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The clinical significance of *Cyniclomyces guttulatus* in dogs with chronic diarrhoea, a survey and a prospective treatment study

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ABSTRACT

This study surveyed the prevalence of massive numbers of *Cyniclomyces guttulatus* in faecal samples from healthy dogs (18%) and dogs with chronic diarrhoea (14%) suggesting that this yeast has no clinical significance. Subsequently, a total of 57 referred dogs with chronic diarrhoea were selected because they excreted massive numbers of *C. guttulatus* and their initial diagnostic work-up yielded no other direct clues explaining their diarrhoea.

Treatment with nystatin did not result in any clinical response in 36 out of these 57 dogs (63%), although they no longer shed the yeast. However, a response was noted in the remaining 21 (37%) dogs: 13 were 'responders', in that their diarrhoea subsided for more than two weeks and the faeces were cleared of the yeast. However, three of these dogs relapsed repeatedly, with signs of diarrhoea and massive shedding of the yeast. The other eight dogs were 'incomplete responders', whereby faecal quality initially normalised, but diarrhoea relapsed within two weeks, whilst still not shedding the yeast. In these cases, further diagnostic work up revealed other co-causes of diarrhoea. It was concluded that there was no direct evidence that *C. guttulatus* is a primary pathogen. However, the results of the prospective treatment study suggest that a possible role in a minority of cases, perhaps as an opportunist, cannot be ruled out.

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1. Introduction

The yeast *Cyniclomyces guttulatus* (formerly called *Saccharomycopsis guttulata*) is commonly found in faeces of rodents and rabbits (Boundy-Mills and Miller, 2011; Richle and Scholer, 1961; Zierdt et al., 1988). It is a normal inhabitant of the gastrointestinal (GI) tract of these animals, residing on the mucous layer in the gastric fundus and pylorus despite the acidic conditions (pH 2–2.5) (Shifrine and Phaff, 1958). Large numbers of *C. guttulatus* are passed with the faeces into the environment (Boundy-Mills and Miller, 2011) where they may persist for a prolonged periods of time due to their ability to form ascospores (Shifrine and Phaff, 1958).

Houwers and Blankenstein (2001) were the first to suggest that the yeast might be of clinical significance in dogs, when they observed large numbers of Cyniclomyces-like organisms in approximately 15% of canine faecal samples submitted to their diagnostic laboratory for parasitological examination, mainly for the investigation of chronic diarrhoea. As some of these patients responded remarkably to oral nystatin, they suggested that the yeast could be of clinical significance, most probably as a direct result of their massive presence in the gut. They also suggested that the yeast probably colonised a niche in the patient's altered intestinal flora. However, no clinical efficacy studies were performed and there was limited follow up of the treated dogs. In a more recent study, large numbers of *C. guttulatus* were found in the faeces and stomach washings of three dogs suffering from recurrent vomiting and diarrhoea, and small numbers in a quarter of the faecal samples from healthy dogs (Flausino et al., 2012). To date, two case reports have documented the successful treatment of chronic diarrhoea associated and massive *C. guttulatus* shedding with nystatin, one in a cat (Peters and Houwers, 2009) and one in a rabbit (Hersey-Benner, 2008).

Therefore, currently, the clinical significance of identifying large numbers *C. guttulatus* in the faeces of dogs with chronic diarrhoea is not known. Hence, a survey on the prevalence of the yeast in healthy and diarrhoeic dogs was performed, followed by a prospective study on the effects of nystatin treatment in dogs with chronic diarrhoea excreting large numbers of *C. guttulatus*. Given that the it was also not clear whether this yeast has the potential to induce inflammation, a hallmark of primary pathogens, histopathological changes in gastrointestinal biopsies taken during investigations were also reviewed. The null hypothesis was that excretion of large numbers of *C. guttulatus* is an insignificant finding, does not any histological changes in the stomach or intestine, and that treatment with nystatin would be ineffective.

2. Material and techniques

2.1. Survey

2.1.1. Healthy dog population

Over a period of 5 months, a total of 140 faecal samples were obtained from healthy dogs at walking services ($n = 4$), veterinary practices ($n = 13$), and animal shelters ($n = 2$), from various regions of the Netherlands. At the time of sampling, breed or type of dog was recorded as well as age, gender and faecal consistency.

2.1.2. Diarrhoeic dogs

These data were obtained by searching the database of the Veterinary Microbiological Diagnostic Center at Utrecht University (VMDC) for submissions of canine faeces samples for parasitological examination of which the history mentioned 'diarrhoea' and 'massive *C. guttulatus*' as the only result. This search covered the period 1-1-2005 to 20-9-2006.

2.1.3. Analytical methods

All faecal samples were analysed within 24–48 h at the VMDC. The analysis was performed with the Centrifuge-analysis Sedimentation-Flotation Method (CSF method) using Zinc Sulphate ($ZnSO_4$) as a flotation fluid with a specific gravity of 1.34. Briefly, 1 g of faeces was suspended in 14 mL of tap water, vortexed and centrifuged for 1 min at $2950 \times g$. The supernatant was removed; the sediment suspended in the $ZnSO_4$ solution, the tube was completely filled (to produce a convex meniscus). A cover slide was then placed on top of the tube, which was then centrifuged for 2 min at $1660 \times g$. The cover slide was carefully removed and placed on a microscope slide and examined.

2.1.4. Microscopic identification

Slides were studied using a magnification of 400×. Five different microscopic fields were observed to identify *C. guttulatus*, morphologically identified by its characteristic 'spectacle case' shape (Fig. 1), and a length of approximately 1/5th of the diameter of a *Toxocara canis* egg. Numbers of *C. guttulatus* were semi-quantitatively assessed as follows:

- No organisms or <50 organisms in total over five microscopic fields (negative).
- 50 to <200 organisms in total over five microscopic fields (intermediate).
- Clear presence, usually in massive – innumerable – numbers (positive); >200 organisms in total over five microscopic fields (positive).

Low numbers (<200) were not regarded as positive scores in view of the possibility that they were just contaminants from oral intake and, thus, not originating from an actual infection.

2.1.5. Culture and polymerase chain reaction

In order to substantiate the morphological identification, an in-house conventional PCR for detection of *C. guttulatus* was developed as described by others (Kurtzman and Robnett, 1998). Rabbit faeces containing *C. guttulatus* was cultured on Sabouraud's dextrose agar, under microaerophilic conditions, at 37 °C for a minimum of 5 days. Light brown colonies were microscopically identified as *C. guttulatus*. DNA from these colonies and DNA extracted from the rabbit faeces was subjected to the PCR; the product had a similar size as the product

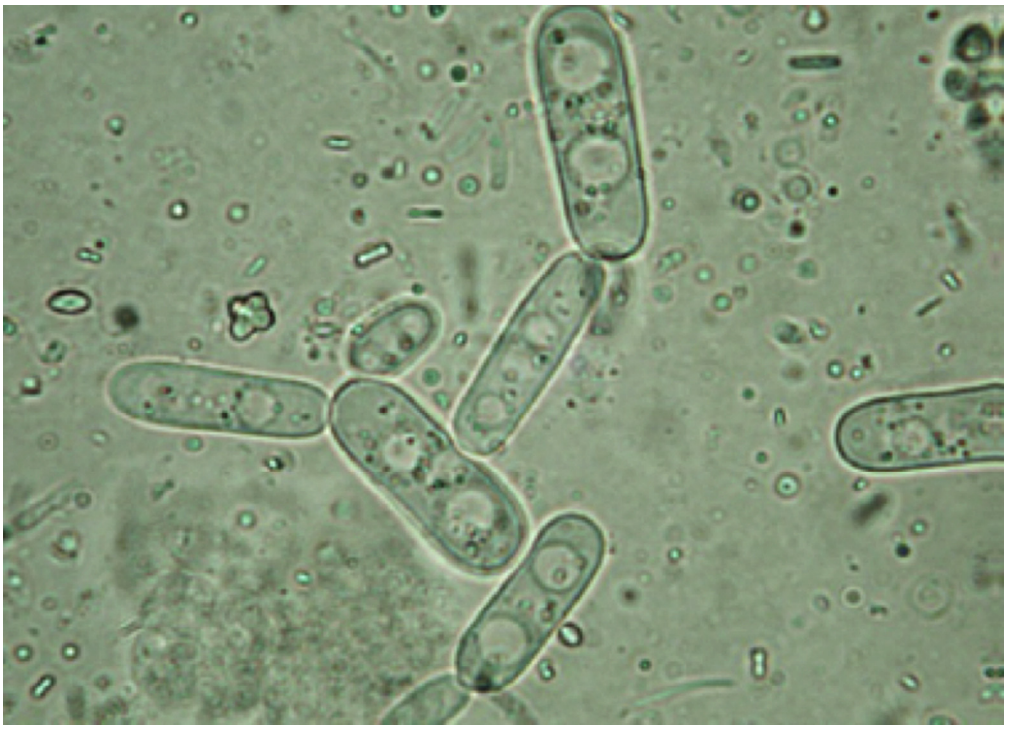

Fig. 1. *C. guttulatus* characteristic spectacle case-shape (photo: Barend Blankenstein, VMDC).

described by Kurtzman and Robnett (1998). Subsequently, six microscopically positive canine faecal samples were subjected to culture and PCR, with the amplicons from the suspect colonies and the faeces having a size similar to that of the rabbit isolate.

2.2. Prospective study of referral cases with chronic diarrhoea with massive excretion of C. guttulatus

2.2.1. Patients

From a cohort of 300 dogs referred between January 2005 and September 2006 for the investigation and management of chronic gastrointestinal disease (Mandigers et al., 2010), a total of 57 patients were selected that appeared to shed massive numbers of *C. guttulatus* in their faeces, based on a standard parasitological examination (CSF) at VMDC. Dogs were included, in this part of the study, if clinical signs had been present for at least three weeks and a complete diagnostic work-up (including endoscopy) had been performed to exclude other causes. Dogs that had received antimicrobials and/or corticosteroid therapy in the three weeks prior to their referral were excluded. All owners were informed of the purpose of the study and consented to their dog participating. All examinations were performed by the same clinician (PJJM).

2.2.2. Diagnostic work-up

A complete history was taken and a physical examination performed. Subsequently, detailed laboratory investigations were performed including a complete blood count, a serum chemistry profile (e.g. urea, creatinine, alkaline phosphatase, alanine aminotransferase, total bilirubin, bile acids, calcium, phosphate, total protein, albumin, globulins, and protein electrophoresis), serum trypsin-like immunoreactivity (TLI), faecal parasitology as described above, and faecal bacteriology (for *Salmonella* spp. and *Campylobacter* spp.). All faecal examinations were performed at the VMDC. If deemed necessary, survey abdominal radiography and abdominal ultrasonography were performed. Finally, upper gastrointestinal endoscopy was performed in all patients, as well as ileum- and colonoscopy in patients where it was deemed to be diagnostically relevant. During this procedure, multiple mucosal biopsies were collected from the stomach (corpus and fundus), upper small intestine (duodenum and, where feasible, jejunum), ileum and colon for standard histopathological analysis. The results were used in the case management by excluding cases not consistent with chronic enteropathy. All slides of the included dogs were subsequently assessed by a European-board-certified veterinary pathologist (SM) and a resident in veterinary pathology (NA) and graded using recent internationally accepted criteria (Jergens et al., 2012; Washabau et al., 2010). Special attention was given to the presence of the yeast and potentially associated histological changes.

2.2.3. Treatment study

All dogs received 150,000 IE nystatin[1] q8h PO for five consecutive days. This dosing regimen was based on the dose suggested for *Candida* spp. infection in dogs (Jenkins

[1] Nystatine Labaz®, oral suspension, 100,000 IU/mL, Sanofi-Aventis the Netherlands BV.

and Boothe, 1987). No concurrent therapy was administered during the treatment with nystatin.

2.2.4. Follow-up

During the study, patient owners recorded their observations with respect to gastrointestinal signs and any changes to the normal routine. The study performer (PJJM) viewed this record, at the evaluation visit, approximately 7 days after the start of the nystatin treatment. Dogs that had responded completely to the nystatin therapy received no additional therapies. All owners were asked to submit a faecal sample 14 days after the start of the treatment. If these samples were positive for *C. guttulatus*, the nystatin therapy was repeated, if a negative result was obtained but diarrhoea still occurred, further diagnostic work-up was performed and other treatments were initiated.

2.2.5. Classification of cases

Classification of cases was based upon the findings of the follow-up evaluations with reference to the information provided by the owner. If the owner observed no change in the frequency and severity of clinical signs after two courses of nystatin, the dog was regarded a 'non-responder'. If a dog responded, i.e. returned for more than two weeks to what the owner classified as normal prior to onset of signs, to the first or second nystatin treatment and the faecal examination was negative for *C. guttulatus*, it was classified as a 'responder'. Dogs that only responded temporarily, i.e. less than two weeks, to the treatment(s) were classified as 'incomplete responders'.

2.2.6. Data handling and statistics

Statistical analyses were performed using a Chi-square test with a computer software program,[2] and descriptive statistics were used to report baseline data and reported as median and range.

3. Results

3.1. Prevalence study

A total of 140 faecal samples of clinically healthy dogs were analysed: 115 (79%) samples were negative or intermediate and 25 (18%) were *C. guttulatus* positive. Dogs housed in animal shelters tended to have a lower number of positive faecal samples (1 out 19; 5%) compared to the dogs sampled from walking services (6 out 30; 20%) and the veterinary practices (18 out 91; 20%) (Table 1). There was no statistical significant difference noted ($P = 0.18$). Between 1-1-2005 and 20-9-2006, VMDC received 1564 canine faecal samples for parasitological analysis. The vast majority originated from dogs with diarrhoea lasting for more than a week. A total of 215 (14%) samples were reported as massive shedders.

There was no statistical significant difference in the prevalence of *C. guttulatus* in samples from healthy dogs and those with diarrhoea ($P = 0.18$). Neither was there a correlation for *C. guttulatus* excretion with age, gender or breed in the 140 healthy dogs.

Table 1
Faecal samples analysed for the presence of *C. guttulatus* and categorised into three groups. The total number of dogs found to shed the yeast massively was 18%. Although the number of dogs, housed in the animal shelters, found positive was low, the difference between the three groups is statistically not significant ($P = 0.18$).

	Negative	Intermediate	Positive
Walking services	21	3	6
Animal shelters	18	0	1
Practitioners	70	3	18
	109	6	25

3.2. Prospective treatment study

Of 300 patients referred during the timeframe of the study, 57 dogs fitted the inclusion criteria. Dog breeds represented included a large variety of breeds such as Akita Inu ($n = 1$), American Bulldog ($n = 1$), Basset ($n = 1$), Bernese mountain dog ($n = 6$), Boerboel ($n = 1$), Bordeaux dog ($n = 1$), Border collie ($n = 1$), Bouvier ($n = 2$), Boxer ($n = 5$), Cane corso ($n = 2$), Dobermann ($n = 2$), Shepherd ($n = 4$), English bulldog ($n = 2$), Fox ($n = 1$), German Shepherd ($n = 4$), Golden retriever ($n = 7$), Great Dane ($n = 1$), Great Swiss ($n = 1$), Greyhound ($n = 1$), Jack Russel Terrier ($n = 1$), Labrador retriever ($n = 2$), Mongrel ($n = 1$), Ploski Owczarek Nizziny ($n = 1$), Rottweiler ($n = 5$), Stabij ($n = 1$) and Weimeraner ($n = 2$). Twenty-seven dogs were female of which 16 were neutered and 30 dogs were males of which six were castrated and their mean age was 5.2 ± 3.43 years (range 3.7 months to 11.1 years; median 5.1 years). Twenty-seven dogs lived in a larger city, 13 semi-rural and 17 rural. Twenty-six of the owners had seen their dog eating rabbit droppings, whilst the others had either not observed this behaviour or were uncertain as to whether such behaviour had occurred. The dogs were fed a variety of diet food (50 out 57) as well as home-cooked food (7 out 57).

3.3. Clinical signs and baseline diagnostic findings

The preceding median duration of the clinical signs was 142 days (range 23–2588 days). Clinical signs of small bowel diarrhoea were seen in 47 out 57 dogs (82%), large bowel diarrhoea in 5 out 57 (9%), and signs of both small and large bowel diarrhoea were seen in 5 out 57 (9%). No significant abnormalities were seen on routine haematological and serum biochemical analysis, except for hypoalbuminaemia, which was identified in 14 out of 57 dogs (25%).

3.4. Histopathology

Gastric biopsies were available of 23 of the 47 dogs that presented with chronic small bowel diarrhoea. Lymphoplasmacytic inflammation was identified in six dogs, with eosinophilic gastritis being identified in a further six. However, *C. guttulatus* was seen on the inner surface of the stomach in only three cases, and there was no evidence of

[2] SPSS 22, IBM company.

mucosal invasion or intracellular organisms. Duodenal/jejunal biopsies were available from all 47 dogs with chronic small bowel diarrhoea and from an additional two dogs with mixed diarrhoea. The biopsies of six dogs showed no abnormalities. All remaining dogs showed various abnormalities, generally mild-to-marked lymphoplasmacytic infiltrates (19), mild-to-marked eosinophilic infiltrates (10), and mild-to-moderate mixed (i.e. lymphoplasmacytic and eosinophilic) infiltrates (13). One dog had a multifocal granulomatous lymphangitis. None in none of the biopsies *C. guttulatus* was seen.

Colonic biopsies were available from 10 dogs, five of which were normal, four had mild eosinophilic colitis, and one had ulcerative colitis. Again, none of these biopsies showed *C. guttulatus*. As result, there was no host inflammatory reaction to the yeast in any of the dogs.

3.5. Effect of nystatin treatment

Thirty-six out of fifty-seven dogs (63%) did not clinically respond to the first treatment with nystatin, yet all but two faecal samples taken 14 days after the start of the treatment were found to be clear of *C. guttulatus*. The two positive dogs were treated again and, although they did not respond, the repeat faecal samples were clear.

According to the owners, clinical signs did resolve in 21/57 (37%) dogs after the first treatment, 13 of which had negative faecal results and were, thus, classified as 'responders'. The remaining eight of these responding dogs, continued to shed large numbers of *C. guttulatus* in their faeces, despite resolution of signs, and diarrhoea relapsed, within two weeks. At this stage, a second nystatin course was administered, and signs resolved in three of these eight dogs, with faecal samples being negative. However, these three dogs returned to the clinic after several months, again with diarrhoea and massive excretion of the yeast. They were treated once more, and again responded with negative faecal samples and resolution of clinical signs. This cycle of events occurred many times thereafter. Hence, these three were also classified as 'responders'.

Four dogs out of these eight responding dogs still shedding *C. guttulatus*, only recovered completely after an additional antibiotic treatment. These dogs were classified as 'incomplete responders'. One dog out of these eight responding dogs still shedding *C. guttulatus*, was subsequently found to be positive for *T. canis* and completely recovered after appropriate therapy with fenbendazol.[3] This dog was also classified as an 'incomplete responder'.

Therefore, of the 21 dogs in which owners reported a clinical response to nystatin, 16 were classified as 'responders', and five as 'incomplete responders'. Six of the 'responders' were followed for more than a year, and they remained free of diarrhoea; three of these dogs returned for review at regular intervals and were each time again shedding the yeast. However, the remaining seven 'responders', subsequently returned after signs of diarrhoea had relapsed after several weeks (at least >4); in all cases, their faecal samples were still free of the yeast, and further examination of these patients revealed various other causes.

[3] Fenbendazol 50 mg/kg for 3 days. Brandname Panacur® MSD.

4. Discussion

Although *C. guttulatus* was initially described more than 50 years ago (Parle, 1956, 1957; Shifrine and Phaff, 1958) it only became the subject of clinical studies in animals during the last decade. Some authors had suggested on the basis of a clinical response to nystatin treatment that the massive presence of the yeast in GI tract could play a causative role in cases of chronic diarrhoea in dogs (Houwers and Blankenstein, 2001) and cats (Peters and Houwers, 2009). One study presented circumstantial evidence that it could act as an opportunistic pathogen in dogs, but it was also found in 21% of wet mounts of the faeces of 60 healthy dogs (Flausino et al., 2012). However, others saw the presence of the yeast as a clinically non-significant finding in dogs (Boundy-Mills and Miller, 2011). This is the first study investigating the prevalence of the yeast *C. guttulatus* in dogs suffering from chronic diarrhoea and those without, combined with data regarding medical intervention to clear this yeast infection. Microscopic detection of the yeast was confirmed using a species-specific PCR.

In the present study in the Netherlands, the observed prevalence in healthy dogs is 18%, which is comparable with the prevalence in Brazil (Flausino et al., 2012). To exclude any regional, sex or breed bias faecal samples were obtained from different regions in the Netherlands, and different breeds and housing situations. It is, therefore, concluded that the yeast has a relatively high prevalence in healthy dogs. Since most samples contained massive numbers it is likely that *C. guttulatus* actually multiplied in the dogs and thus that the dogs were truly colonised. It should be noted that the CSF method used in this study leads to considerable enrichment and may, therefore, be a more sensitive method than wet mount detection.

The environmental source of this organism is unclear, but one possibility is exposure to rabbit faeces. This is in line with finding that the organism was less commonly isolated from dogs in animal shelters that usually only have access to secluded outdoor areas. Given that rabbits are ubiquitous in areas of countryside, it is likely that at least some of the pet dogs not excreting *C. guttulatus* were also exposed. So, the negative dogs were almost certainly exposed but did not become infected, possibly as a result of absence of particular conditions in the stomach lining or the lack of a niche in the gastrointestinal environment.

The prevalence of the yeast in dogs suffering from chronic diarrhoea was 14%, which corroborates the 15% in the earlier study (Houwers and Blankenstein, 2001), and is statistically not significantly different. This finding, supports the null hypothesis that massive excretion of *C. guttulatus* may not have clinical significance. Thus, the presence of chronic diarrhoea, whatever the cause, does not increase the likelihood of infection with the yeast, which is interesting. As it has been suggested that extensive colonisation with *C. guttulatus* may exacerbate an existing imbalance in gastrointestinal function and

trigger or exacerbate diarrhoea (Houwers and Blankenstein, 2001).

The effect of monotherapy with oral nystatin was prospectively studied in a group of 57 dogs with chronic diarrhoea and massive faecal shedding of *C. guttulatus*. The overall response rate observed in this study was 21/57 (37%) of which 16 were categorised as 'responders', after one or repeated treatment courses, and five as 'incomplete responders' that became diarrhoeic again within weeks. Notably, a *Cyniclomyces*-negative faecal sample was seen in every 'responder', whereas 'incomplete responders' once again showed massive faecal shedding after several weeks. Conversely, the fact that only 2/36 'non-responders' shed the yeast after a single nystatin course, suggested that the therapy was at least effective in eliminating it from the gastrointestinal tract. Therefore, the five 'incomplete responders' probably relapsed because either the yeast was not entirely eliminated or re-infection occurred rapidly, which is obviously hard to prevent in the average dog walking situation.

In the case 6/16 'responders', the clinical response was long-lasting suggesting that the *C. guttulatus* might have had a causal role (either primary or secondary) of in the clinical signs. In another three dogs, a clear association between signs if diarrhoea and the presence of *C. guttulatus* in the faeces, in that massive yeast shedding was noted only when these dogs had diarrhoea. Such a finding again suggests a possible role for *C. guttulatus* in some cases of chronic diarrhoea. However, in the remaining seven 'responders', the diarrhoea relapsed after more than two weeks without massive yeast excretion. Further investigation of these patients revealed various other causes, which were subsequently successfully leading to clinical resolution. The significance of *C. guttulatus* in these cases is less clear, be it an incidental finding or a possible exacerbating factor.

It is possible that the nystatin was acting against other elements of the gastrointestinal microbiota and not against *C. guttulatus*. However, since nystatin affects ergosterol-binding sites which are only present in fungal cell wall, it is unlikely that bacterial components of the gastrointestinal microbiota were affected. Although a recent molecular analysis of the fungal microbiome demonstrated that the DNA of many fungal species can be present in canine faeces (Foster et al., 2013), only low numbers of yeasts can usually be cultured from jejunal chyme and faeces of healthy dogs (Mentula et al., 2005). Further, if substantial numbers of other fungal species (including yeasts) were a causative factor, they arguably should have been identifiable in either faecal samples or tissue biopsies. Thus, if the nystatin were having a genuine effect, it is most likely to have been due to eradication of the *C. guttulatus*. That said, the trial was uncontrolled, and it is possible that the improvement were coincidental to this therapy. Arguably, a prospective randomised controlled trial would be a better means of verifying that the response was genuine.

Although inflammatory changes were identified in the histological examination of both gastric and intestinal biopsies, the lack of mucosal invasion suggested that they were not the direct effect of *C. guttulatus*. Thus, if this yeast does have a pathogenetic role in cases of chronic diarrhoea, it is probably not through invasion into, or adherence to, the inner surface layers of the GI tract. It is of course possible that the presence of massive numbers of *C. guttulatus* might lead to diarrhoea through an alternative mechanism. However, further studies would be needed to examine such a possibility.

5. Conclusion

In conclusion, the survey results and the histology supported the null hypothesis, and there was no direct evidence that *C. guttulatus* is a primary pathogen. However, the results of the prospective treatment study did demonstrate that some dogs with chronic diarrhoea and massive *C. guttulatus* shedding improve after nystatin therapy. Thus, a possible role in a minority of cases, perhaps as an opportunist, cannot be ruled out. Further studies, including randomised controlled trials, would now be recommended in order better to define the true significance of this organism and the benefit of therapeutic eradication.

Conflict of interest

The authors report no conflict of interest.

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