UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

THE SENSITIVITY OF DIRECT FAECAL EXAMINATION, FAECAL FLOTATION AND CENTRIFUGAL SEDIMENTATION/ FLOTATION IN THE DIAGNOSIS OF CANINE SPIROCERCOSIS

By

Jevan Christie

A dissertation submitted to the Faculty of Veterinary Science, Department of Companion Animal Clinical Studies, University of Pretoria, in partial fulfillment to the requirements for the degree MMedVet (Med)

Pretoria

June 2011

TABLE OF CONTENT

Acknowledgements

The author would like to thank the following people for their assistance and guidance to this project.

- Prof Robert Kirberger for your dedication, help and assistance in the initial protocol layout and development.
- Ms Loini Bodenstein and Ms Jaqui Sommerville from the Department of Statistics, Faculty of Natural and Agricultural Science for your help in the statistical analysis of the data.
- Ms Dawn Durand from the Department of Tropical Diseases, Faculty of Veterinary Science for your hard work in the centrifugal sedimentation/flotation faecal examination methods performed and your dedication to the project.
- The Onderstepoort Veterinary Academic Hospital and Faculty of Veterinary Science, University of Pretoria for their support and financial assistance towards this project.
- All the support staff at the Onderstepoort Veterinary Academic Hospital for your help and assistance to this project.

Supervisor: **Dr LL van der Merwe, BVSc, MMedVet (Med)**

Co-supervisor: **Dr EV Schwan, DVM (Hannover), MVSc (Applied Parasitology) (Liverpool), Dr. med.vet. (Hannover), PhD (Pretoria)**

List of abbreviations

Epg = Eggs per gram

G = Gravity

$MgSO_4$ = Magnesium Sulphate

NaCl = Sodium Chloride

$NaNO_3$ = Sodium Nitrate

OVAH = Onderstepoort Veterinary Academic Hospital

PCR = Polymerase Chain Reaction

SG = Specific Gravity

S. lupi = *Spirocerca lupi*

$ZnSO_4$ = Zinc Sulphate

List of Figures and Tables

Summary

A variety of faecal examination methods have shown variable sensitivity in identifying larvated *Spirocerca lupi (S. lupi)* eggs. The purpose of this study was to determine which faecal examination method, including a novel modified centrifugal flotation method, was most sensitive in the diagnosis of spirocercosis.

Faeces were collected from 33 dogs diagnosed with spirocercosis by oesophageal endoscopy at the Onderstepoort Veterinary Academic Hospital between 2008 and 2009. If the first evaluation was negative, a second faecal sample was evaluated 24-48 h later. Ten faecal examinations using 1 g aliquots of faeces were performed per sample. Four faecal examination methods were evaluated; direct faecal examination using saline, direct faecal flotation, a modified faecal centrifugal flotation and a laboratory performed faecal sedimentation/flotation. The direct and modified centrifugal flotation methods were each performed using four faecal flotation solutions; $NaNO_3$ (Specific gravity (SG) 1.22), $MgSO_4$ (SG 1.29), $ZnSO_4$ (SG 1.30) and saturated sugar (SG 1.27). The sedimentation/flotation method utilized $MgSO_4$ (SG 1.29). The modified centrifugal flotation method required centrifugation (1400 G) of a prepared faecal suspension (1 g faeces suspended in 5 ml of flotation solution) after which 0.1 ml of the supernatant was aspirated from the surface using an adjustable volume micropipette for microscopic examination. The 10 faecal examination tests were statistically analysed using the Friedman test (nonparametric equivalent of analysis of variance) $p=0.000$, z value = 0.05.

The sensitivity of the tests ranged between 42 % and 67 %, with the $NaNO_3$ solution showing the highest sensitivity in both the direct and modified centrifugal flotation methods.

The modified $NaNO_3$ centrifugal method ranked first with the highest mean egg cell count (45.24 ± 83). The modified centrifugal $NaNO_3$ method was found to be superior

(*i.e.* higher egg counts) and significantly different ($p<0.001$) compared with the routine saturated sugar, $ZnSO_4$ and $MgSO_4$ flotation methods. The direct flotation method/technique using $NaNO_3$ flotation fluid was also superior and significantly different ($p<0.001$) when compared to the same technique using $ZnSO_4$ or $MgSO_4$ flotation fluids.

Neoplastic transformation of oesophageal nodules was confirmed in 15 % (n=5) of dogs and a further 18 % (n=6) had both neoplastic and non-neoplastic oesophageal nodules. *S. lupi* eggs were demonstrated in 40 % of dogs with neoplastic nodules and in 72.9 % of dogs with non-neoplastic nodules. The mean egg count in the non-neoplastic group (61) was statistically greater ($p=0.02$) than that of the neoplastic group (1).

The results show that faecal examination using the direct and modified centrifugal flotation methods with the $NaNO_3$ flotation fluid are the most sensitive methods in the diagnosis of spirocercosis. The modified centrifugal flotation method using this solution has the highest mean egg count. The study also found that dogs with neoplastic nodules shed significantly fewer eggs than dogs with non-neoplastic nodules.

Keywords: Spirocerca lupi; spirocercosis; dog; faecal examination; egg

CHAPTER 1

Justification

1.1 Introduction

Spirocerca lupi (S. lupi) is a nematode of the superfamily Spiruroidea and follows an indirect life cycle which may include a paratenic host. The predominant definitive host is the dog which passes larvated eggs with the faeces and to a lesser extent with their vomitus[2,5]. Following ingestion of an infected intermediate host (coprophagous beetles) or paratenic host (birds, lizards, frogs, snakes, mice, rabbits and rats) infective third stage larvae (L3) larvae are liberated within the stomach[1,2,31]. Soon after ingestion the L3 penetrate the stomach mucosa and migrate, within the artery walls, towards the aorta[2,4,14,22]. This initial part of the migration process takes approximately 3 weeks[2,11]. Further development of the L3 to immature adults occurs in the wall of the thoracic aorta. These immature adults then migrate in the mediastinum from the wall of the aorta towards the oesophagus at about 102–124 days post infection[2,11]. In the oesophagus the adults provoke the development of a fibrous nodule in which they undergo further maturation. These nodules may become neoplastic[2,24]. The adult spirurid nematode is a relatively large worm, reddish in colour with males and females reaching 3-4 cm and 6-7 cm in length respectively and 1 mm in width[11]. The prepatent period in the dog is 4 to 6 months[2,13].

Oesophageal nodules may have a small opening or multiple small openings/ opercula into the oesophageal lumen through which larvated eggs are passed[12]. Large numbers of larvated eggs are passed with the faeces of infected dogs[2]. If the nodule has no opening the infection is not yet patent[31]. Owing to the complex migration route, worms are sometimes found in aberrant sites within the dog which include the skin, lungs, trachea, pleura, diaphragm and spinal cord [2,4,7,13,16,23,27].

1.2 Clinical signs associated with spirocercosis

Clinical signs associated with spirocercosis are variable but are usually as a result of the parasites' effect on the oesophagus, mediastinum or aorta. In early infections the parasite may cause no clinical signs (subclinical spirocercosis) and infection is diagnosed incidentally on faecal examination or thoracic radiography[8,15]. Peracute death may occur due to rupture of the aorta or other major blood vessel secondary to aneurysm formation due to damage caused by larval development and migration[2,11]. Classical spirocercosis clinical signs result from the spirurid nodule obstructing the oesophagus and compressing the intrathoracic structures and include vomiting, regurgitation, coughing, dysphagia, sialorrhoea, pyrexia and melaena[18,20,32].

Less frequent clinical presentations include mediastinitis, pleuritis and pyothorax[2,32]. Weakness and weight loss become apparent with chronicity and neoplastic transformation. Neoplastic spirocercosis results from the neoplastic transformation of the parasitic nodule, usually into an osteosarcoma, fibrosarcoma or undifferentiated sarcoma[17]. The clinical signs of regurgitation and dysphagia are similar to the classical form but hypertrophic osteopathy, anaemia, leukocytosis and thrombocytosis are often found concurrently[9]. Atypical manifestations of spirocercosis including haemopericardium, paraparesis and subcutaneous nodules can be associated with aberrant migrations[2,7,13,23,27].

1.3 Diagnosing spirocercosis in dogs

Oesophageal endoscopy is considered the diagnostic test of choice for spirocercosis[20,25]. Nodules are typically smooth, round and sessile proliferations which protrude into the oesophageal lumen. The overlying epithelium is intact and these nodules cannot be endoscopically biopsied as the biopsy punch does not biopsy significant layers of the epithelium and often slips off during biopsy. Neoplastic nodules usually are pedunculated and show a roughened, ulcerated, necrotic surface and should be biopsied to determine if they are neoplastic[9,32]. Radiography is also a sensitive diagnostic test with the dorso-ventral and right lateral thoracic views superior for diagnosing a caudal mediastinal mass. Radiological features regarded as pathognomonic for spirocercosis include a caudal oesophageal opacity, an undulating descending aorta and spondylitis of the 6th to 12th thoracic vertebrae[8,11]. The sensitivity of coproscopical techniques for the diagnosis of spirocercosis varies significantly. *Spirocerca lupi* eggs are small-sized (22-37 x 11-15 µm), thick-shelled, elongated with parallel sides and larvated[5,6]. Coproscopical techniques previously described include direct faecal smears, faecal flotation, faecal sedimentation and recently the *FLOTAC* method[3,15,21,25]. Owing to the erratic shedding of eggs, faecal flotations should be repeated if negative**.** Mazaki-Tovi *et al.* (2002) found that a second faecal examination conducted several days after a negative result was obtained, identified a larger number of infected animals (80 % compared to 57.5 % in 40 *S. lupi* infected animals).

1.3.1. Spirocercosis faecal examination

Reche-Emonot *et al*. (2001) found that direct faecal examination was superior in detecting *S. lupi* eggs compared to routine $MgSO_4$ and sugar flotation techniques. Cabrera and Bailey

(1964) and Evans (1983) concluded that these nematode eggs were heavier than other nematode eggs and thus their demonstration on faecal flotation was variable; Cabrera and Bailey (1964) suggested that these eggs require a flotation solution with a high specific gravity (SG) to enable them to float. They also found that eggs did not concentrate efficiently in the salt and sugar solutions usually recommended. Evans (1983) reported that a $ZnSO_4$ flotation solution (SG 1.32) was satisfactory for egg flotation. Chhabra and Singh (1972) found that a $ZnSO_4$ flotation solution (SG 1.36) damaged the eggs and the NaCl or $MgSO_4$ flotation fluids (SG 1.18-1.22) were unsatisfactory for the flotation of these eggs. Harrus and Harmelin (1996) found that a sugar solution (SG 1.27) was very efficient for the detection of *S. lupi* eggs. Reche-Emonot and Beugnet (2001) determined that flotation with a sugar or $MgSO_4$ solution gave comparable results. Markovics and Medinski (1996) described a centrifugal sedimentation/flotation technique using a sugar flotation fluid (SG 1.27) and found that it was sensitive for detecting *S. lupi* eggs in faecal samples containing few eggs, and consequently recommended its use for routine diagnosis. Improved egg recovery has also been demonstrated using a modified Stoll technique where artificial gastric juices were added to faeces [3]. Traversa *et al.* (2008) found that the *FLOTAC®* technique scored the highest number of identifying positive faecal samples compared to the routine $ZnSO_4$ flotation and the Markovics/Medinski technique.

Considering the contradictory findings in the published literature, there is a clear need to compare and assess the overall sensitivity of faecal examination techniques in the diagnosis of spirocercosis and to determine the most suitable in terms of sensitivity and practicality.

CHAPTER 2

Study Objectives

2.1 Problem Statement

Reports on various coproscopical techniques for detecting *S. lupi* eggs are diverse and provide no practical guidelines for the veterinary diagnostician.

2.2 Purpose of the study

1 To compare four published coproscopical techniques and to determine the most sensitive one for detecting *S. lupi* eggs in dogs with spirocercosis confirmed by oesophageal endoscopy.
2 To evaluate a novel modified faecal flotation examination test using a commercial flotation fluid.
3 To determine the percentage of *S. lupi* infected dogs confirmed by oesophageal endoscopy that shed eggs.
4 To determine whether the number of *S. lupi* eggs shed differs between dogs with non-neoplastic and neoplastic nodules.

CHAPTER 3

Materials and Methods

3.1 Ethical considerations

This study was approved by the Animal Use and Care Committee of the University of Pretoria. Protocol reference V079/07.

The study utilised cases which were part of a larger study evaluating a variety of clinical and diagnostic aspects of canine spirocercosis. This allowed information regarding nodule transformation, response to therapy and outcome to be readily available.
Protocol name: Epidemiology, clinical presentation and diagnostic methods (ante and post mortem) for spirocercosis in dogs.
Protocol: Reference V037-07.

Owner consent was obtained for faecal collection from dogs. diagnosed with spirocercosis at the Onderstepoort Veterinary Academic Hospital (OVAH).

3.2 Patient Selection

Faecal samples were collected from dogs that presented to the OVAH that met the following criteria:

1. Spirocercosis was confirmed by oesophageal endoscopy.
2. None of the dogs had received any medication containing macrocylic lactones in the preceding 6 months.

Selected dogs were further divided into one of three groups:

1. The non-neoplastic group, where the oesophageal nodule(s) showed the typical smooth, sessile endoscopic characteristics and regressed after treatment with doramectin as assessed through follow-up endoscopy at 6 weeks.
2. The neoplastic group, where the surface of the oesophageal nodule(s) appeared roughened cauliflower-like and ulcerated and where neoplastic transformation was confirmed by histopathology of the endoscopically or surgically obtained biopsy.
3. The mixed group, consisting of dogs with multiple oesophageal nodules including both non-neoplastic and neoplastic sub-types, as determined by the above-mentioned criteria.

3.3 Sample collection

Faeces were either obtained directly from the rectum or fresh stool samples were collected from dogs that were seen to defaecate. The sample was stored at 4℃ until processed. All faecal samples were analysed within 24 hours. If faecal samples were found to be negative for *S. lupi* eggs, a second sample was collected 24-48 h later for analysis (see appendix 1 for data collection sheet).

3.4 Faecal examination methods

All faecal samples were examined using a combination of methods and flotation fluids. A total of 10 faecal examination tests were performed on each sample.

Four different faecal examination methods were used:

- Direct faecal examination
- Direct faecal flotation
- Modified centrifugal flotation
- Centrifugal sedimentation/flotation

Four different flotation fluids were used in the direct and modified centrifugal flotation methods. These flotation fluids included:

- Sugar solution (SG 1.27)
- Zinc sulphate ($ZnSO_4$) solution (SG 1.30)
- Sodium nitrate ($NaNO_3$) solution (SG 1.22)
- Magnesium sulphate ($MgSO_4$) solution (SG 1.29)

Only the $NaNO_3$ flotation solution (SG 1.22) was commercially available, under the trade name, Faecalyser® (Kyron). The remaining solutions were made up in a laboratory by the author using de-ionised water and the corresponding solute. A 40% formaldehyde solution was added to the sugar solution (at a ratio of 40 ml/l) to preserve it. The SG of all the flotation solutions was determined using a hydrometer. For a quality assurance purposes, the SG of the made-up solutions was also monitored at 3-month intervals. All solutions were kept sealed at room temperature, away from light.

3.4.1 Direct faecal examination technique

One gram of faeces was placed into a plastic test tube to which 5 ml of saline was added. The mixture was manually agitated using a wooden spatula for 30 s. An aliquot of 0.1 ml of the suspension was aspirated using an adjustable micropipette and placed onto a microscope slide and coverslipped with a 22 mm x 22 mm cover-slip and examined under a light microscope at 100x magnification. All the *S. lupi* eggs under the coverslip and those seen at the edges of the cover-slip were counted.

3.4.2 Direct faecal flotation

One gram of faeces was placed into the receptacle of a commercial direct faecal flotation test device, OvaTector® (Kyron). The flotation solution was added and the mixture was agitated with a wooden spatula for 30 s. The faecal flotation test kit's strainer was inserted and the tubular receptacle was then filled to the rim and a 22 mm x 22 mm cover slip placed on top. Twenty min was allowed to elapse to maximize egg flotation after which the cover slip was removed and placed onto a microscope slide and examined at 100x magnification (see appendix 2 for *Ovatector®* instruction manual). All the *S. lupi* eggs under the cover slip and those seen at the edges of the cover slip were manually counted. Faecal flotations were conducted separately using each of the four flotation fluids, namely sugar, $ZnSO_4$, $NaNO_3$ and $MgSO_4$.

3.4.3. Modified centrifugal flotation

One gram of faeces was placed into a plastic test tube to which 5 ml of the flotation solution was added. The mixture was agitated using a wooden spatula for 30 s. The test tube was capped and placed into a fixed-arm centrifuge. The sample was centrifuged at 1400G for 10 min. Two aliquots of 0.05 ml of the supernatant were aspirated, using an adjustable micropipette and placed onto a microscope slide. A 22 mm x 22 mm cover-slip was applied and the slide was examined under a light microscope at 100x magnification. All the *S. lupi* eggs under the cover slip and those seen at the edges of the cover slip were manually counted. The modified centrifugal flotation method was conducted separately using each of the four flotation fluids, namely sugar, $ZnSO_4$, $NaNO_3$ and $MgSO_4$.

3.4.4 Centrifugal sedimentation flotation

One gram of faeces was put into a 50 ml graduated tube and mixed with 30 ml of artificial gastric juice (2 % pepsin and 1 % concentrated hydrochloric acid). The faecal suspension was agitated for 5 min at room temperature with the aid of a magnetic stirrer prior to being strained through a fine tea strainer. The strained suspension was centrifuged at 1400 G for 10 min after which the supernatant was removed. The $MgSO_4$ solution was then added to the sediment which was re-suspended and centrifuged for a further 10 min. The tube was then filled completely to form a meniscus with additional $MgSO_4$ solution and a 22 mm x 22 mm cover slip was floated on the meniscus for 10 min. The cover slip was then placed onto a microscope slide and examined under a light microscope at 100x magnification. All the *S. lupi* eggs under the coverslip and those seen at the edges of the cover-slip were manually counted. This was a modification of the Markovics and Medinski (1996) method as our initial attempts to place coverslips over the test tubes in the swing-out rotor for the centrifuge were unsuccessful and had to be abandoned. This method was conducted by the Helminthology Laboratory at the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

3.5 Egg count per gram

The total egg count per gram (epg) of faeces per dog was calculated using the egg count obtained from the direct faecal examination method. In this method 1g of faeces was mixed with 5 ml of saline for 30 s. 0.1ml of the sample was removed for microscopic examination and all the eggs were counted.

Calculation:
Eggs per gram (EPG) = total egg count (0.1ml) x 50

CHAPTER 4

Results

4.1 Data selection

A total of 33 dogs with spirocerosis confirmed by oesophageal endoscopy were included in this study. The faecal samples were collected between April 2008 and December 2009. Ten faecal examinations were performed on each faecal sample.

4.2 Statistical analysis

BMDP Statistical software (BMDP Statistical software, Inc. Los Angeles, USA) was used to compute the statistics.

4.2.1 The Friedman non-parametric repeated measures analysis of variance

Egg counts for the 10 different faecal examinations were compared using the Friedman non-parametric repeated measures analysis of variance. Multiple comparisons, using a 0.05 level of significance with the Bonferroni correction, were used to determine which pairs of methods were statistically different.

4.2.2 Mann-Whitney U test

The non-neoplastic and neoplastic groups' egg counts were statistically compared using the Mann-Whitney U test.

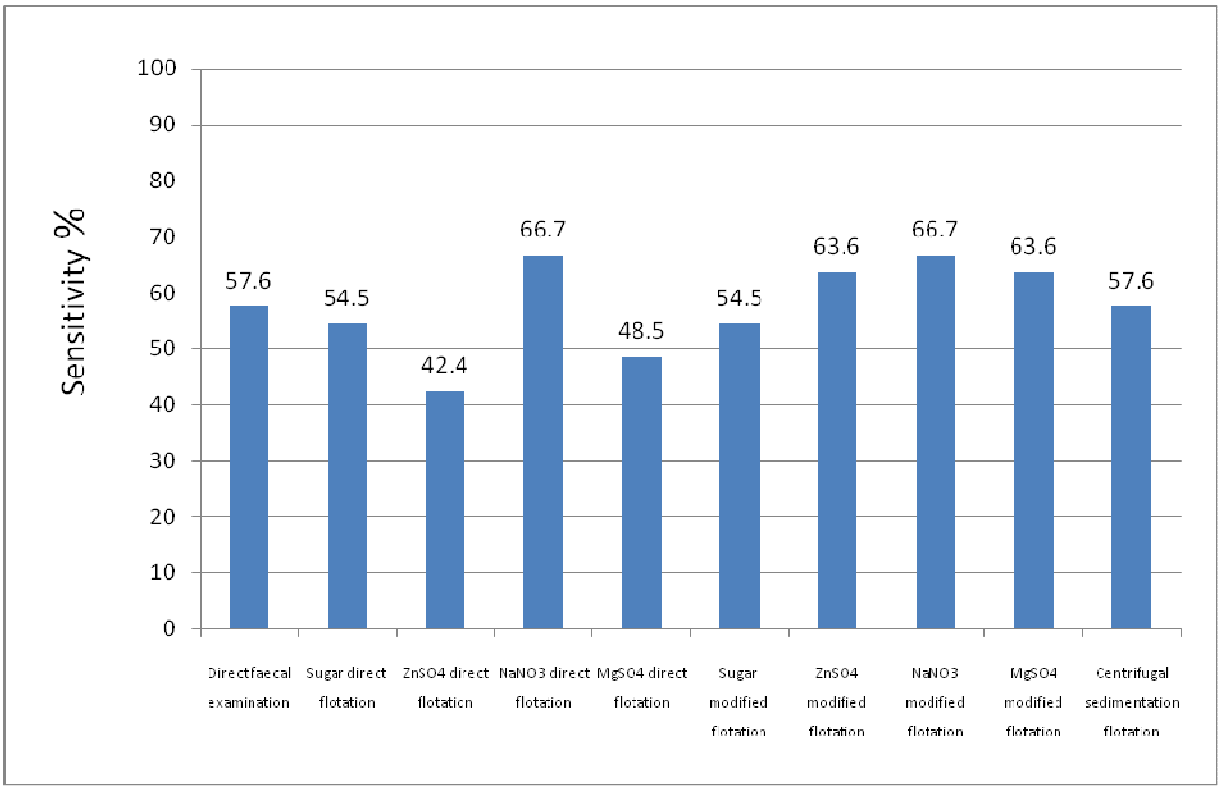
4.3 Data analysis

4.3.1 The sensitivity of the faecal examination methods to detect *S. lupi eggs*

The sensitivity of faecal examination in the detection of *S. lupi* eggs (Fig. 1) ranged between 42.4 % and 66.7 %, with the direct and modified centrifugal $NaNO_3$ flotation tests showing the highest sensitivity at 66.7%. No eggs were found in 12 of 33 (36.6%) initial faecal samples. The second sample collected 24 to 48 hours later revealed only one additional dog to be shedding eggs. In this patient only the routine and modified $NaNO_3$ flotation test were positive. Thus a total of 11 of 33 (33.3 %) dogs were negative for *S. lupi* eggs on flotation. The direct $ZnSO_4$ flotation test was found to be the least sensitive with a sensitivity of 42.4%. The direct faecal

examination showed a sensitivity of 57.6% while the remaining faecal flotation tests showed a sensitivity of 48.5% and 54.5% using the $MgSO_4$ and sugar flotation fluids, respectively. The modified centrifugal flotation method resulted in an increased sensitivity for both the $ZnSO_4$ and $MgSO_4$ flotation fluids/tests but did not increase the sensitivity of the $NaNO_3$ or the sugar flotation fluids. The centrifugal sedimentation test showed a sensitivity of 57.6%.

Figure 1: The sensitivity of the ten faecal examination methods in detecting *S. lupi* eggs.



4.3.2 Statistical comparison of the faecal examination methods evaluated in the detection of *S. lupi*.

The egg counts of all 10 examination methods are summarized in table 1. The mean egg count varied between 12 and 45 eggs per sample. The modified $NaNO_3$ flotation test ranked first (Fig. 2) with the highest mean egg cell count of 45. Table 2 shows the comparison between the different faecal flotation tests evaluated. It was found that the $NaNO_3$ modified faecal flotation test was superior (higher egg count) and statistically different to the sugar, $ZnSO_4$ and $MgSO_4$ direct flotation tests ($p<0.05$). The $NaNO_3$ direct faecal flotation test was also found to be

superior and statistically different to the $ZnSO_4$ and $MgSO_4$ direct flotation tests ($p<0.05$). When applying a 0.1 level of significance, the $NaNO_3$ direct flotation was also found to be superior and statistically different to the direct sugar flotation test. No further statistically significant differences were found between the techniques.

Table 1: Statistical summary of the faecal examination methods evaluated showing: the number of samples; mean egg count; standard deviation; median egg count, minimum egg count and maximum egg count.

Variable	Number of samples	Mean egg count	Standard Deviation	Median egg count	Min. egg count	Max. egg count
1. Direct faecal examination	33	21.970	74.472	1	0	425
2. Sugar direct flotation	33	14.788	41.148	1	0	226
3. $ZnSO_4$ direct flotation	33	17.515	76.862	0	0	442
4. $NaNO_3$ direct flotation	33	27.000	52.681	6	0	244
5. $MgSO_4$ direct flotation	33	11.758	25.308	0	0	113
6. Sugar modified flotation	33	16.242	32.061	2	0	155
7. $ZnSO_4$ modified flotation	33	23.848	69.935	4	0	368
8. $NaNO_3$ modified flotation	33	45.242	83.172	4	0	331
9. $MgSO_4$ modified flotation	33	23.636	75.003	2	0	425
10. Centrifugal sedimentation flotation	33	35.212	93.192	4	0	504

Figure 2: The mean number of *S. lupi* eggs present using the different faecal examination methods evaluated. Error bars represent the standard error.

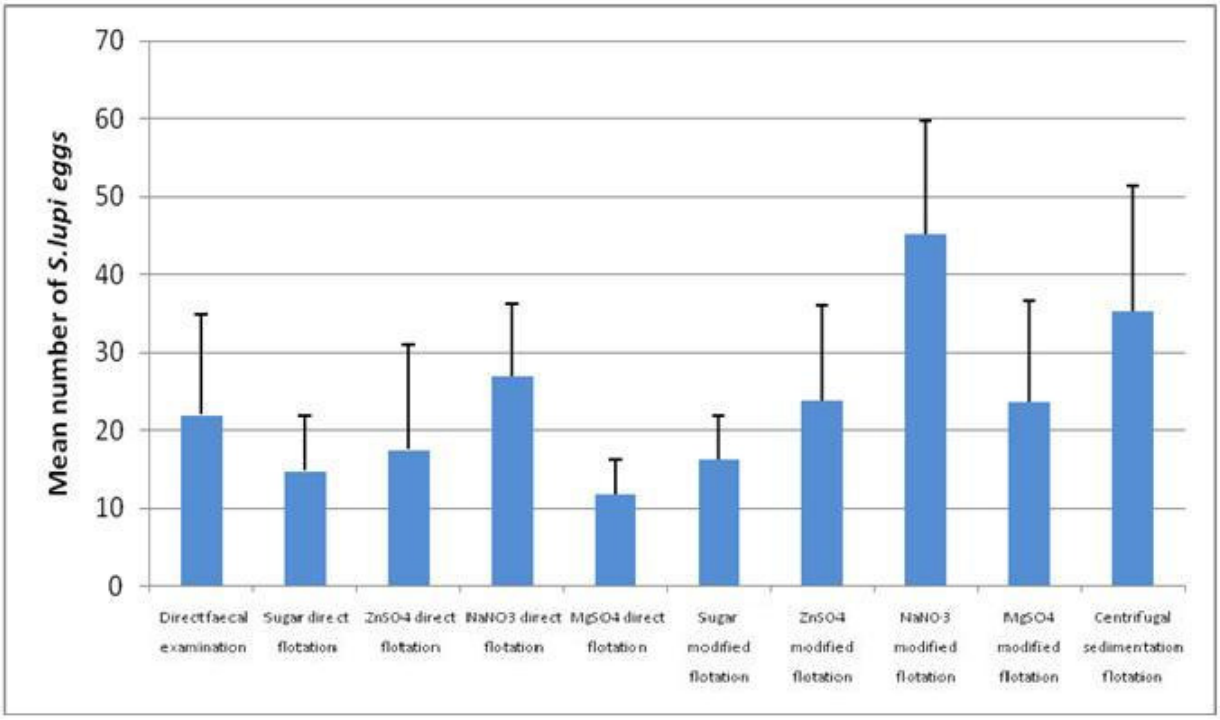


Table 2: A summary of the significant comparative results. Friedman two-way analysis of variance test results:

The critical Z values are:

3.06 for the overall significance of 0.1 (*)

3.26 for the overall significance of 0.05 (**)

Comparisons	Z-Stat	DIF	SE
Sugar direct flotation – $NaNO_3$ direct flotation	**3.07 ***	**-75.50**	**24.60**
Sugar direct flotation – $NaNO_3$ modified flotation	**3.66 ****	**-90.00**	**24.60**
$ZnSO_4$ direct flotation – $NaNO_3$ direct flotation	**3.94 ****	**-97.00**	**24.60**
$ZnSO_4$ direct flotation – $NaNO_3$ modified flotation	**4.53 ****	**-111.50**	**24.60**
$NaNO_3$ direct flotation – $MgSO_4$ direct flotation	**3.44 ****	**84.50**	**24.60**
$MgSO_4$ direct flotation – $NaNO_3$ modified flotation	**4.02 ****	**-99.00**	**24.60**

Chi-square distribution with 9 degrees of freedom

Kendall coefficient of concordance 0.1298

The null hypothesis is rejected if the Z-Stat is larger than the critical value ZC, where 1-ZC = Alpha/(k[k-1]). Alpha is the overall significance level and K is the number of groups compared (10 groups).

See table 1 for faecal examination method description.

See appendix 3 for the complete statistical comparative results.

4.3.3 The average EPG

The average EPG of faeces was calculated at 1100 (22 eggs per 0.1ml faecal suspension) with the highest EPG being 21 250 (425 eggs per 0.1ml faecal suspension).

4.3.4 The sensitivity to detect *S. lupi* eggs in the non-neoplastic and neoplastic group of patients

The non-neoplastic group of patients (n=22) accounted for 67 % of the samples, the neoplastic group (n=5) for 15% and the mixed group (n=6) for 18% of the samples. The sensitivity of faecal flotation (using the $NaNO_3$ modified flotation method) to detect *S. lupi* eggs was 72.7 % in the non-neoplastic group and 40% in the neoplastic group. The mixed group was excluded in this assessment. The mean egg count using this method was 61 ± 95.8 in the non-neoplastic group and 1 ± 1.7 in the neoplastic group. This difference was significant (p=0.024, Mann-Whitney U test, one sided test).

CHAPTER 5

Discussion and conclusion

5.1 Discussion

5.1.1 Sensitivity of coproscopical techniques for the detection of *S. lupi* eggs

This study demonstrated that the sensitivity of faecal examination for detecting *S. lupi* eggs in dogs diagnosed with spirocercosis is highly variable and depends on which faecal examination technique and flotation fluid is utilized. The sensitivity in this study ranged between 42 % and 67 %, which confirms that faecal flotation *per se* is not a sensitive tool to confirm a diagnosis of spirocercosis and it should therefore not be used as a screening test for infection. Egg shedding from *S. lupi* infected dogs is variable and does not occur in all infected individuals.
Reasons for this are prepatent infections, the absence of an operculum in the nodule, postpatent infection or neoplastic transformation of the nodule where the worm is usually no longer present, aberrant migration with the nodules present in organs other than the gastrointestinal tract and therefore have no patent opening to the oesophageal lumen, infection with female or male only nematodes and the typical intermittent shedding of eggs by the female worm. Fox *et al.* (1988) claimed that the diagnosis by faecal analysis is only possible when eggs are passing in the faeces and this passage can occur for an unpredictable, relatively short period of time in the adult worm.

This study also demonstrated that the second faecal sample collected 24-48 h after an initial negative faecal flotation test was only able to detect eggs in an additional 8.4 % of patients. Mazaki-Tovi *et al.* (2002) found that in a study of 40 *S. lupi* positive dogs, using the sugar flotation examination technique, a repeat faecal examination increased the sensitivity by 22.5%. The sensitivity to detect *S. lupi* eggs rose from 57.5 % on the first faecal flotation, to 80% on the subsequent faecal flotation. However, the authors did not specify how many days elapsed between the two samples. For ethical reasons the client-owned dogs in this study had to be treated with doramectin as soon as possible after diagnosis was confirmed which accounted for the 48 h timeframe. Doramectin has shown good therapeutic effect in the treatment of non-neoplastic spirocercosis[17].

5.1.2 Egg count analysis

Sen and Anataraman (1971) showed that peak egg production occurred between 140 and 205 days post infection with a maximum of 2 100 eggs per gram of faeces being detected. Bailey

(1972) showed that egg counts ranged from 2 000 to 11 000 epg in laboratory dogs inoculated with 175 infective larvae. This study demonstrated that egg production can be very high, with 21 250 epg of faeces recorded in one naturally infected dog. Two additional dogs were found to have egg counts above those reported by Sen & Anataraman (1971), with 2 650 and 3 150 epg respectively. In the present study the average egg count was 1100 epg, thus even though shedding is intermittent, infected dogs do contaminate the environment and this may explain the increasing incidence of the disease in regions of South Africa. The importance of rapid removal of faeces from the environment is thus emphasized as a practical control measure. Treatment with doramectin causes a 99.3 % decrease in egg counts within 10 days after the first dose, thus early diagnosis and treatment is imperative to decrease environmental contamination[17].

5.1.3 Sensitivity of coproscopical techniques in the diagnosis of non-neoplastic versus neoplastic nodules in canine spirocercosis

The initial assumption was that the typical early spirocercosis cases with or without clinical signs would, have higher egg counts on faecal examination than those with more advanced neoplastic disease, as the worms were younger and more prolific. It was hoped therefore that faecal examination would be a more sensitive screening tool for these early and often sub-clinical cases. Unfortunately, the sensitivity of the modified centrifugal $NaNO_3$ faecal flotation as a screening test did not increase significantly when only these early cases were included in the analysis. The sensitivity of faecal egg counts was different in the non-neoplastic (72.7 %) *versus* the neoplastic group, (40 %) but not statistically significant. Furthermore a sensitivity of 72.7% is inadequate for a screening test. The study also showed that the mean number of eggs shed by the dogs in the neoplastically transformed group (1 ± 1.7) was significantly lower than in the non-neoplastic group (61.1 ± 95.8). This may indicate that either these neoplastic nodules contain fewer worms and/or that these worms shed fewer eggs. In support of this idea, neoplastic nodules identified at post mortem do contain fewer or no worms at all. [2]. Although the difference between the mean egg counts between the non-neoplastic and neoplastic group was statistically significant there is no real clinical relevance as both groups shed eggs. Furthermore 18% of dogs in this study had both neoplastic and non-neoplastic nodules present in the oesophagus, another major reason why faecal flotation cannot to used to differentiate between non-neoplastic and neoplastic canine spirocercosis.

5.1.4 Comparative faecal analysis

This study demonstrated that $NaNO_3$ (SG 1.22) had the highest sensitivity when utilized in both the direct and modified centrifugal flotation tests**.** This was unexpected as the prior literature clearly stipulates that *S. lupi* eggs have a higher specific gravity than other nematode eggs, which consequently requires the use of flotation solutions with higher specific gravities to improve recovery of eggs. In this study the $ZnSO_4$ (SG 1.30) and the $MgSO_4$ (SG 1.29) flotation fluids damaged the eggs as was previously reported by Chhabra and Singh (1972). The eggs became rectangular in shape and the edges appeared to fold in on themselves. The sugar flotation solution crystallized rapidly, impairing visualization of the eggs and was sticky and difficult to work with compared to the other solutions. The $NaNO_3$ (SG 1.22) solution was easy to work with and the solution was also extremely clear, facilitating easy visualization of the *S. lupi* eggs.

The results of this study demonstrate that flotation fluids with a high specific gravity are not required to detect *S. lupi* eggs. Markovics and Medinski (1996) reported on a sugar flotation method to be 100 % sensitive in detecting *S. lupi* eggs in eight samples with low egg numbers (100 epg), whereas with direct faecal examination only 50 % were found to be positive. This study demonstrated equal sensitivities (57.6%) for the direct faecal examination and the centrifugal sedimentation/flotation, but the centrifugal sedimentation/flotation method gave a higher mean egg count of 35 compared to the direct faecal examination with 22. The study also showed that the addition of artificial gastric juice did not enhance the sensitivity of the Markovics/Medinski method. A recent study by Traversa *et al* (2008) found that the detection of *S. lupi* eggs using $ZnSO_4$ (SG 1.35) flotation fluid and the Markovics/Medinski technique was unsatisfactory since eggs were detected in only 7 out of 31 and 4 out of 31 *S. lupi* PCR-positive faecal samples. They also showed that the FLOTAC® apparatus was more sensitive as it detected eggs in 10 of these 31 *S. lupi* PCR-positive faecal samples. The study found that the PCR was more sensitive to detect positive faecal samples (49 out of 94) when compared to a combination of faecal flotation methods (19 out of 94).

5.2 Conclusion

The sensitivity of coproscopical techniques to detect canine spirocercosis was relatively poor. The highest sensitivity (67%) was obtained with $NaNO_3$ flotation fluid. The modified flotation method using $NaNO_3$ (SG 1.22) solution resulted in the highest egg counts and it proved to have an equal sensitivity to that of direct faecal flotation using the same flotation fluid. Both methods are simple to perform, do not require specialized equipment, utilize a commercially available flotation fluid and can be completed in less than 20 min, which make them suitable for the private practice environment. The relatively low SG of the commercially available flotation fluid is also easily maintained as it does not crystallize as much as the more concentrated flotation fluids.

A limitation of this study is that the volume of fluid in which the eggs were counted under the cover-slip was not consistent. In the modified centrifugal and direct faecal examination tests 0.1ml of supernatant or fluid was used, while in the direct faecal flotation and centrifugal/ sedimentation tests the drop adhering to the cover-slip was used. However this was not considered to be too significant as the volume of flotation solution under the cover-slip in any group faecal flotation test is not consistent between tests.

This study also demonstrated that dogs with neoplastic spirocercosis do shed eggs although far fewer eggs are detected compared to patients with non-neoplastic spirocercosis.

References

1. Anataraman M, Sen K 1966 Experimental spirocercosis in dogs with larvae from a paratenic host, *Calotes versicolor*, the common garden lizard in Madras. *Journal of Parasitology* 52: 911-912
2. Bailey W S 1972 *Spirocerca lupi*: a continuing inquiry. *Journal of Parasitology* 58: 3-22
3. Cabrera D J, Bailey W S 1964 A modified Stoll technique for detecting eggs of *Spirocerca lupi*. *Journal of the American Veterinary Medical Association* 145: 573-575
4. Chandraskharon K P, Sastry G A, Menon M N 1958 Canine spirocercosis with special reference to the incidence and lesions. *British Veterinary Journal* 114: 388-395
5. Chhabra R C, Singh K S 1972 On the life cycle of *Spirocerca lupi*: Preinfective stages of the intermediate host. *Journal of Helminthology* 46: 125-137
6. Dixon K G, McCue J F 1967 Further observations on the epidemiology of *Spirocerca lupi* in the southeastern United States. *Journal of Parasitology* 53: 1074-1075
7. Du Plessis C J, Keller N, Millward I R 2007 Aberrant extradural spinal migration of *Spirocerca lupi*: four dogs. *Journal of Small Animal Practice* 48: 275-278
8. Dvir E, Kirberger R M, Malleczek D 2001 Radiographic and computed tomographic changes and clinical presentation of spirocercosis in the dog. *Veterinary Radiology and Ultrasound* 42: 119-129
9. Dvir E, Kirberger R M, Mukorera V, Van der Merwe L L, Clift S J 2008 Clinical differentiation between dogs with benign and malignant spirocercosis. *Veterinary Parasitology* 155: 80-88
10. Evans L B 1983 Clinical diagnosis of *Spirocerca lupi* infestation in dogs. *Journal of the South African Veterinary Association* 54: 189-191
11. Fox S M, Burns J, Hawkins J 1988 Spirocercosis in dogs. *Compendium of Continuing Education of the Practicing Veterinarian* 10: 807-822
12. Georgi J R, Georgi M E 1990 Parasitology for Veterinarians (5th edn). W.B. Saunders, Philadelphia, USA
13. Harrus S, Harmelin A, Markovics A, Bark H 1996 *Spirocerca lupi* infection in the dog: aberrant migration. *Journal of the American Animal Hospital Association* 33: 125-130
14. Hu C H, Hoeppli R J C 1936 The migration route of *Spirocerca sanguinolenta* in experimentally infected dogs. *Chinese Medicine Journal-Peking Supplement* 1: 11-99

15. Kaschula V R, Malherbe W D 1954 The incidence and diagnosis of spirocercosis in dogs in the Transvaal. *Journal of the South African Veterinary Association* 25: 53-59
16. Kirberger R M, Zambelli A 2007 Imaging diagnosis: aortic thromboembolism associated with spirocercosis in a dog. *Veterinary Radiology and Ultrasound* 48: 418-420
17. Lavy E, Aroch I, Bark H, Markovics A, Aizenberg I, Mazaki-Tovi M, Haga A, Harrus S 2002 Evaluation of doramectin for the treatment of experimental canine spirocercosis. *Veterinary Parasitology* 109: 65-73
18. Lobetti R G 2000 Survey of the incidence, diagnosis, clinical manifestations and treatment of *Spirocerca lupi* in South Africa. *Journal of the South African Veterinary Association* 71: 43-45
19. Markovics A, Medinski B, 1996 Improved diagnosis of low intensity *Spirocerca lupi* infection by the sugar flotation method. *Journal of Veterinary Diagnostic Investigation* 8: 400-401
20. Mazaki-Tovi M, Baneth G, Aroch I, Harrus S, Kass P H, Ben-Ari T, Zur G, Aizenberg I, Bark H, Lavy E 2002 Canine spirocercosis, clinical, diagnostic, pathological and epidemiological characteristics. *Veterinary Parasitology* 107: 235-250
21. Minnaar W N, Krecek R C, Fourie L J 2002 Helminths of dogs from a peri-urban resource-limited community in the Free State Province, South Africa. *Veterinary Parasitology* 107: 343-349
22. Murray M 1968 Incidence and pathology of *Spirocerca lupi* in Kenya. *Journal of Comparative Pathology* 78: 401-405
23. Pereira W L A, Guimaraes F A B D A, Martins A K P, Peixoto P C 1995 Haemopericardium in a dog associated with hyperparasitism by *Spirocerca lupi*. *Boletin Faculdade de Ciencias Agrarias do Para* 23: 45-51
24. Ranen E, Lavy E, Aizenberg I, Perl S, Harrus S 2004 Spirocercosis-associated esophageal sarcomas in dogs. A retrospective study of 17 cases (1997 – 2003). *Veterinary Parasitology* 119: 209-221
25. Reche-Emonot M, Beugnet F, Bourdoiseau G 2001 Étude épidémiologique et clinique de la spirocercose canine à l'Il de le Réunion à partir de 120 cas. *Revue de Médecine Vétérinaire* 152: 469-477
26. Sen K, Anataraman M 1971 Some observations on the development of *Spirocerca lupi* in its intermediate and definitive hosts. *Journal of Helminthology* 45: 123-131
27. Singh B, Juval P D, Sobti V K 1999 *Spirocerca lupi* in a subcutaneous nodule in a dog in India. *Veterinary Parasitology* 13: 59-60

28. Stephens S C, Gleiser C A, Jardine J H 1983 Primary pulmonary fibrosarcoma associated with *Spirocerca lupi* in a dog with hypertrophic pulmonary osteoarthropathy. *Journal of the American Veterinary Medical Association* 182: 496-498
29. Soulsby E J L 1982 *Helminths, arthropods and protozoa of domestic animals*. Lea and Febiger, Philadelphia, USA
30. Traversa D, Avolio S, Modrý D, Otranto D, Iorio R, Aroch I, Cringoli G, Milillo P, Albrechtová K, Milhalca A D, Lavy E, 2008 Copromicroscopic and molecular assays for the detection of cancer-causing parasitic nematode *Spirocerca lupi*. *Veterinary Parasitology* 157: 103-116
31. Urquhart G M, Armour J, Duncan J L, Dunn A M, Jennings F Q 1994 *Veterinary Parasitology.* Longman Scientific and Technical, Essex, England
32. van der Merwe L L, Kirberger R M, Clift S J, Williams M, Keller N, Naidoo V 2008 *Spirocerca lupi* infection in the dog: a review. *The Veterinary Journal* 197: 294-309

Appendices

Appendix 1: Data collection form template:

PATIENT DETAILS	
Patient Number: **Patient Name:** **Breed:** **(PATIENT STICKER)** **Age:** **Sex:**	**Date:** **Faecal sample:** Stool sample Rectal sample
DIAGNOSIS	
Oesophagoscopy (List findings)	**Performed Yes No**
Thoracic radiography (List findings)	**Performed Yes No**
FAECAL EXAMINATION	
1st faecal sample	**2nd faecal sample required**
METHOD	**Number of *Spirocerca lupi* eggs found**
A) Direct faecal examination	•
B) Direct faecal flotation • **Sugar flotation fluid (SG 1.27)** • **$ZnSO_4$ flotation fluid (SG 1.30)** • **$NaNO_3$ flotation fluid (SG 1.22)** • **$MgSO_4$ flotation fluid (SG 1.29)**	 • • • •
C) Modified centrifugal flotation • **Sugar flotation fluid (SG 1.27)** • **$ZnSO_4$ flotation fluid (SG 1.30)** • **$NaNO_3$ flotation fluid (SG 1.22)** • **$MgSO_4$ flotation fluid (SG 1.29)**	 • • • •
D) Centrifugal sedimentation/flotation	•

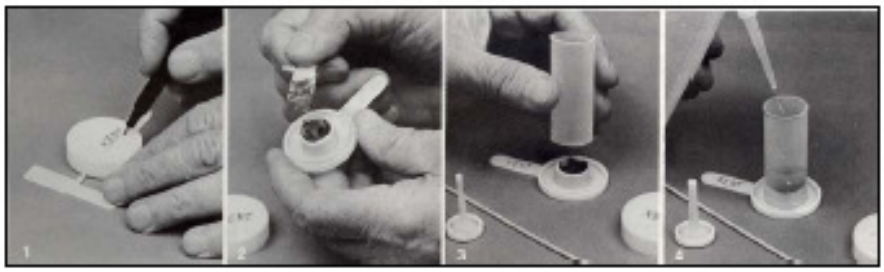
Appendix 2: Ovatector routine faecal flotation method; manufacturer's instructions (Kyron)

KYRON

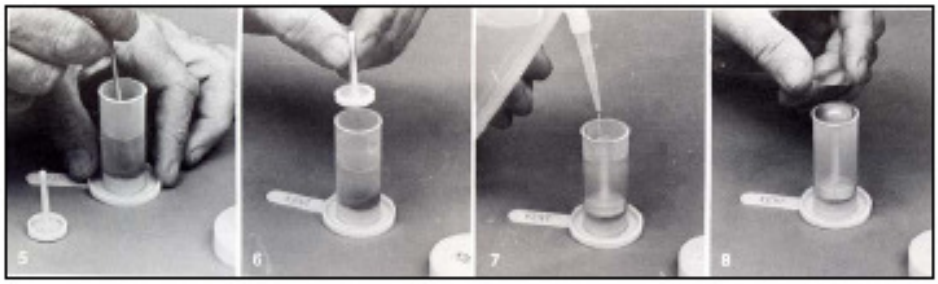
OVATECTOR® disposable fecal diagnostic system

The original standard of excellence.

Follow this simple procedure to set up the OvaTector ® system in less than 45 seconds



). Using a marking pen, identify the faecal collection container and dispense to client. **(2).** Client separates atula from container and fills centre receptacle with faecal matter (holds a 2g sample). **(3).** When faecal ntainer is returned, the cylinder with lip-end up, is placed over the centre receptacle and snapped into sition forming the floatation system. **(4).** Fill the cylinder halfway with *Kyron Egg Flotation Fluid*.



(5). Mix the faecal specimen and solution thoroughly with applicator stick provided. **(6).** Push strainer gently down into cylinder until handle is below top lip. **(7).** Add more *Kyron Egg Flotation Fluid* until convex meniscus is formed at the top of the cylinder. **(8).** Float a 22mm cover slip on the meniscus. Allow to stand at least 15 min. for ova to float through the strainer and adhere to the cover slip. Lift cover slip with a smooth motion and place on microscope slide. Examine under low power and 100x for ova.

Copyright permission granted from Kyron laboratories (08/09/2011)

Appendix 3: Friedman two-way analysis of variance, complete test results:

The critical Z values are:

3.06 for the overall significance of 0.1 (*)

3.26 for the overall significance of 0.05 (**)

Comparisons	**Z-Stat**	**DIF**	**SE**
Direct faecal examination – Sugar direct flotation	0.67	16.50	24.60
Direct faecal examination – $ZnSO_4$ direct flotation	1.54	38.00	24.60
Direct faecal examination – $NaNO_3$ direct flotation	2.4	-59.00	24.60
Direct faecal examination – $MgSO_4$ direct flotation	1.04	25.50	24.60
Direct faecal examination – Sugar modified flotation	0.81	-20.00	24.60
Direct faecal examination – $ZnSO_4$ modified flotation	1.32	-32.50	24.60
Direct faecal examination – $NaNO_3$ modified flotation	2.99	-73.50	24.60
Direct faecal examination – $MgSO_4$ modified flotation	1.18	-29.00	24.60
Direct faecal examination – Centrifugal sedimentation flotation	1.06	-26.00	24.60
Sugar direct flotation – $ZnSO_4$ direct flotation	0.87	21.50	24.60
Sugar direct flotation – $NaNO_3$ direct flotation	**3.07 ***	**-75.50**	**24.60**
Sugar direct flotation – $MgSO_4$ direct flotation	0.37	9.00	24.60
Sugar direct flotation – Sugar modified flotation	1.48	-36.50	24.60
Sugar direct flotation – $ZnSO_4$ modified flotation	1.99	-49.00	24.60
Sugar direct flotation – $NaNO_3$ modified flotation	**3.66 ****	**-90.00**	**24.60**
Sugar direct flotation – $MgSO_4$ modified flotation	1.85	-45.50	24.60
Sugar direct flotation – Direct faecal examination0	1.73	-42.50	24.60
$ZnSO_4$ direct flotation – $NaNO_3$ direct flotation	**3.94 ****	**-97.00**	**24.60**
$ZnSO_4$ direct flotation – $MgSO_4$ direct flotation	0.51	-12.50	24.60
$ZnSO_4$ direct flotation – Sugar modified flotation	2.36	-58.00	24.60
$ZnSO_4$ direct flotation – $ZnSO_4$ modified flotation	2.87	-70.50	24.60
$ZnSO_4$ direct flotation – $NaNO_3$ modified flotation	**4.53 ****	**111.50**	**24.60**
$ZnSO_4$ direct flotation – $MgSO_4$ modified flotation	2.72	-67.00	24.60
$ZnSO_4$ direct flotation – Direct faecal examination0	2.6	-64.00	24.60
$NaNO_3$ direct flotation – $MgSO_4$ direct flotation	**3.44 ****	**84.50**	**24.60**
$NaNO_3$ direct flotation – Sugar modified flotation	1.59	39.00	24.60
$NaNO_3$ direct flotation – $ZnSO_4$ modified flotation	1.08	26.50	24.60
$NaNO_3$ direct flotation – $NaNO_3$ modified flotation	0.59	-14.50	24.60

$NaNO_3$ direct flotation – $MgSO_4$ modified flotation	1.22	30.00	24.60
$NaNO_3$ direct flotation – Centrifugal sedimentation flotation	1.34	33.00	24.60
$MgSO_4$ direct flotation – Sugar modified flotation	1.85	-45.50	24.60
$MgSO_4$ direct flotation – $ZnSO_4$ modified flotation	2.36	-58.00	24.60
$MgSO_4$ direct flotation – $NaNO_3$ modified flotation	**4.02 ****	**-99.00**	**24.60**
$MgSO_4$ direct flotation – $MgSO_4$ modified flotation	2.22	-54.50	24.60
$MgSO_4$ direct flotation – Centrifugal sedimentation flotation	2.09	-51.50	24.60
Sugar modified flotation – $ZnSO_4$ modified flotation	0.51	-12.50	24.60
Sugar modified flotation – $NaNO_3$ modified flotation	2.18	-53.50	24.60
Sugar modified flotation – $MgSO_4$ modified flotation	0.37	-9.00	24.60
Sugar modified flotation – Centrifugal sedimentation flotation	0.24	-6.00	24.60
$ZnSO_4$ modified flotation – $NaNO_3$ modified flotation	1.67	-41.00	24.60
$ZnSO_4$ modified flotation – $MgSO_4$ modified flotation	0.14	3.5	24.60
$ZnSO_4$ modified flotation – Centrifugal sedimentation flotation	0.26	6.5	24.60
$NaNO_3$ modified flotation – $MgSO_4$ modified flotation	1.81	44.50	24.60
$NaNO_3$ modified flotation – Centrifugal sedimentation flotation	1.93	47.50	24.60
$MgSO_4$ modified flotation – Centrifugal sedimentation flotation	0.12	3.00	24.60

P-value = 0.0000

Chi-square distribution with 9 degrees of freedom

Kendall coefficient of concordance 0.1298

The null hypothesis is rejected if the Z-Stat is larger than the critical value ZC, where 1-ZC = Alpha/(k(k-1)). Alpha is the overall significance level and K is the number of groups compared (10 groups).

See table 1 for method description.