The relation between a raw meat diet for dogs and a patent infection with *Sarcocystis* spp.*, Isospora* spp*. and Neospora caninum* in dogs in the Netherlands and Belgium.

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Abstract

By reading discussion forums on internet, pet owners become more interested in an alternative way of feeding their dogs besides commercially available dog food. Based on the life cycle of *Neospora caninum, Sarcocystis* spp. and *Isospora* spp., eating of a paratenic host or an intermediate host is an additional risk of getting an infection. In this study, research was done to see whether a relationship exists between feeding dogs raw meat and the development of a patent infection with *N. caninum, Sarcocystis* sp. and *Isospora* sp. in the dog. For this study owners in the Netherlands were asked to participate voluntarly, and send in faeces samples every month. The faecal samples have been processed using centrifuge sedimentation flotation method with sucrose (1,27-1,30 kg/dm$^{3)}$ as flotation medium (4). The slides were examined microscopically for oöcysts of *Isospora* spp *Sarcocystis* spp. and *N. caninum.* Also examination was don for eggs of *Toxocara canis,* Strongyle (*Uncinaria), Trichuris* spp*., Capillaria* spp. *and Tenia* spp..
A significant association was found between dogs that did eat raw meat, fresh or frozen and a patent *Sarcocystis* sp*.* infection. Fresh raw meat was defined in this study as meat that had not been frozen before being fed to the dog. The origin of the meat is probably of great importance. No difference was found between dogs that ate raw meat from intensive livestock farming and meat from organic/ free range animal husbandry. In one sample small oöcysts in the size of *N. caninum* was found. No significant association between dogs that did eat raw meat and a patent *Isospora* spp*.* infection has been found. Neither dogs that did eat raw meat from intensively kept meat producing animals and free-range meat producing animals nor the group that has been fed with fresh meat versus frozen meat and patent infection with *Isospora* ssp. was found.

Introduction

By reading discussion forums on internet pet owners seem to become more interested in an alternative way of feeding their dogs besides commercially available dog food. Mostly because of the idea that the dog must be fed like they would feed themselves in nature. Other arguments are the distrust against commercial dog food, specific medical demands for the pet and involvement in choosing the ingredients for their own dog's meal (5). Since the dog originates from the wolf that eats prey, people seem to find it necessary to feed their animal according to their origin (6). There is a diversity in feeding raw meat to dogs. The first principle is the BARF (Bones and Raw Food of Biologically appropriate Raw food) principle. This principle has been devised by the Australian vet Ian Billinghurst (7). The BARF diet contains 60% raw meat with bones, supplemented with products (cereals) that a dog would eat in the wild. The second principle is derived from Tom Lansdale, who promotes feeding whole prey because the proportions of bone, flesh and organs are already well balanced (8). The NRV (Natural Raw Food) principle is the same as the principle of Tom Lansdale and is based on the dog eating prey. The NRV diet contains no supplements, but solely whole carcasses (9). In the Netherlands, raw meat is also commercially available as a complete pet food. This is

called KVV, which stands for 'complete fresh food'. There also is the Volhard diet, which consists of a mixture of yoghurt and grains in the morning and raw meat, vegetables and fruit in the evening (10).

Dog owners in the Netherlands were asked to participate voluntarily for two years. Every month the participating owners collected faecal samples and sent them to the Faculty of Veterinary Medicine of Utrecht University. A questionnaire was sent by e-mail to dog owners in order to gather information about possible risk factors causing a patent *N. caninum, Sarcocystis* spp. and *Isospora* spp. infection.

The definitive host of *N. caninum* are canids like dogs and coyotes, intermediate hosts are cattle, sheep, horse and goats. *N. caninum* and *Toxoplasma gondii* are morphologically and biologically similar coccidians, with a structural similarity of the asexual stages of the two parasites. This is why *N. caninum* was previously confused with *T. gondii. N. caninum* have canids as final host and the final hosts for *T. gondii* are felids. A range of animals could be infected by ingesting sporulated oöcysts including cattle. Dogs are not solely a final host, they could also act as intermediate host (11). 8-23 days after infection, small numbers of resistant oöcysts in faeces have been observed. The oöcysts are up to 10-11 µm(38). These are the environmentally resistant stages of the parasite, are excreted in the faeces of the dogs and coyotes in an unsporulated state (12) (13) (14). Outside the host oöcysts sporulate in 24 hour(14). Herbivores are intermediate host and probably become infected by sporulated *N. caninum* oöcysts after the ingestion of contaminated food or drinking water. Carnivores possibly get infected by ingesting tissues containing bradyzoites (1). Viable parasites are isolated from placenta and aborted foetuses of cows. Tissues of infected meat or prey of dogs may represent a logical source of infection, however viable parasites have not been isolated from potential dog's prey, such as birds or rodents (15). *N. caninum* infection can cause illness in dogs and cattle (16). In the definitive host *N. caninum* causes mild diarrhoea or no symptoms (11). It is unclear whether the immunity which is built during infection leads to elimination of the excretion of oöcysts in the further live (11). Because *N. caninum* and *T. gondii* are morphologically and biologically similar it is assumed that the environmental resistance of *N. canincum* oöcysts is similar to that of *T. gondii* oöcysts (17).

When the dog acts as an intermediate host neosporosis can occur. Neosporosis is characterised by a progressively ascending paralysis, particularly of the hind limbs and occurs in transplacentally infected puppies. Hepatitis and polymyositis may also occur. At 1-6 months, the first clinical signs could be noticed. These signs could be observed in adults and older dogs as well. Sudden death due to myocarditis has been regularly reported. The diagnosis is based on a history of neurological signs and muscle weakness with a progressively ascending paralysis. In order to confirm the diagnosis, an indirect antibody fluorescent test (IFAT) is available. Lesions are most commonly seen in the spinal cord, brain, skeleton muscles and nerve roots. However, any organ could be affected including the skin. The grey matter in the brain is most severely affected, while in the spinal cord the submeningeal white matter tends to be most severely affected. Tachyzoite proliferation is associated with granulomous reaction, suppuration and focal malacia. Particularly in submeningeal areas of the cerebrellar cortex a marked fibrosis may develop. Parasitised muscle fibres undergo rapid necrosis and massive infiltrations of lymphocytes, macrophages and plasma cells occur. Tissue cysts are usually found only in the CNS and are scarce. The mechanisms of repeated congenital transmissions are unknown at present. Treatments with trimethoprim, sulphadiacine, pyrimethaminde and clindamycin could be useful if canine neosporosis is diagnosed at an early stage (11).

In the intermediate hosts stages of tachyzoites and tissue cysts are found and they occur intracellularly (18). Extra neural tissues, especially muscles, may contain tissue cysts. When tachyzoites are transmitted from an infected dam to her foetus during pregnancy transplacental infection could occur. *N. caninum* is a major cause of abortion in beef and dairy cattle (11). Seropositive animals have been shown to suffer a higher risk of abortion than seronegative animals in the herd (11). Abortion could happen at any stage, although most abortions occur at 5-6 months of

pregnancy. Foetuses could die in the uterus and be reabsorbed, mummified or aborted. If the calves are born alive with the infection, neurologic symptoms such as ataxia, decreased reflexes and exophthalmia could occur. In adult dairy cows an infection could reduce milk production through its effects on fertility and higher abortion rates. Histological examination of freshly aborted foetuses could contribute to come up with the diagnosis of *N. caninum*. Lesions in the central nervous system(CNS) and in the heart are strong indications for the diagnosis. With IFAT, the diagnosis is confirmed. In the retina and CNS of affected calves tachyzoites and tissue cysts are found. The most common site is the brain, although infection can be found in many organs. In the brain, spinal cord or heart microscopic lesions of encephalitis, neuromyelitis and myocarditis could be perceived in aborted foetuses. Transmission in cattle could happen naturally through ingestion of food and water contaminated with dog faeces containing *N. caninum* oöcysts, or vertically from dam to calf in utero. There is no effective treatment in cattle. Prevention of *Neospora*- induced abortions in cattle depends on prevention of contamination with faeces of dogs.

Isospora is known for its host-specificity. *Isospora* comprises about 200 species. Transmission is usually faecal –oral by ingestion of oöcysts. The prepatent period is 8-11 days and the patent period is 4-28 days. Oöcysts in the size between 20-42µm-36-43µm can be found in the faeces of the dogs, depending on which *Ispospora* sp. it is. *I. canis, I. ohioensis* and *I. burrowsi* which infected dogs are between 20-42µm-14-36µm(11,29). *Isospora* spp. differs from *Eimeria* spp., which contains four sporocysts. The process by which sporozoites are released from the sporocysts/oöcysts is called excystation. For all mammalian *Isospora* sp., the process is basically the same, and similar to what occurs in *Sarcocystis* spp and *T. gondii* (19). Coccidial life cycles are complex with both exogenous and endogenous cycles present.

The exogenous phase of the coccidian life cycle is sporogony, which usually occurs outside the host. Sporogony depends on temperature, moisture and adequate oxygen (19). During sporogony, structural events occur which are similar for all species. Oöcysts are excreted in the faeces, and they usually have a contracted sporont. During sporogony a sporoblast becomes 2 sporocysts and eventually in this sporocysts develop 4 sporozoit, and the oocysts is considered to be sporulated. Sporulated oöcysts contain two sporocysts (21). In the sporocysts the sporozoites become motile and tumble or glide around one another. The sporocysts wall eventually opens along four plate-like junctions and the sporozoites will exit through the opening that is formed. Through indentations or fractures formed in one or both ends of the oocyst wall, sporozoites exit.

In the endogenous life cycle of mammalian *Isospora* spp., sporozoites enter cells in the intestine. In order to form two daughter merozoites, intestinal sporozoites may retain their elongate sporozoite shape, become binucleate and divided by endodyogeny. These daughter merozoites, are divided an definite number of times by endodyogeny and form eventually multinucleate meronts. These meronts retain their merozoite shape and elongate. In the same host, several meronts may occur, and with time, sexual stages will be formed. Microgamonts produce biflagellated microgametes and are multinucleate (22). Microgamonts and macrogamonts may coexist in the same host cell.

Members of the genus *Isospora* could complete their entire life cycle in a single host (a definitive host). However, they have also evolved towards the ability to use a paratenic host in their development life cycle (19). When a definite host ingests an infected paratenic host, patent infections occur (2). The prepatent period may be shortened for infections initiated by consumption of paratenic hosts (19). Dogs, cats, mice, rats, cattle, camels and sheep have been shown to be paratenic hosts for several *Isospora* sp.(23) (24-27). Sporozoites invade extraintestinal tissues, such as mesenteric lymph nodes in the paratenic host. Tissues, such as the spleen, liver and tracheobronchial and mediastinal lymph nodes could be infected, the mesenteric lymph nodes are most often involved(23). Disease like fever, anorexia, diarrhoea, weight loss can occur (28)(22). For at least 23 months, parasites remain viable in extraintestinal tissues of mice (28).

In the tissues of the definitite host, the extraintestinal stages occur in dogs and cats (23,24). Some sporozoites leave the intestinal tract and invade extraintestinal sites in the final host instead of undergoing the normal intestinal developmental cycle. The number of oöcytes produced by the definite host and the patent period are similar to those in oöcyst-induced infections. Because dogs build op immunity, young dogs are more likely to show patent infection. Stray dogs become more infected than dogs with an owner, because stray dogs must hunt for food. Therefore, they are generally more exposed to infected paratenic hosts (19). *I. canis* can cause diarrhoea in dogs, from other *Isospora* spp. it is unclear if they cause diarrhoea (2,29). Diarrhoea associated with the presence of coccidian oöcysts in young dogs occurs, however the clinical significance has not been established due to the possibility of concurrent viral or bacterial infections (19). Further study on natural cases is necessary prior to draw firm conclusions. Experimental infections have usually not been associated with disease.

Sarcocystis spp. are intracellular protozoan parasites with a prey-predator (intermediate-definite) host relationship, an obligate two-host life cycle (3). After ingestion of oöcysts or free sporocysts by the intermediate host, they move to the small intestine. The plates forming the sporocyst walls separately release the four sporozoites held inside. Organisms replicate asexually in the intermediate host, motile sporozoites migrate through the gut epithelium to form schizonts in endothelial cells in small arteries throughout the body. In the endothelial cells they undergo the first of four asexual generations (called schizogony or merogony), producing numerous merozoites about 15 to 16 days after ingestion of sporocysts. Merozoites are released from the schizonts, developing downstream in the direction of blood flow arterioles, capillaries, venules, and veins throughout the body and then develop the final asexual generation in muscles. Merozoites from this generation are subsequently released from the schizonts and enter muscle cells to form metrocytes, and then initiate the formation of sarcocysts. Sarcocysts begin as unicellular bodies containing metrocytes. Numerous metrocytes accumulate through repeated asexual multiplication and the sarcocysts increase in size. The small, rounded, non-infectious metrocytes give rise to infectious crescent-shaped bodies called bradyzoites, as sarcocysts mature. Variation in maturation is different for each species. It could take 2 months or more until bradyzoites are formed and sarcocysts become infectious for the definite host (30). Not all infected animals are infectious, it depends on the age and how long they are infeceted.The sarcocyst is ingested by the definite host. Bradyzoites are released from sarcocysts and transform into sexual stages (female and male gamonts) in the lamina propria of the small intestine after ingestion by the definite host. After fertilization formation of zygote, a oocyst wall around the zygote is formed. In the lamina propria oöcysts sporulate, and are released into the intestinal lumen and excreted in faeces. Dogs are final hosts for numerous species of *Sarcocystis* (11). Subsequently, diarrhoea in young puppies is likely to occur(11). The prepatent period of *Sarcocystis* is 1-2 weeks, during 8-10 weeks oöcysts are excreted in faeces. Dogs are not likely to build up immunity and stay sensitive for patent reinfection.

Coccidian species	Intermediate host	Final host
S.bovicanis	Cattle	Dog,fox, wolf, coyote
S. ovicanis	Sheep	Dog
S. capracanis	Goat	Dog
S. hircicanis	Goat	Dog
S. suicanis	Pig	Dog
S.equicanis	Horse	Dog
S. fayeri	Horse	Dog
S. hovarthi	Chicken	Dog
S. cameli	Camel	Dog

Because of the discussed life cycle of *N. caninum, Isospora* spp. and *Sarcocystis* spp., feeding dogs raw meat carry a higher risk of developing a patent infection.

Preparation of meat at higher temperatures then 30°C in combination with low relative humidities (53% RH and 62% RH), leads to the death of *Isospora suis* tissue cysts within 24h (31). For *Sarcocystis* spp. it appears that freezing to -18°C and -24°C for 5 days and cooking are effective methods for inactivating *SarcocystisI* spp. in guanaco meat (32). Dogs did not shed *sporocysts,* when they were fed buffalo heart muscle heated at 65-75°C containing *sarcocysts* of *Sarcocystis levenei,* whereas other dogs who were fed infected heart muscle heated between 40 and 60°C did shed sporocysts. When the infected heart muscle was freezed at -4°C during 48h dogs did not shed sporocysts, but those fed similar infected tissues stored at -2°C for 24h did shed sporocysts (33). The viability of *Sarcocystis gigantean* was tested and the findings showed that macrocysts were still viable after 10 min at 52,5°C but not after 20 min at 55°C or 10 min at 60°C . Freezing at -14°C for 60 days showed that they remained infective. After being stored for 13 days at 10°C or 20 days at 4°C, cysts were still vigorously metabolized (34). These results indicate that for different *Sarcocystis* spp. different temperatures are able to inactivate them. These studies show that feeding dogs fresh meat (meat that was not frozen) could be an risk factor for developing a patent *Sarcocystis* spp. infection. Frozen meat is of less risk. *N. caninum* bradyzoites cysts within tissue are killed when they are freezed at -20°C for one day (35). Therefore, heated meat is not a risk factor for a patent infection in dogs while fresh raw meat is. Temperatures of at least -20°C and less (which kill the parasites) will not be reached in an ordinary freezer, where the temperature is usually -18°C. Temperatures in an ordinary freezer do not kill the parasites. Subsequently, they will stay infective.

Materials and methods

Faecal samples:

For the research of this project, samples have been used that were collected fresh from the ground by dog owners and sent to us. In 2011, dog owners were asked to participate in an independent research at the faculty of Veterinary Medicine at the Utrecht University concerning patent infections of the roundworm *Toxocara canis* in dogs. These dogs should be older than 6 months of age and the participants had to send faecal samples every month during two years.

Registration:

A unique number was used to link every participant and the name of the dog. Every faecal sample, which was sent in was provided with a unique number. The results are saved under this unique number in order to couple all the information of the participant. Participants received materials to send and collect the faeces safely to the lab. Furthermore, the participants received instructions how to pack and send the faecal samples. All dogs were living in the Netherlands and Belgium when the samples were collected. The faeces were packed in a plastic bag, a little plastic box, tissues, a seal bag and a bubble wrap padded envelope. Most samples were sent by mail, uncooled. Some samples were delivered directly to our laboratory, or were collected in a container which was checked twice a week, packed in one or two plastic bags. All the incoming faecal samples were registered directly on the weekly laboratory list and received a laboratory number starting with number 1 on Mondays and the last number ends on Friday. The laboratory numbers were written in the laboratory list on the plastic boxes with the faecal samples . This way samples could be found quickly, if necessary the faecal samples were stored in a refrigerator at 5°C until examination for a maximum of 4 days.

Questionnaire:

The faecal samples arriving at the lab were registered in the system with the date of delivery. When their package has been arrived, participants did receive an e-mail with the request to fill in a questionnaire. The first time the participants sent a faecal sample the questionnaire contained questions about possible factors of risk that could be associated with a patent infection. Questions were asked about health status of the dog, food, demographical data, the environment the dog lives in etc. When the participants sent in their following faecal samples, they were asked to fill in a questionnaire about changes in health status, food, demographical data, the environment of the dog

and so on. All these questionnaires with answers were collected and linked to the unique participants number.

Parasitological examination:
The faecal samples were processed the day they arrived at the laboratory. Only when there were too many samples to process in one day, some faeces were kept in the refrigerator to be processed the next day. Faeces of two dogs will be examined together, which means that faecal samples are initially pooled. We worked together with the VMDC (Veterinary Microbiologial Diagnostic Centre). When pooled faeces samples were tested positive, the VMDC split the samples for us. This way we had the opportunity to process a larger number of samples in one day. Faeces samples of less than 6grams (otherwise there is not enough faeces to examine for a second time at the VMDC) , faeces samples for which the examination had to be repeated t because they were positive the last time and faeces samples of dogs that have been positive and were dewormed are processed individually. In order to screen for oöcysts of *Isospora* spp., *Sarcocystis* spp., N. caninum and *Giardia duodenalis* and eggs of *Toxocara*, *Strongyle* types (*Uncinaria), Trichuris* sp., *Capillaria* sp., *Taenia* sp., the centrifuge-sedimentation –flotation method (CSF method) with sucrose solution (specific gravity of 1,270-1,330 kg/dm^3) used (4). The faeces itself is macroscopically judged for parasites and consistency, which will be noted in the laboratory list. A clean spatula is used to transfer 3-5 gram of faeces per dog from the bag that has been sent in to a piece of plastic on a scale. The weighed faeces were put in a mortar together with 55 ml tap water in case of a single faecal sample, and 110 ml tap water in case of pooled faecal sample. Using a pestle, the mixture of water and faeces was made into a suspension, the suspension was then poured through a sieve that was placed on the top of a plastic cylinder. The lager parts stay in the sieve and the smaller parts, including worm eggs *,* cysts and oöcysts *(N. caninum, Sarcocystis* spp*.* and *Isospora* spp*.)*, are collected in the cylinder. The sieve was shaken a little to get some of the remaining fluid into the filtrate. Bones, lager worms or other dietary elements could be observed in the sieve. After swinging the cylinder in different directions, the collected suspension with possible worm eggs was poured into a centrifuge tube. The tube was placed in the Rotofix 32 Hettich centrifuge for 2 minutes at 3000 *rpm,* and the supernatant was discarded.

After the supernatant was discarded, a sucrose solution with a specific gravity of 1,27-1,30 kg/dm^3 was added to the sediment, until the tube was filled for approximately one third. The test tubes were suspended again, using a vortex mixer. The centrifuge tube was filled up with sucrose solution to the top until a slight positive meniscus appeared. A coverslip was placed on the top of each of the centrifuge tubes, which were then centrifuged again at 3000 *rpm* for 2 minutes. After centrifugation the coverslips were lifted straight up and placed on a slide. They were marked and examined. Pooled monsters that are found positive for eggs or oöcysts were split and examined by the VMDC, and the results were written on a form. These forms were eventually filled into the system, in order to easily access the results Differentiation of detected objects was limited to: *Toxocara* eggs, Strongyles type eggs, oöcysts of either *Eimera* spp., or *Isospora* spp., or *Sarcocystis* spp., *Giardia duodenalis* cysts, *Trichuris* spp. eggs and nematode larvae.

Processing of the results:
In a Microsoft Excel file ,the results of the microscopic examination were processed. Via the e-mail results were communicated to owners of the dog(s) after they had filled in the questionnaire. When the pooled samples were negative for eggs and oöcysts, owners received a mail saying that the faeces of their dog(s) were negative and they were asked to send in a new sample the following month. When the pooled faeces sample appeared positive, the samples were examined separately by the VMDC using zincsulpate (specific gravity of 1,34 kg/dm$^{3)}$ (36).

Supplementary online questionnaire:
A supplementary online questionnaire was sent to owners who were feeding their dog(s) raw meat, because the aim of this research is to find out if there is a relationship between the origin of the raw meat and a patent infection with *N. caninum, Sarcocystis* spp. and *Isospora* spp. Some additional questions are necessary about the origin of the meat that is being fed, questions that were asked were dealing with the kind of meat the dog eats (fresh or frozen meat), how the animals were kept, from which species the meat came from, if the dogs ate animals of prey and how the meat had been preserved. The same questions were asked about snacks. This way, more information was gathered about the origin of raw meat and this ensured to get an indication about the possible risk for a patent *N. caninum, Sarcocystis* spp. and *Isospora* spp. infection.

Statistical analysis

We evaluated the association between dogs eating raw meat and a patent *N. caninum, Sarcocystis* spp. and *Isospora* spp. infection. We also looked for an association between a patent infection and fresh versus frozen meat and the origin of the meat.
The outcomes were score binary, but in some groups the values were beneath N=5 so for these groups a Fisher's Exact Test has been used, for the other groups a Pearson Chi-Square has been used. A P-value of <0.05 was considered significant. SPSS has been is used to analyze the data.

Results

There were 1005 participating dogs in this research we received at least one time a faecal sample from. From 137 dogs data was missing so these dogs were excluded from the statistical analysis.
In this study risk factors for a patent *N. caninum, Sarcocystis* spp. and *Isospora* spp. infection associated with raw meat was statistically analyzed. Overall in one faecal sample was small oöcyst possible of *N. caninum* was found.
A patently infected dog we called when several oöcysts were found in a single sample. We found 7,1% dogs positive for a patent *Isospora* spp. infection, and 1,4% positive for a *Sarcocystis* spp. infection.
63,9 % of the 868 participating dogs ate raw meat.
For *Isospora* spp. we found in the raw meat eating dogs 8,5% a patent infection. For dogs that did not eat raw meat 6.1% were patently infected with *Isospora* spp.. We found no significant association (P=0.198) between dogs eating raw meat and a patent *Isospora* spp. infection.

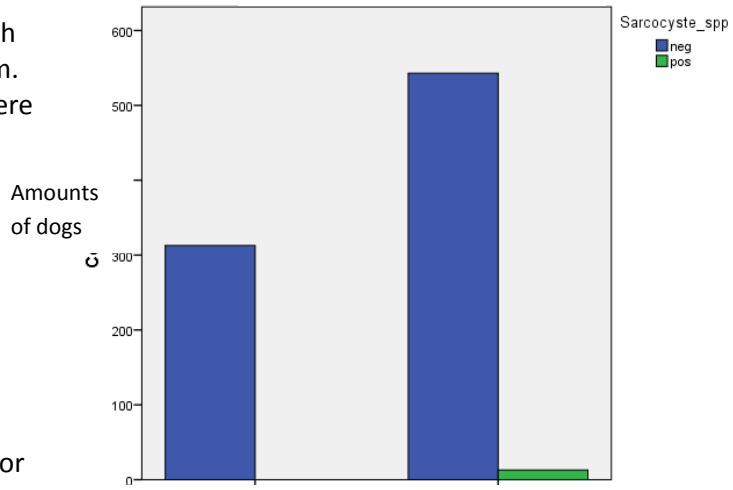

Fig. 1– Number of dogs that do not eat raw meat and dogs that do eat raw meat.

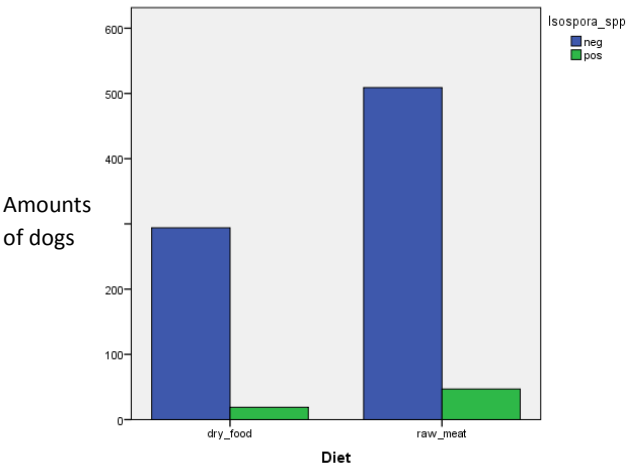

Fig. 1– Number of dogs that do not eat raw meat and dogs that do eat raw meat.

For *Sarcocystis* spp. 2,3% of the raw meat eating dogs, we found a patent infection. For dogs that did not eat raw meat 0% were patently infected with *Sarcocystis* spp.. We found a significant association (P=0,006) between dogs eating raw meat and a patent *Sarcocystis* spp. infection.

In one faecal sample small oöcyst in the size of *N. caninum* was found during this research in the faeces of an raw meat eating dog. In the literature, different values of seroepidimiological study and oöcyst shedding were found between the 1.5% and

4,9%(42,43,44). Based on these percentages we calculate the confidence interval to see if our dog population was big enough. We calculate with the highest prevalence of 5% with an confidence interval of 95%, Win episcoop calculate that we needed at least 73 dogs in this study. Our dog population was N=868, big enough for the possibility to find *N. caninum*

In the additional questionnaire owners of dogs who fed their animals raw meat were asked about the housing systems these meat producing animals were kept in and the origin of the meat concerning the risk of infected of meat producing animals. From the group of dogs that ate raw meat 14,8% eat raw meat from intensive livestock farming, 19,6% from free-ranging animals, 54,7% did not now the origin of the meat and 10,9% filed in otherwise. From the dog owners who filed in otherwise 4,4% filed in biological this dogs did not solely fit into one group. No association was found between a patent *Sarcocystis* spp. infection and the origin of the meat, which means there was no significant difference found between dogs that were feed with free-ranging animals and dogs feed with intensively livestock farmed animals (p=0786). Also no association was found between a patent *Isospora* spp. infection high risk meat and low risk meat, which means there was no significant difference found between dogs that were feed with free-ranging animals and dogs feed with intensively livestock farmed animals (P=0,729). In this study is also looked if there are other possible factors of risk concerning the raw meat. No significant association between frozen meat or fresh meat for a patent infection with *Sarcocystis* spp. was found. No significant association between frozen meat or fresh meat for a patent infection with *Isospora* spp. was found. A significant association (P<0.001) between dogs eating fresh meat and a patent *Sarcocystis* spp. infection was found. 5,4% of the dogs that eat fresh meat have a patent *Sarcocystis* spp. infection. No significant association between dogs eating fresh meat and a patent *Isospora* spp. infection(P=0,258) was found.

Discussion

Dog population:

It could be questionable whether the results are influenced by the defined group of dogs used in this research, when considered the extent of the group of dogs used in this research this can be considered unlikely. In the literature, different values of oöcyst shedding for *N. caninum* was found between 1.5% and 4,9% (40-42). With Win Episcoop the confidence interval was calculated to see if our dog population was big enough to make a statement about the results. Calculating with the highest prevalence of 5% with an confidence interval of 95% and desired absolute precision of 5%, Win episcoop calculates that at least 73 dogs are needed for a reliable outcome. For *Isospora* spp. values of oöcyst shedding was found between 4,1% and 31,5% (43,48,49). With a confidence interval of 95% and desired absolute precision of 5% 384 dogs are needed for a reliable outcome (50). For *Sarcocystis* spp. values of oöcyst shedding was found between 1,8% and 17%. With a confidence interval of 95% and desired absolute precision of 5% 246 dogs are needed for a reliable outcome (50). So we can conclude the dog population in this study was big enough.

For this study we used a random dog population. We asked owners in the Netherlands to participate voluntary in this research. Owners had to send in a faecal sample every month for two years. No selection was made based on breed or gender. The sole restriction of partitioning in this research was the dog being older the 6 months of age. According one of the nutritionist from University Utrecht 15% of dogs in the Netherlands eat raw meat. In our dog population a striking large number of dogs, 63,9% ate raw meat. In this group dogs catching and eating their prey, dogs that would eat any amount of raw meat, and dogs eating dry food and raw meat combined are included. We had a bigger group of raw meat eating dogs in our study, probably because the way we defined the group of dogs that eat raw meat is different. Another possibility is the way people were invited to participate in this research, especially forums on the internet who reached a lot of people feeding their dog(s) raw meat. People who participate in this research were possibly more motivated than less critical dog owners. Maybe is this also the group who feed their animals raw meat. Al these fact

might have led to a bigger percentage of raw meat eating dogs than the nutritionist specialist of Utrecht University calculates.
The group for this study consist of dogs of which the owners have send in at least one faecal sample during the research project and had filled in the questionnaire. Dogs with missing data were excluded from this research. By excluding these dogs from the research project we might have attracted a specific group of people that might not be representative for the dog-owners in the Netherlands.

The only selection in age which was made for this study is that dogs had to be older than 6 months of age. Our dog population had a wide range of age variation from 6 months to 13 years. For *Sarcocystis spp.* this is not important since they have no or pour immunity building (20). Dogs stay sensitive for re-infection so the change to detect *Sarcocystis* spp. in dogs is bigger than when they build up immunity. It is unclear whether the immunity which is built during infection of *N. caninum* leads to elimination of excretion of oöcysts in the further live (11). For *Isospora* spp. especially young animals excrete oöcyst, indicating the development of specific immunity or age resistance (20). It is unclear whether the immunity which is built during infection leads to elimination of excretion of oöcysts in the further live (11). Many older dog participated in this study so they may have already experienced the infection at an early age. It could be so these dogs have built up immunity and infection during our research no longer got through. It is unclear when only young dogs where used more infection with *N. caninum* and *Isospora* spp. where found. Maybe if a younger dog population was used that a significant difference was found for *Isospora* spp. and *N. caninum*.

Samples:
It could also be questionable the way faeces samples were collected for this study could affect the outcome of the results in this study.
It is unknown how old the samples where when they arrived in the laboratory. The faeces used for this research was collected from the ground and there was no information about what happened with the samples between collecting by the dog owner and receiving for diagnosis. It is possible that the owner didn't send in the faeces immediately after collecting it. The circumstances in which the packages were kept during the period they were send true the mail was not known. Most protozoa like *Isospora, Sarcocystis* and *N. caninum* will remain findable for a long period and under variable circumstances. So for these protozoa we do not expect a underreporting due to the influence of the transport of the samples. We should however consider a over reportage due to environmental contamination.

Our dog population is checked only 1 time per month. In one faeces sample of a dog small oöcysts of possible *N. caninum* was found. Other publications show that the prevalence of oöcyst shedding of *N. caninum* is very low 1,5%-4,9%, so there is a small change to detect N. *caninum* (40,41)(42). In this dog population 7,1% dogs were positive for *Isospora* spp., and 1,4% for *Sarcocystis* spp. The prevalence of oöcyst shedding for *Isospora* spp. in other publication is between the 4,1% and 31,5%. For Sarcocystes other publications show prevalence of 1,8%-17% (43,48,49).
If the patent period is less than a month there can be a underreporting of the protozoa. Infection of *N. caninum* is hard to find, because dog pass small numbers of oöcysts in faeces from 8-23 days after infection (11). For *Isospora* spp. the patent period is 3-35 days (20). So for *N. caninum* and *Isospora* spp. we probably had to examine faeces samples of dog more than once per month. Because of this there is a change that we have an underreporting of these parasites. The patent period of *Sarcocystis* spp. is 8-10 weeks, so for this parasite faecal examinations one time per month should be sufficient for detection of these protozoa (20). Another point of discussion is that there is a change that at the beginning of an infection and at the end of an infection less oöcysts are excreted in the faeces. This also could lead to an underreporting of oöcysts. In one faeces sample small oöcysts of possible *N. caninum* was found, this dog ate raw meat but no conclusions could be made. Parasites were identified based on morphology alone, thus in many instances identification beyond the genus level

may not always be accurate. *N. caninum* oöcyst must be differentiated from oöcysts of related coccidians such as *Hammondia heydorni (*and *Toxoplasma gondii)* and are rarely found in faeces (39). Molecular tools like PCR can help to identify *N. caninum.* Oöcysts with the size of 35-42x36-43 µm with a smooth, pale wall without a micropyle were called *Isospara sp.,* but a lot of *Eimeria sp.* have the same size and sometimes it is hard to see if there is a micropyle or not (11). If the *Isospora sp.* were sporulated and contains two sporocyst each with four sporozoites it was sure that it was *Isospora spp.* under the microscope. To be sure if it was Isospora spp. the oöysts had to be culturing until they sporulate, to look how they sporulated, this was not done in this study. Maybe for this reason in this study was an over reporting of *Isospora* spp..

In this study a Centrifuge Sedimentation Flotation (CSF) technique was used with a sucrose solution as flotation medium with a SG of 1,27-1,30 kg/dm³. This particular flotation medium is used for detection of oöcyst such as *Isospora* spp*., Sarcocystis* spp*.* and *N. caninum* and parasite eggs such as *T. canis*, *Trichuris* spp, *Uncinaria*, *Capillaria* spp., *(1).* We send pooled faecal samples to the VMDC when we find oöcysts like *Isospora* spp*., Sarcocysts* spp*., N. caninum* and parasites like *T. canis, Trichuris* spp, *Giardia duodenalis.* In the samples. The VMDC uses as flotation medium with a specific gravity of 1,34 kg/dm³. It could be questionable whether this difference in work method could be of influence on the results in this study. The literature show that zinc sulphate will find the same oöcyst and parasites eggs as the sucrose solution, so to look for *Isospora* spp*., Sacrocystis* spp*.* and *N. caninum*, the difference in flotation medium should not be a problem (4).
However, it is possible that the CSF-technique does not find all *Isospora* spp*., Sarcocystis* spp*.,* and *N. caninum.* When we examine the faeces is it possible that the little piece of faeces we examine will contain very little or no oöcyst, it may be that the dog is infected but we miss this infection, so a dog could be false negative.

Laboratory:
The fact that many different people worked on this project makes this research not continuous. The work in the laboratory most of the time was done by students who stayed at the project for 3-5 months and who were no experts, especially in the beginning of there projects. Protozoa like *N. caninum, Isospora* spp*.* and *Sarcocystis* spp*.* are not easy to distinguishability like *T. canis* eggs, recognizing these protozoa is hard to learn quickly and easily to miss because of their small size. In this study samples will be checked by a expert for at least 3 weeks to prevent eggs or protozoa being missed. So for this reason it is unlikely that we have a underreporting duo to incompetence. It is possible that in this study oöcysts have mistakenly diagnosed as *Isospora spp..* In the case *Isospora* spp. were found there is a possibility it were passaging oöcysts. By the fecal-oral transmission route it could be that unsporulated oöcyste eaten by faeces leave the dog with the faeces, so there is only a passage of these oöcysts (2). When dogs eat (un)sporulated oöcysts from birds these oöcyst also can passage and leave the dog with the faeces (11). These dogs are positive but have no patent infection. To be sure that it was a patent infection, another sample could have been asked to send in by owner. For this sample the dog has to be on a leash for three consecutive days to prevent from eating faeces. Afther these three days faeces had to be collected and send in again. This is not done in this study for protozoa otherwise it was too much work with the sample stream every day. Although all materials are thoroughly flushed with water, it is possible that the faecal samples are contaminated during the process in the lab.

Questionnaire:
There are many risk factors that influence the occurrence of a patent infection with *Isospora* spp*., Sarcocystsis* spp. and *N. caninum.* Preparation of meat at higher temperatures or freezing meat for a sufficient time below certain temperatures (see introduction) will inactivate cyst of these protozoa*.* That is why eating raw meat is a potential factor of risk for developing a patent infection with *Isospora* spp*., Sarcocystsis* spp. and *N. caninum* (31,35)*.* In the questionnaire no questions were asked about, for example, the origin of the meat. To get a good image for risk factors within the

group of dogs that eat raw meat a supplementary questionnaire was send to owners who feed their dog(s) raw meat. Some questions ware not correctly interpreted the way we intended. For example, we asked if their dog(s) eat(s) animals of prey. What was mend by this question was if their dogs caught wild animals like mice, rabbits or birds and ate these prey. In Some cases this was interpreted as if the dogs owners would be catching the prey and then feed them to their dogs. So this question was not interpreted the way it was intended. In order to make an estimate of how large the risk of infection is, questions were asked about the way people preserve the meat and for what period in time. Sometimes these questions were also misinterpreted. To get an indication of the chance of survival of *Isospora* spp.*, Sarcocystis* spp. and *Neospora caninum* oöcysts we wanted to know whether meat was kept in the freezer, refrigerator or at room temperature and for what time. Most of the time all these possibilities were answered while most people preserving the meat in the freezer but defrosted it at room temperature and then kept it in the refrigerator. By the not correctly interpreted questions the results may not be reliable and better formulated questions should have been asked.

The statistical analysis showed that there is no significant association (P=0,198) between dogs eating raw meat and a patent infection of *Isospora* spp. infection. The statistical analysis showed that there is a significant association (P=0,006) between dogs eating raw meat and a patent infection with *Sarcocystsis* spp*.* This means that dogs that eat raw meat are more often positive for a patent infection with *Sarcocystsis* spp*.*
For *Sarcocystis* spp. it is in line with our expectations, because it is a obligatory prey-predator (intermediate-definitive) host relationship, a requisite two-host life cycle. Dogs cannot be infected by ingestion of oöcyst from the environment (3).
For *Isospora* spp*.* it is not in line with our expectations. Dogs can be infected by fecal-oral route or by ingestion of paratenic host containing extraintestinal monozoic cyst (2). Dogs who eat raw meat have more change to exposure to infected paratenic host. It is possible that the hygiene of meat producing animals is very good so *Isospora* spp. have no change in these animals to develop. It is also possible that *Eimeria spp.* have mistakenly designated as *Isospora* spp.. *Eimeria* spp. can be easily eaten from the environment. There is a change for dogs who did not eat raw meat but are positive diagnosed for *Isospora* spp. they were misdiagnosed if we excluded passing oöcysts by coprophagy. The prevalence of *Isospora* spp. in meat producing animals in the Netherlands is not known. The prevalence of *Sarcocystis* spp*.* in meat producing animals in the Netherlands is not known in other countries they have been found frequently (44,45).

During this study different factors within the group of raw meat eating dogs were checked. Looking at the life cycle of *Sarcocystis* spp*.* and *Isosopora* spp*.* free-ranging animals could be at higher risk of getting infected with tissue cysts (3,19). If dogs eat raw meat of free-ranging animals they could be at higher risk of getting a patent infection. However no significant association was found between dogs that did eat meat from free ranging animals and a patent infection with *Sarcocystis* spp. and *Isosopora* spp*.*. However 72,7% of the people who filled in the additional questionnaire did not know the origin of the meat and the way these meat producing animals are kept. So it is hard to conclude anything from these data because many data was missing.
In this study the difference between frozen meat and fresh meat was examinated. No difference was found for *Isospora* spp*.* between frozen meat and fresh meat. For *Sarcocystis* spp. a significant difference (P<0.001) was found between the frozen meat and fresh meat group*.* 0% of the dogs that ate frozen meat showed a patent *Sarcocystis* spp*.* infection and 5,4% of the dogs that eat fresh meat have a patent *Sarcocystis* spp. infection. In this study parasites were identified based on morphology alone, thus in many instances identification beyond the genus level may not always be accurate. \

Conclusion

The results show that raw meat does only influence the change of getting a patent *Sarcocysts spp.* infection. Also the difference in the fresh meat versus frozen meat has been studied. Fresh meat is a potential risk, frozen meat not. For Isospora spp. and N. caninum no potential risk factors could be detected. This study looked for al *Isospora* spp.. There is a change that if there was only looked for *Isospora canis, Isospora ohioensis* and *Isospora burrowsi* that a significant difference could be found.

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