat. no. Koelkast 4

Kwaliteits controle

- Positieve controle positief gespiked feces (2)
- MilliQ
-

	Smelttemperatuur	Product grootte
Tritrichomonas	81 ºC	200bp

Werkwijze

DNA Isolatie met behulp van QIAamp® DNA Stool Mini kit:
Gebruik werkformulier.

- Weeg 180-200 mg feces af in een 2,0 ml epje, of 200 µl bij vloeibare feces.
- Voeg hieraan 1,4 ml ASL buffer toe en vortex 1 minuut
- 15 minuten incuberen bij 95 ºC
- 15 seconde vortexen
- Centrifugeer 1 minuut bij 14000 rpm.
- Pipeteer 1,2 ml supernatant in een nieuw 2,0 ml epje.
- Voeg hieraan 1 InhibitEX tablet toe, 1 minuut vortexen, 1 minuut incuberen bij kamertemperatuur.
- Centrifugeer 3 minuut bij 14000 rpm.
- Pipeteer al het supernatant in een 1,5 ml epje.
- Centrifugeer 3 minuut bij 14000 rpm.
- Pipeteer 15 µl proteinase K in een 1,5 ml epje.
- Voeg hieraan 200 µl supernatant toe.
- 200 µl AL-buffer toevoegen, vortex 15 seconden.
- 10 minuten incuberen bij 70ºC in hitteblok
- 200 µl Ethanol toevoegen en goed vortexen
- Breng alles over in een QIAamp -spin kolom.
- Centrifugeer 1 minuut bij 14000 rpm. Verwijder doorgestroomde vloeistof en opvangtube
- Plaats de spin-kolom in een nieuwe opvangtube en voeg 500µl AW1- buffer toe
- Centrifugeer 1 minuut bij 14000 rpm. Verwijder doorgestroomde vloeistof en opvangtube
- Plaats de spin-kolom in een nieuwe opvangtube en voeg 500µl AW2- buffer toe
- Centrifugeer 3 minuut bij 14000 rpm. Verwijder doorgestroomde vloeistof en opvangtube.
- Plaats de spin-kolom in een steriel 1,5 ml epje en voeg 200 µl AE buffer toe
- Incubeer 1 minuut bij KT
- Centrifugeer 1 minuut bij 14000 rpm
- Verwijder spin-kolom en bewaar DNA bij 4ºC in koelkast 4.

Real-time PCR;
Real-time PCR m.b.v. Lightcycler 1.5 (Roche)

PCR Mix:	Takara	10 µl
	MgCl2 (25mM)	2,4 µl
	TFITS R (10 µM)	1,0 µl
	TFITS F (10 µM)	1,0 µl
	Milliq	0,6 µl
	Template	5,0 µl

Het is verstandig om meer mix te maken dan dat er nodig is.

- Maak een werklijst van de monsters (Bijlage 2: werkformulier Lightcycler) G:\CLIF\Diagn Admin\Werkvoorschriften en werkformulieren

Werken in W327C.
- Zet minimaal 15 min. voor begin werkzaamheden de UV-lamp aan
- Maak de PCR mix
- Zet het benodigde aantal LightCycler Capillairen (Roche) in het koelblok
- Pipeteer 15µl mix in iedere capillair
- Pipeteer 5µl template in het juiste capillair.
- Sluit af met de bijbehorende dopjes en de Lightcycler Capping tool (Roche)

Ruimte W345
- Plaats de capillairtjes in de carrousel
- Centrifugeer de capillairen in de LC Carousel Centrifuge 2.0 (Roche).
- Plaats de carrousel in de Lightcycler en sluit het deksel
- Run de PCR volgens onderstaand programma. Tritrichomonas.EXP

Ruimte W327B
- Haal de capillairen uit de carrousel m.b.v. de Lightcycler Capillary Releaser

Ruimte W327C
- Na werkzaamheden: werkblad schoonmaken met RNA-away
- UV-lamp 15 minuten laten branden.

Gemaakt/gewijzigd op	Gemaakt door	Geautoriseerd door
30-10-2006/20-6-2007/16-2-2009	Anky Schoormans	