Les parasites gastro-intestinaux chez le cheval, revue des méthodes de diagnostic et étude de terrain sur des chevaux sauvages retrouvés morts au Cap Toi, Japon

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Objectif du travail :

Mon travail s'est déroulé en deux parties distinctes. La première est de faire un état des lieux des connaissances scientifiques sur les parasites gastro-intestinaux chez le cheval. Il sera également fait état des différents outils de diagnostic qu'il est possible de réaliser, en particulier pour les parasitoses à *Strongylus spp*. et à *Cyathostomum spp*. Les problèmes de résistances aux antihelminthiques seront également abordés.

La deuxième partie est axée sur une étude de terrain et des méthodes diagnostiques mises en œuvre lors de mon stage de recherche en pathologie à l'université de Miyasaki.

En alliant stage externe et TFE, j'ai pu aller au bout d'un processus scientifique qui allait d'une étude de terrain à de la recherche documentaire tout en essayant de garder un esprit critique. Le but est d'avoir une vue d'ensemble sur les parasites gastro-intestinaux et les méthodes diagnostiques en n'oubliant pas que ce sujet est très vaste et les techniques de diagnostiques en constante évolution.

Résumé :

En médecine équine, les parasites gastro-intestinaux peuvent induire des tableaux cliniques lésionnels qui peuvent être mortels. Les principaux parasites sont présentés dans ce travail. Les démarches diagnostiques sont essentielles afin de mettre en place une prévention et un traitement parasitaire adéquat en terme d'efficacité et de lutte contre la résistance aux antihelminthiques.

Dans ce TFE, la partie pratique détaille les démarches diagnostiques appliquées lors de l'étude de parasites retrouvés sur deux chevaux retrouvés morts au Cape Toi en 2019. Le type de lésions macroscopiques et histopathologiques nous a incité à investiguer deux types de parasites, les grands strongles et les petits strongles. Des tests de flottaison de matière fécale, comptage et identification des œufs, identification morphologique des vers prélevés, PCR et séquençage d'ADN ont été effectués. L'identification morphologique, les PCR et le séquençage d'ADN ont permis d'identifier la plupart des parasites comme appartenant à la famille des cyathostomes qui sont de nos jours les parasites rencontrés les plus fréquemment en raison de leur résistance aux antihelminthiques. L'histopathologie, l'identification d'un adulte *Strongylus vulgaris* et le résultat positif d'un échantillon pour *Strongylus equinus* laisse à penser qu'il s'agit d'une parasitose mixte et qu'une stratégie de traitement plus adaptée devrait être mise en place.

Gastrointestinal parasites in horses, review of diagnostic methods and field study on wild horses found dead in Cape Toi, Japan.

Objectives:

My work took place in two separate parts. The first is to make a scientific review on parasites in horses. The various diagnostic procedures that can be carried out, in particular for the parasitosis caused by *Strongylus spp.* and *Cyathostomum spp.*, will also be reported. The problems of resistance to anthelmintics will also be mentioned.

The second part focuses on a field study and diagnostic methods that I was fortunate enough to put into practice during my pathology research internship at Miyasaki University.

By combining external internship and thesis, I was able to complete a scientific process that went from a field study to documentary research while trying to keep a critical mind. The goal is to get an overview of gastrointestinal parasites and diagnostic methods, keeping in mind that this subject is very wide and the diagnostic techniques constantly evolving.

Summary:

In equine medicine, Gastrointestinal parasites in horses can induce clinical and lesional pictures which can be fatal. The main parasites are presented in this work. Diagnostic procedures are essential in order to implement adequate parasite prevention and treatment and struggle against anthelmintic resistance.

In this work, the practical part highlights the diagnostic procedures applied for the study of parasites found on two horses found dead in Cape Toi in 2019. The type of macroscopic and histopathological lesions prompted us to investigate two types of parasites, the large strongyles and the small strongyles. Faecal flotation tests, fecal egg count (FEC) and eggs identification, morphological identification of collected worms, PCR and DNA sequencing were performed. Morphological identification PCR and DNA sequencing allowed us to identify parasites belonging to the cyathostomae family who are actually the most prevalent parasites in the world due to their anthelmintic resistance. The histopathology, the identification of an adult *Strongylus vulgaris* and the positive result of a sample for *Strongylus equinus* suggests that it is a mixed parasitosis and that a more appropriate treatment strategy should be implemented.

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Table of content

1. Foreword :

I would like to thank Miyasaki University and especially Hira sensei and his team for letting me use their infrastructures and who taught me so much.

This paper is very interesting to me, for it shows all the the literary research undertaken and the practical steps carried out during my internship.

Parasitosis in horse is a serious subject especially for domestic horses. Working on parasitosis on wild japan horses gave me an overview on this problem and the wide range of research filed possibilities. Beside, my internship and this thesis increased my knowledge on this special subject.

2. Parasitosis in horses: a litterature review:

2.1 Introduction:

Gastrointestinals nematods (GINs) are present ubiquitously in horse and are a major concern in veterinary medicine. There are over 50 species of equine strongyles belonging to two subfamilies, the Strongylidae (‘large strongyles’) and cyathostominae (‘small strongyles’ or ‘cyathostomes’) (Gasser RB, *et al.*, 1999). They are the main intestinal nematode parasites of horses, constituting 75% of the total parasite fauna (Saeed MA, *et al*., 2019; Lichtenfels JR, *et al*., 2008). The most important non strongylid nematodes found in horses are ascarids *(Parascaris equorum, and Parascaris univalens),* Threadworms *(Strongyloides westeri),* Oxyurids *(Oxuyris equi)* and stomach worms *(Draschia megastoma, Habronema muscae, Habronema majus and trichosctrongylus axei).*

Theses parasites are a worldwide concern and have difference prevalence depending on climate and geographic conditions. They are responsible of a wide range of clinical signs such as diarrhea, abdominal pain and distention, colic, emaciation, fever, weakness, reduced stamina, and a lack of immunocompetence that can lead to death. It should be noted that helminth infections have been demonstrated as a direct cause of 30% of chronic weight loss syndrome cases in Europe, 75% of which have been attributed to larval cyathostominosis (Corning S, *et al*., 2009) which has a mortality of up to 50% and has an impact similar on horses across the world (Collobert-Laugier C, *et al*., 2000).

Parasites are considered to be one of the major contributor of colic in equines (Enbavelan *et al*. 2015). Therefore, it is important to be able to make a correct diagnose in order to choose an appropriate treatment and prevention strategies.

2.2 Strongylid nematodes:

The nematodes considered as parasitic in equids fall into 7 suborders, 12 families, 29 genera, and 83 species. The great majority (64 of 83) are members of a single family, the Strongylidae. The 64 species, in 19 genera, of the family Strongylidae, are the most common and economically important nematode parasites of horses (Lichtenfels, *et al*., 2008).

2.2.1 Large strongyles:

The subfamily of strongylinae is composed by five genera of strongyle; *Strongylus vulgaris, Strongylus edentatus, Strongylus equinus, Oesophagodontus robustus and Triodontophorus serratus.* The large strongyles are parasites, at the adult stage, of the colon and the cecum of horses. Those nematodes have a well-developed buccal capsule, a mouth collar with two leaf-crowns and a copulatory bursa (Lichtenfels, *et al*., 2008). They are medium to large size with respectively 1 cm to 1.5 cm for *Triodontophorus serratus*, 1.5 cm to 2.5 cm for *S. vulgaris and Oesophagodontus robustus*, 2.5 cm to 4.5 cm for *S. edentatus* and 2.5 to 5 cm for *S. equinus*. The adults can be recognized by morphological differentiation but the task is not easy. For example, *S. vulgaris* possess 2 teeth in the buccal capsule, *S. edentatus* and *O. robustus* possess none, *S.equinus* has 3 conic teeth and T. serratus possess 3 bifid teeth (Figure 1).

Each parasite has a different migratory pattern and pathogenicity. However, their cycle has certain points in common. Their life cycle has 2 phases, an endogenous phase (from the ingestion of L3 to the elimination of eggs via the stool) and an exogenous phase (from the elimination of eggs to the ingestion of L3). During the exogenous cycle the eggs are emitted in the feces, favorable to the development of the eggs. These will hatch and molt until stage L3 where they will leave the feces and end up in the grass (following various factors such as rain, trampling...) and be ingested by a horse. The endogenous cycle has differences specific to each species and consequently their pathogenesis is different.

Strongylus vulgaris: The L3 larva is ingested and will penetrate the intestinal mucosa and migrate into the intestinal wall to moult around the 7th day. This larva has a tropism for the

blood vessels and more precisely for the arteries. It will migrate towards the intima of the great vessels until the large mesenteric artery and in particular the right branch of this one, where it will cause great damages. During this migration, the larva molts into L5 (120 days after ingestion). It will then return to the large intestine after 3-4 months to settle in it with its buccal capsule and lay eggs. The pre-patent period is 200 days.
It is responsible for clinical symptoms such as colic, pain and fever, anorexia and changes in peristalsis.

Strongylus edentatus: the L3 larva is ingested and will penetrate the mucosa at the level of the cecum and the ventral colon. This larva also has a tropism for the vessels, but this time for the veins. It will therefore migrate from the veins of the submucosa to the portal vein and then it will go to the liver. Moulting of L3 into L4 will take place 15 days after ingestion of the larvae. From the liver it will migrate under Glisson's capsule and reach the parietal peritoneum via the hepatic ligament. This will cause lesions on the migration path but may also cause hepatic hemorrhage and septic complications. The pre-patent period is 300 days.

Strongylus equinus: the L3 larva will penetrate the wall of the large intestine where it will moult into L4 after 1 week. The migration will be towards the liver through the peritoneum. A prolonged intrahepatic migration will cause lesions. The larva will continue to progress to the pancreas and make its last L5 mutation. *S. equinus* will return to the cecum, cross its wall to find itself in the lumen, mate and lay eggs. This type of migration can cause hepatic and pancreatic hemorrhage. The pre-patent period is 250 days.

Oesophagodontus robustus: to few details about its pathogenesis. In general he is described along with the other large strongyles.

Triodontophorus serratus: it has an endogenous cycle similar to cyathostominae ("small strongles"). In fact, this type of parasite performs less migration than large strongles and remains in the wall of the digestive tract, leading to typhlitis and granulomatous or chronic colitis. These attacks can lead to catarrhal, hemorrhagic and fibrinous enteritis which can be complicated by septicemia. This type of cycle will be explained below for small strongyles.

2.2.2 Small strongyles:

Cyathostomins became the most common of the GINs infecting horses and are now considered the most important in prevalence terms (Love et al., 1999). This change is due to a decrease in the prevalence of large strongyle infections which is the result of an increase of

anthelmintic treatments. Their prevalence is very high, whatever the climatic and management differences are. They can be found in a tropical climate as much as in a cold climate. These parasites can be held responsible directly or indirectly by paving the way to other disease as it lowers down the immunity of the host. Knowing their biological cycle and their clinical importance is essential in order to allow the implementation of an anthelmintic treatment strategy. Around 57 species of cyathostomes have been reported throughout the world (Eslami and Kiai, 2007). Those species are grouped into 14 genera inclunding *Cyathostomum, Cylicocyclus, Cylicodontophorus, Cylicostephanus, Cylindropharynx, Gyalocephalus, Hsiungia, Parapoteriostomum, Petrovinema, Poteriostomum, Caballonema, Coronocyclus, Skrjabinodentus and Tridentoinfundibulum* (Lichtenfels J, *et al*., 2008, Bredtmann C, *et al*., 2017).

As the cyathostomins have similar morphology, clinical signs and epidemiology, they will be discussed as a single entity in this paper.

Small strongyles are also known as "small red worms". The reason is that they are usually less than 2.5 cm in length and are more coloured than white. They have a direct life cycle and no intermediate host. The life cycle is represented in Figure 2. The stage 3 larva, which is the infectious stage, will be ingested and will end up in the intestine. At this point Cyathostomins differ from other parasites in that they are able to stop their maturation and protect themselves by encysting in the lining of the large intestine. Up to 90% of cyathostomins can be found encysted and whose development during the L3 phase is inhibited. They can therefore remain in the intestinal wall for a period ranging from 2 months to 4 years. The climate is an important parameter for this inhibition. For temperate climates, the larva will accumulate during the grazing period and will become encysted during the cooler months of the year. When the temperature rises in the spring the larvae will emerge massively. The reverse is true for tropical climates where the larvae encyst in the warmer months and emerge during the fall. The strength of small strongyles is that they are able to survive in the host and on the pasture for very long periods. Once the eggs are expelled with the feces on the pasture, their development will be directly proportional to the temperature. During warm weather, the larva can develop from L1 to L3 in 3 days. Once the L3 infectious stage is reached, the larvae will surround themselves with a protective membrane and will be able to survive even in freezing conditions (Chapman M, *et al*., 2002).

This parasite has a preference for the colon and the cecum. Which may explain their pathogenicity. They are damaging as soon as they enter the intestine after ingestion. Cysthostomins can be more pathogenic in the larval stage than in the adult stage. In fact, L3

can already cause severe damage to the intestinal mucosa but a large number of encysted larvae can cover the mucosa wall, damaging it, reducing its peristalsis and reducing nutritional metabolism (Collobert-Laugier C, *et al*., 2000). A large number of adult parasites can lead to clinical signs of lethargy, sudden weight loss, debilitation and diarrhea (Chapman M, *et al*., 2002). In addition, the shell around the larva protects it from conventional antiparasitics. However, the most damage is done by L4 when cysts emerge and develop into adults in the intestinal lumen. In spring or in the fall there will be a massive disencystment called "larval cyathostominosis" causing diarrhea, severe colic, granulomatous colitis and mortality of up to 50% (Love S, *et al*., 1999; Abbott E, *et al*., 1998 and Corning S, *et al*., 2009). Although young horses are the most vulnerable (Abbott E, *et al*., 1998), adults remain sensitive to cyathostomins throughout their lives. It is therefore important to set up an anthelmintic treatment which can eliminate the encysted and non-encysted forms effectively.

2.2.3 Non-Strongylid parasites:

There are many types of non-strongylid parasites such as stomach worms, ascarids, threadworms, oxyurids and tapeworms. The most important are detailed in the dedicated part to the annexes related to the bibliographic review (Addendum 1: Non strongylids parasites).

3. Diagnosis of Strongylus spp and Cyathostomins spp:

3.1 Clinical signs and Hematology :

It is difficult to establish an accurate diagnosis based on clinical signs or hematology only. However, this can lead to a suspicion of a parasite infestation. As mentioned above, the clinical signs of parasitosis caused by cyathostomins or strongyles are not pathognomonic. It is therefore useful to associate them with other data such as the horse's history of deworming, its age, its grazing grounds, the season, the presence of other horses or other species and finally hematology. Horses infected with these parasites often develop hypoalbuminemia, and / or neutrophils. According to Jeremy D. Hubert *et al.* (2004), who made a study examining helminth-naive ponies the effects of infection with *Strongylus vulgaris*, has noted in some groups a significant increase in total leukocyte count, fibrinogen and protein concentrations plasma, significant reductions in erythrocyte counts and hematocrit. It is also sometimes

possible to have anemia with or without eosinophilia and /or lymphocytosis (Tomczuk K, *et al*., 2014). It is therefore important to notified as many parameters as possible. For example, for Smets K. *et al.* (1999) a diagnosis based on clinical signs and hematological findings can be made if in winter a horse has a low body score with diarrhea, a serum albumin concentration of less than 20g / L and a ration of albumin: globulin of less than 0.7. In this type of case it is very likely that the horse is infected with adult cyathostomins or in the L4 larval stage.

3.2 Fecal flotation test:

It is one of the most widely used and recommended coprological methods, with a sensitivity over 50% (Tomczuk K, et al., 2014; Williamson *et al*. 1998). This test involves mixing feces with a solution which will be centrifuged in order to bring the parasite eggs to the surface while the organic matter remains in the bottom. The solution used will be chosen according to the type of parasite sought (Table I). They have different compositions (Table II) and are intended to have a specific gravity greater than that the one of the eggs. Once the eggs are collected, they will be analyzed by microscopy. This technique is used very frequently but unfortunately the absence of eggs does not rule out parasitic disease (Wolf D, *et al*., 2014). There are 2 types of flotation test, one qualitative and one quantitative. The purpose of the qualitative test is to determine whether or not there is the presence of parasite larvae. This test is valid for all species. It requires 10gr of feces which will be centrifuged twice on a sugar solution, (1.33 specific gravity for the Animal health diagnostic center for example). It is best if the feces are fresh, but if stored in 10% formalin or 70% alcohol, the sample can still be submitted. However, the preservation method can compromise the quality of detection of the parasite. The quantitative test (FEC) identifies the presence of the parasite with the number of eggs per gram of feces (EPG) or the absence of parasites. It is based on the same technique as the qualitative test but in addition allows to see if a treatment is effective and if there is the development of a drug resistance. However, if the sample is diarrheal, it cannot be quantified and the qualitative test will be performed. It is this quantitative technique will be preferred for the horses.

3.2 Fecal worm egg count analysis:

Fecal worm eggs count analysis (FWEC) is a relatively important diagnostic method. It can be used to measure the level of contamination by identifying the quantity of eggs excreted in the feces and thus select an appropriate anthelmintic treatment. It is important to take measures

to limit resistance to anthelmintic. Using FWEC in a treatment program would reduce the frequency of anthelmintic administration by 75-82% (Gasser RB, *et al*., 2004; Wood E, *et al*., 2012). With this technique it is also possible to determine the effectiveness of a treatment by observing a decrease in FWEC (Wood E, *et al*., 2012). However, it should be remembered that the FWEC only detects eggs excreted by mature females and does not take into account adult males, larval forms and in particular encysted cyathostomins larvae (Kaplan R, *et al*., 2002). There are several forms of FEC analysis. There are the McMaster methodologies, the FECPAK methodology, FLOTAK methodology and Ovatec methodology. After the fecal sample has undergone the flotation test, the eggs will be collected and analyzed under a microscope (x40 magnification).

Each test has its own analytical sensitivity in terms of time to carry out the test, laboratory equipment and the level of expertise to perform the test accurately (Wood E, *et al*., 2012). These tests have a notion of "detection limits" in order to describe their sensitivity (Table III, Lester H, *et al*., 2014). Indeed, a low detection limit means that the method will be more sensitive for it will use a low multiplication factor (or equal to 0). This implies a lower margin of error than if the test has a high detection limit. This multiplication factor converts the number of eggs observed into an estimate of the number of EPG and therefore if this factor is high it risks truncating the final result (Matthews J, *et al*., 2011). It is therefore preferable to use a test with a low detection limit, especially when evaluating resistance to anthelmintic (Morrison D, *et al*. 2004).

McMaster methodologies:

The McMaster Method is the most universal FEWC technique and is currently recommended by the World Association for the Advancement of Veterinary Parasitology. This technique can assess the efficacy of an anthelmintic drug (Torgerson P, *et al*., 2012) and can also detect resistance to anthelmintic (Kaplan R, *et al*., 2010). The McMaster technique has many variations. They can be found in the literature and many scientists are still bringing modifications. What differs between the McMaster test variants are the weight of the feces examined, the type and volume of flotation solutions, dilutions, flotation times, additional centrifugations, centrifugation time and speed, number of sections of the McMaster slide counted and of the different interpretation coefficients (Wood I, *et al*., 1995; Coles G, *et al*., 1992; Vadlejch J, *et al*., 2011).

Pereckiene A. *et al*. (2007) and Vadlejch J. *et al.* (2011) tried to determine which variant of McMaster technique is the most reliable (Table IV). The first study evaluated 7 different

variants of McMaster techniques by comparing the estimated results in EPG of *Ascaris suum* eggs of 30 samples of feces. The second compared the reliability of 3 methods using feces artificially contaminated with *Teladorsagia circumcinta* eggs at 3 different concentrations (low, medium and high). Both studies demonstrated that variants of the McMaster technique that using a larger amount of fecal matter and a lower dilution ratio hence a lower multiplication factor and thus have a more reliable result with better sensitivity.

The FECPAK methodology: (addendum 2)
The FLOTAC methodology: (addendum 3)
The Ovatec methodology: (addendum 4)

3.4 Morphological differentiation:

Morphological differentiation is a complicated task and requires extensive knowledge. While interspecies recognition is easier, intraspecies differentiation is very subtle and complicated. The 14 species of Strongylins are easier to identify than the 50 species of cyathostomins (Lichtenfels JR, *et al*., 2008). It is possible for some authorities using comparative anatomy to identify adult nematodes. However, the larvae are extremely difficult to identify and it is impossible to identify the eggs at the subfamily level (Lichtenfels JR, *et al*., 2008). It is therefore important to continue research on the identification of parasites in parallel with molecular diagnostic methods. Some books such as the "Illustrated Identification Keys to Strongylid Parasites Strongyllidae Illustrated Identification Keys to Strongylid Parasites Strongyllidae Nematoda of Horses Zebras and Asses Equidae" have collected identification keys that can aid in the recognition of certain parasites, for example large strongyles (figure 4 to 8). It is advisable to prepare the parasites in order to visualize them as well as possible. The classic method for identifying adult parasites by micro-examination is the iodine mount method. It allows identification through morphological characteristics and details of internal structures. This technique is simple to perform, fast, inexpensive and allows a good visualization. On the other hand, the preparation will dry quite quickly which does not allow the slide to be kept for a future demonstration or a discussion and therefore requires the manipulation to be repeated several times and is therefore time consuming (Estevez E, *et al*., 1986). It is also possible to use methylene dye and glycerol combination. This type of wet mount is a more economical substitute than iodine, lasts longer and allows better visualization and allows easier differentiation of organic debris that may be present on the slide. In

addition, glycerol is hygroscopic in nature and will absorb the surrounding water molecules thus avoiding the drying out of the wet mount. In addendum 5 are described the 3 types of preparation that can be done, published by Vinay Khanna *et al*., (2014). These techniques allow observation of ova, cysts, larvae and adults.

Cyathostomins are the most difficult strongyles to identify. However, by focusing on certain morphological characteristics such as the mouth collar, cephalic papillae, internal leaf-crown (ILC) and external leaf-crown (ELC), extra-chitinous supports (S) of the ELC, buccal capsule, and esophageal funnel (figure 9) these species of small to medium-sized strongylids are readily recognizable (Lichtenfels J, *et al*., 2008). It is also possible to use the lower part of males and females to identify the species

3.5 Molecular test: PCR, PCR-ELISA, Reverse line blot assays:

Because of the difficulty in identifying certain stages (larvae or eggs) by morphological differentiation, it is necessary to use other techniques in order to ensure a correct diagnosis. That is the reason why the DNA technology such as conventional and real time PCR, PCR-ELISA and reverse line blot assay (RLB) is currently used. According to studies already carried out, conventional PCR and Real time PCR have high specificity and sensitivity (Nielsen MK. *et al*., 2008; Campbell AJD. *et al*., 1995; Hung G-C. *et al*., 1996). Campbell *et al*. (1995) conducted the first study of the nuclear ribosomal (rDNA) of the strongyles by amplifying and sequencing the second internal transcribed spacer (ITS-2) of rDNA using polymerase chain reaction (PCR) technique (figure 10). The advantage of working with ribosomal DNA sequences as a target for the definition of species markers is that rDNA tends to maintain sequence homogeneity within a species. Another advantage is that the rDNA sequences are repetitive and therefore abundant in an organism, thus giving the test a high sensitivity (Gasser R. *et al*., 1996). The ITS-2 sequence of *S. vulgaris*, *S edentatus* and *S. equinus* is 217 to 235 nucleotides and the analysis of the sequence shows a very low degree of intraspecific variation (0 to 0.9%), while the degree of interspecific variation ranges from 13% to 29% (Gasser R. *et al*., 2003). As the PCR-coupled restriction fragments length poly-morphism (PCR-RFLP) were able to identify the 4 strongles species (including *S. Asini*) this proves the potential of the ribosomal spacers as genetic markers. In addition, Gasser R. *et al* (1996) managed to characterize 11 species of cyathostominae using PCR-RFLP techniques on rDNA with ITS-1 and ITS-2 and with the 5.85 rRNA gene. Thanks to these species-specific features, Hung GC. *et al*., (1999), were able to develop specifics PCR for the 5 important species of Strongyloids.

The small difference of nucleotides between species led to the construction of specific PCR primers based on a single different nucleotide.

This ability to be able to amplify rDNA the 4 most abundant cyathostomins species (*Cy. Catinatum, Cylicocylus. Nassatus, Cs.longibursatusand, Cylicostaphanus Goldi*) is an essential diagnostic tool in order to study theirs biological, epidemiological and pathological aspects (Gasser R. *et al*., 2004). Regarding the high specificity and sensitivity of these PCR methods using these markers, they prove to be very useful in diagnosing larval cyathostominosis from DNA extraction from intestinal biopsy samples. These methods are also very important in the detection of resistance to anthelmintics (Gasser R. *et al.,* 2004).

Another way to investigate the relative importance of individual species is to use the species-specific DNA probes developed for the six common cyathostomins (*Cylicocyclus ashworthi, Cylicocyclus insigne, Cc. nassatus, Cyathostomum catinatum, Cs. goldi* and *Cylicostephanus longibursatus*) by Kaye JR, *et al*., (1998) and Hodgkinson J, *et al*., (2001). This probes are based on intergenic spacer (IGS) region sequences. Kaye JR, *et al.,* (1998) chose as specific markers, not ITS-1 and ITS-2 but the 26S-18S rDNA IGS in order to compare 16 species of cyathostomins. This IGS region, which is less conserved between species than rRNA, has been widely used to identify relatively closely related species. The intrapsecific variation of this region is 0.2 to 2.2% with an interspecies variation of 3 to 60%. This shows that this region could be useful for differentiating species (Hodgkinson J. *et al*.,2001).

With IGS it is also possible to perform a PCR-ELISA. This system is more advantageous than the classic hybridization technique because it has a higher sensitivity, verification of integrity of amplicon and rapid analysis of multiple PCR assays simultaneously, as well as being suitable for routine diagnosis and automation (Hodgkinson JE. *et al.,* 2003).

The PCR-RLB hybridization assay is faster and more convenient than other molecular techniques. It allows on the one hand the simultaneous identification of the most common cyathostoma species and on the other hand the discrimination of the three *Strongylus spp.* (Traversera D. *et al*., 2007). It is only this technique that allows the identification of such a number of taxa at a species level at the same time (Gasser R. *et al*., 2004; Hodgkinson J. *et al*., 2006). In addition, the other methods are relatively expensive, time-consuming and potentially inaccurate in the presence of several species of strongyles (Traversera D. *et al*., 2007). Indeed, it is possible to screen up 43 samples at the same time and the membrane can be reused between 10 and 15 times without loss of signal. It is a powerful diagnostic tool from a practical and clinical perspective. Especially regarding mixed infections because we do not know yet the

pathogenesis of each cyathostome (Traversera D, *et al*., 2007). Unfortunately, none of these assays have been developed to quantitatively reflect the parasite burden.

With the PCR products it is also possible to send it to a specialized laboratory which will carry out DNA sequencing in order to see to which species the helminths analyzed belong. Once the data received, it is possible to make a phylogenetic tree.

3.6 Necropsy and histopathology:

When performing an autopsy on a horse it is always important to have an overview of its medical history and the circumstances of its death. Knowing the pathogenicity and clinical signs of cyathostomins or strongyles infestation can help us focus on certain organs. In fact, special attention should be paid to the liver, lungs, peritoneal cavity, blood vessels (such as the cranial mesenteric artery and veins), the jejunum, the ileum, the colon and the cecum. It will be useful to note the consistency of the feces, the presence of parasites and take a sample.

As a reminder, the clinical signs that put us on the path to cyathostoma infection are; sudden weight loss, recurrent diarrhea with or without fever, colic and subcutaneous edema. These clinical signs are caused by inflammatory enteropathy which can lead to typhlitis and severe colic associated with either the penetration of larvae or the emergence of larvae from the intestinal mucosa (Murphy and Love, 1997; Thamsborg *et al*., 1998). According to Love S. *et al*., (1999), the nodular larval cyathostomosis was classified by Velichkin PA., (1952), as" a catarrhal and fibrinous inflammation of the cecum and colon accompanied by diffuse hemorrhagic foci in the mucosa". There are of course other abnormalities present such as: mucosa edema; larva cyathostomes on the mucosal surface, in the intestinal content and in the feces; necrotic nodules in the mucosa as a sequel of larval emergence and enlargement of mesenteric lymph nodes (Love S. *et al*., 1999).

Once the macroscopic observation is complete, it is advisable to think of which samples will be taken for laboratory analyzes (molecular tests and histopathology). The conservation of the samples will not be the same depending on the test to be performed later. For example, tissues taken for histopathology will be put 10% formalin solution. For molecular tests the samples will be put in a dry pot and put in the freezer (-20°C) in order to preserve the tissue as long as possible. If parasite larvae are visible they can be stored in an 10% formalin or 70% ethanol solutions. It is also important to take the contents of the intestine in different sections (jejunum,

ileum, caecum, colon) because if adult larvae are not visible this does not mean that there are not eggs or larvae mixed with the feces.

Histopathologically, there are changes occuring as a generalized mucosal disruption along with cellular infiltration. Once the larva has penetrated the basement membrane of the epithelial cells of the tubular glands it causes a fibroblastic reaction. When the larvae enter the submucosa it creates the formation of a dense fibroblast and collagen capsule. The more the larvae grow, the more they cause deformation of the goblet cells and hyperplasia and hypertrophy. Around the encapsulated larva, there will be a predominantly lymphocyte infiltration with a few plasma cells, epithelioid macrophages and sometimes a large number of eosinophils. Other microscopic observations are submucosal edema, scattered foci of submucosal hemorrhage with eosinophil infiltrates (Love S. *et al*., 1999).

For *S. equinus*, one to four weeks after infection, small hemorrhagic nodules may appear on the serosa of the ileum and colon. White dots appear on the liver a week after infection and turn into a tortuous groove about three weeks later. Many granulomas containing larvae are found in the flanks, diaphragm, omentum and sometimes under the visceral pleura (McCraw B. *et al*., 1985).

About the *S. vulgaris*, the association with thrombosis and lesions of the walls of arteries, especially the cranial mesenteric artery, is well known. Fourth (L4) and fifth (L5) stage larvae are responsible for arteritis, necrosis, and fibrosis of the cranial mesenteric artery and its branches (Borji A. *et al*., 2014). Une rupture du rectum peut aussi se produire lors d'une infestation a S. vulgaris. En effet, la migration des larves à travers l'artère mésentérique caudale peut causer a vascular disease and thrombosis dû à une dévitalisation et une nécrose ischémique du tissu rectal. (Guglick M, *et al*., 1996).

For an infestation with *S.edentatus* white foci were observed in the liver from two to 56 days. According to McCraw B. *et al.,* (1974) they contained mononuclear cells and eosinophils and later necrotic cores of eosinophils. By one month foci were replaced by tortuous tracks of migrating larvae. Aberrant larvae in the lungs were confined in granulomas. Massive granulomas in the wall of the cecum and colon contained small larvae which were probably inhibited by antibody associated with the third molt. Severe disruption of omental architecture and adhesions involving the intestine occurred several weeks after infection. (McCraw B. *et al.,* 1974). Hemomelasma ilei est une lesion possible lors d'infection par S. edentatus. Il s'agit de multiples dark hemorrhagic fibro-vascular plaques sur la séreuse de l'ileum. Ces lésions peuvent être causées par S. edentatus en particulier ou toutes larves de Strongyles spp (Jubb K, *et al*., 2016).

4. Treatments:

4.1 Anthelmintic treatment :

Three major of anthelmintic classes are worldwide used for GINs control: benzimidazole (*e.g.* fenbendazole, ofendazole); macrocyclic lactones (MLs; *e.g.* abamectin, ivermectin and moxidectin); and tertahydropyrimidines (*e.g.* morantel and pyrantel). Each of these classes has variable anthelmintic activity, efficacy and time to effectiveness (Table VI, Saeed M. *et al*., 2019). According to Hunt E. *et al*., (1996) and Mercier P. *et al*., (2001) an oral formulation of ivermectin (0.2 mg / kg) can reduce to below 90% for up to 6 weeks post administration the burden of strongyles (cyathostomins, *Triodotonphorus spp*). Ivermectin has a broad spectrum of activity against a good number of larval and adult stage of GINs, however, the encysted cyathostomin larvae are refractory to it even at a higher concentration (Rolfe PF. *et al*., 1996). Moxidectin (0.4mg / kg) has the advantage of being effective against all stages of cyathostomin larvae. For example, moxidectin gel (0.4mg / kg; oral) has proven its effectiveness in reducing strongyles eggs below 100 EPG for a period of at least 12 weeks post administration, despite the continued reinfection of horses during treatment (Holm-Martin M. *et al.*, 2005). In addition, moxidectin requires a longer administration interval, thus reducing the frequency of treatment, and may therefore be of benefit in the fight against anthelmintic resistance (AR; Corning S. *et al*., 2009). It is also possible to use other MLs such as abamectin in combination with praziquantel alone or in combination with oxfendazole or morantel. This combination has been shown to be highly effective against strongylins, cyathostomins and Parascaris spp, while abamectin alone shows poor efficacy (Rolfe PF. *et al*., 1996, Wilkes EJA. *et al*., 2017, Barger I. *et al*., 1979). The use of anthelmintics is worldwide, but the FEC threshold which should justify treatment is not set and remains controversial. For example, there are 100 EPGs in the literature for Boersema J. *et al*. (1996) and Holm-Martin M. *et al*. (2005), 200 EPGs for Mercier P. *et al*. (2001) and Jacobs D. *et al*. (1995) and between 200 and 500 EPG for Lester HE. *et al*. (2014). It is therefore essential to find a definition and a common value for ERP and cut-off FECRT value in horse worldwide (Saeed MA. *et al*., 2019).

4.2 Anthelmintic resistance:

The implementation of anthelmintic treatment strategies to control *S. vulgaris* has been very effective and has reduced morbidity and mortality. But on the other hand, it allowed a selection of resistant parasites such as cyathostomins. Large strongyles are currently less of a concern because they have given way to cyathostomes which are therefore considered to be the main pathogenic parasites worldwide. Resistance of cyathostomes to benzimidazole drugs is highly prevalent globally and resistance to pyrantel is becoming increasingly common (Kaplan RM, 2002). This resistance is due to the concern that owners want their horses to have a near zero fecal eggs count (FEC) and thus refuse to skip an unneeded treatment. Therefore, there is a regular use of ineffective drugs which will result in an increase of the resistance.

In the 1960s an epidemiological approach was introduced to control *S. vulgaris* infestations with benzimidazole (BZ) with an administration interval of 6 to 8 weeks. This program, which has been used widely, has proven to be very effective because in the 1980's *S. vulgaris* was recognized as a low infesting species in horses. However, on the other hand, a drastic increase in cyathostomins infestations was observed (Herd R.P. *et al*., 1981). Subsequently, the use of ivermectin was the final blow to S. *vulgaris*, this drug being highly effective against migrating larvae. At the same time as the interval dose treatment was started, cyathostomins, regarded as a minor problem at the time, showed their first resistance to thiabendazole. This has resulted in resistance to other BZs and more recently to pyrantel. Nowadays cyathostomins have developed resistance to all the most common anthelmintics except avermectin and mylbemycins (Chapman MR, *et al*., 1996, Craven J. *et al*., 1999, Sangster NC, *et al*., 1999, Kaplan RM, 2002).

It is important to have methods in place to diagnose anthelmintic resistance. For example, the fecal egg count reduction test (FECRT) is considered to be the gold standard for clinical diagnosis of anthelmintic resistance. The World Association for the Advancement of Veterinay Parasitology (WAAVP) has published recommendations to standardize the procedures used to detect resistance (Coles G. *et al*., 1992). Although most of these recommendations apply to goats and sheep, they may be useful for horses (Kaplan RM, 2002). According to WAAVP, when using FECRT, resistance is defined as the reduction in FEC of less than 95% with a lower confidence limits (LCL) of less than 90%. It is only when these two criteria are met that one can suspect resistance.

In order to fight against this resistance, it is important that before setting up a treatment with BZ or pyrantel, the veterinarian does an FECRT to eliminate any resistance already present. It

would also be helpful if the veterinarian did regular FECs to measure the effectiveness of a worm control program and to identify horses that do not require treatment from those that do. According to some studies (Uhlinger C.A. *et al*., 1992), the practice of rotation drugs does not slow down the development of resistance. Therefore, an effort should be made to set up a single anthelmintic annual rotation (Coles G.C. *et al*., 1992). And of course it is advisable to always use the correct dose.

5. Report case of two feral horses found dead in Cape Toi, Japan:

5.1 Abstract:

Two Misaki feral horse were found dead in October in Cape Toi Japan. This was an opportunity to study the type of parasitic infection of these horses. The lesions present at the autopsy and the histopathology leads us to an infection with *Strongylus spp*. and/or cyathostomins. Eggs of small and large strongyles were observed during the FEC. Adult parasites were collected from the intestinal contents of the ileum, cecum, colon and were subjected to morphological identification and PCR. The use of the NC1-NC2 primers gave us positive results for 5 samples proving that they were Strongyloid-type parasites. Nested PCR, using Forward primers; Sv, Seq, Sed and reverse NC2, did not allow us to confirm or disprove the presence of *S. vulgaris*, *S. equinus* and *S. edentatus*. Although, the pit 2 of the *S. equinus* showed a different result so it was sent with the other samples for a DNA sequencing followed by a phylogenetic tree. The samples A19-41 colon, A19-41 colon1many, A19-40 cecum, A19-41 colon1 belong to the family of cyathostomins. The Sample from A19-40 equinus gel, A19-40 equinus and A19-40 colon was closer from the Strongylinae. It was therefore found that the cyathostomins were more visible, but it is not possible to exclude infection with *Strongylus spp*. regarding the histopathologic lesions, the identification of a specimen and the results of the DNA sequencing.

5.2 Introduction :

The Misaki feral horse (Equus Caballus) is one of the eight breeds considered native to Japan and designated as a National Natural Treasure (Dong JB. *et al*., 2013, Niazmand MH. *et al*., 2019). About a hundred horses live in Cape Toi in the south of the island of Kyushu in Japan (figures 11 and 12). In 1697, the lord of the Takanabe domain (from the Akitsuki clan) used the Cape Toi region in order to breed Misaki riding horses for the ruling samurai class (Kobayashi I. *et al*., 2019). Over the years the number of horses decreased as a result of the decrease in grazing areas and the lack of care, until the council for the protection of Misaki horses was created in 1968 with the objectives of the protection of Cape Toi horses and the control of its population (control of parasites, tickets and inedible grass). Several times a year, the university's pathology and parasitology department visits Cape Toi to monitor the horses. The horses are gathered in two paddocks and will be lined up in a bamboo installation leaving them as little movement as possible. Each horse is numbered and subjected to a blood test, ticks samples and general condition analysis. This is where deworming also takes place. These horses are dewormed only once or twice a year, which is not enough time to break the life cycle of internal parasites (Drudge and Lyons, 1966). Therefore, Misaki feral horses may still be prone to infestations with *Strongylus spp*. So when two horses were found dead during October 2019, it was important to determinate what species of parasites is currently infecting the japanese wild horses.

5.3 Materials and Methods:

Necropsy and Histopathology:

The necropsies were made according the protocol and pictures were taken of the organs with macroscopic lesions. Samples come from the stomach, duodenum, jejunum, ileum, cecum, colon, cranial mesenteric artery, renal artery, both kidneys, liver, lungs, heart, lymph nodes (axillary, inguinal, superficial cervical) and bone marrow. The samples were fixated in a formalin solution 10% for 48 hours. All materials were serially sectioned into segments of 3-4 µm thickness and slides were prepared in hematoxylin and eosin stain (figure 13,14).

Adult worm collection and egg collection:

Adult worms were collected from the intestinal contents of the ileum, cecum, and colon. To make it easier to visualize the parasites, water has been added and after collection is complete, the supernatant was stored for PCR. They were stored in a 70% alcohol solution and kept at - 20 °C.

For the FEC 1g of feces was diluted in 50 ml of mineral water for 20 minutes and then centrifuged at 2500 rpm for 5 min. The supernatant was discarded. A saturated sugar solution with a specific gravity of 1.20 (sugar 100 gr + water 155 ml + phenol 2 ml) was added, then centrifuged again at 2500 rpm for 5 min. A saturated sugar solution 1.20 was added again to make a positive meniscus and was left to stand for 30 min. 200 µl was aspirated, put on a slide and covered. EPG was calculated for each sample and pictures were took for morphological identification. The rest of the solution were kept at -20 °C for further analysis.

Morphological identification of adult parasites:

Morphological identification was performed by observing worms treated with lactophenol solution using an Olympus BX51 microscope equipped with an Olympus DP Digital Camera 12. Our attention focused on the mouth collar, papilla, buccal capsule, esophagus, vulva, anus, tail and speculum.

PCR and sequencing:

10% homogenate was prepared by making an homogenous solution with 0.12-0.15g of worms from ileum, cecum and colon of each horses and 250 µl of Dulbecco's Modified Eagle Medium (DMEM). 750 µl were added then the solution was vortexed, spine down and finally centrifuged at 3000 rpm at 4°C for 10 min. The supernatants were collected and used for Polymerase Chain Reaction and nested PCR.

DNA was extracted with the following steps; incubation of 50 µl of the 10% homogenate solution with 2µl of proteinase K for 2 hours at 37°C, an incubation at 100°C in a thermos aluminium bath for 20 minutes, a centrifugation at 15000 rpm at room temperature (20°C) for 5 minutes. The supernatants were collected, put in new tubes and then stored at -30°C to 80°C.

A nested PCR was made to detect *Strongylus spp.*, with the primers NC1-NC2 for the amplification of the ITS-2 segment (forward NC1>: 5'-ACGTCTGGTTCAGGGTTGTT-3' and reverse <NC2: 5'-TTAGTTTCTTTTCCTCCGCT-3'). And the second PCR used specific primers for *S. vulgaris, S. equinus* and *S. edentatus* with respectively Sv>: 5'-GTATACATTAAATAAGTGTCCCCCATTCTAG-3' (forward), <NC2: 5'-TTAGTTTCTTTTCCTCCGCT-3' (reverse), Seq>: 5'-TACACAGAATGG-CATAACATCG-3' (forward), <NC2 (reverse) and Sed>: 5'TACACAGAATGG-CATCACATCG-3' (forward), >NC2 (reverse). PCR reaction (25 µl) were performed using the following PCR mix: GoTaq 12.5µl, 05µl of each primer, 10 µl of demineralised water (DW) and 1µl of DNA under the following conditions: 94°C 5 min (initial denaturation); 94°C, 30s (denaturation); 55°C, 30s (annealing), 72°C, 30s (extension) for 30 cycles and 72°C, 5 min for the final extension. The amplification products were put in an agarose gels for viewing the results. The PCR was made on the extraction of single parasite from colon, cecum and abdominal cavity of A19-40 and A19-41.

DNA sequencing was made using PCR product after checking the DNA concentration with the nanodrop. The preparation of the sample was according FastGene^{TM} (figure 15). One sample was taken from the agarose gel and the other samples were from PCR products.

Results:

The first horse found dead (A19-40) was a young female of unknown age with emaciation, pressure sores and a significant number of ticks especially around the genital and anal area. Bleeding was observed in the right front leg. At organ level, adhesions were found on the diaphragmatic side of the liver and white spot of 2-3 mm were found (figure 16). The pyloric area of the stomach had ulcers (figure 17). In the colon, red lesions are distributed throughout the mucosa and parasites were found (figure 18). The cranial mesenteric artery has thickened and a clot adherent to its lumen (figure 19). In the lungs we could observe a parasite-like path on the edge of the right caudal lobe (figure 20). The aorta has a lesion 1.5 cm in diameter (figure 21).

The second autopsied horse (A19-41) is a 17 years old female, with severe emaciation. She has a tick infestation. In the liver, brown spots are observed on the serosa as well as fibrin debris (figure 22). The left kidney presented in its serosa a black cystic structure (figure 23). The stomach has petechial hemorrhages in the mucosa with the presence of small red worms (figure 24). In the small intestine and colon small white worms were found in the intestinal contents and other white intestinal parasites were found attached to the mucosa (figure 25). In

the thoracic cavity, adhesions of the pleura to the thoracic wall have been observed (figure 26). Fibrin covered the pulmonary serosa and white spots were found multifocal distribution (figure 27). There was a foamy content in the trachea (figure 28). A pericardial effusion with a yellowish fluid (figure 29). And osteoarthritis was present in many joints in all four limbs.

For the histopathological analysis I only took one case, the A19-41. The duodenum, jejunum, ileum, cecum and colon had infiltration of inflammatory cells in the lamina propria especially lymphocytes et eosinophils. In lumen of the ileum, the cecum and the colon segments and proglottids of tapeworm were found (figure 30). Some were also found in the lamina propria. About the cranial mesenteric artery thrombosis was found in the tunica intima as granulation and calcification. Eosinophils, lymphocytes, neutrophils and macrophages were also present (figure 31). A parasite was found in the thrombus area (figure 32). The renal artery had the same pathologic lesions. A cystic formation surrounded with fibrous tissue was present in the left kidney. The liver had a suppurative granulation in the portal triad and fibrosis. Suppurative granulation and mineralisation were presents in the lungs as neutrophils, eosinophils, macrophages and lymphocytes in the bronchiolar and interstitial area. The heart had a serous atrophy and a fibrosis. A lymphadenitis was observed in the axillary, inguinal and superficial cervical lymph nodes. The bone marrow presented osteomyelitis. The histopathological diagnosis are; severe eosinophilic gastroenteritis, thromboarteritis with dystrophic mineralization, suppurative granulomatous hepatisis with fibrosis, suppurative granulomatous bronchopneumonia, lymphadenitis and osteomyelitis. The final diagnostic is a gastroenteritis with thromboembolic due to parasitic infestation. The pathological and histopathological leads us to search for *strongylus spp.* due to their pathogenesis.

The next step was the fecal egg count and eggs identification. We focused on the small and large strongyles. We found 870 EPG in the colon sample A19-40. 30 EPG were counted in the Caecum and in the colon of A19-41.

For the morphological identification of the adults, one has the length and the morphological traits of *Strongylus vulgaris* (figure 33). Only a bilobed thoot was present in the buccal cavity and the external leaf-crowns were numerous and prominent. The other parasites collected seemed to belong to the cyathostomins, for their size and appearance, but it was very difficult to identified them (figure 34 to 36).

The first PCR gave 5 positive results with the NC1-NC2 primers, 1 from the extraction of a single parasite from A19-41 colon 1 and 4 positives from the extraction of several parasites from A19-40 colon, A19-40 cecum, A19-41 and A19-41 cecum 2 (figure 37). The next step was to make the nested PCR to know if *Strongylus spp.* were presents using the forward

primers Sv, Seq, Sed and the reverse primers NC2. The results were positive with many bands (figure 38 and 39). However, the well 2 from the A19-40 colon sample appears to display a different result than the others so the gel was collected, purified and sent for DNA sequencing along the PCR product from A19-40 colon and the 4 other PCR product. A phylogenetic tree was created and showed that four samples seemed to belong to the Cyathostominae family (figure 40) and the A19-40 colonequinusgel, the A19-40 equinus and the A19-40 colon does not belong to the cytahostominae family but was closer from the strongylinae family.

Discussion:

In this present study, regarding the macroscopic lesions and histopathological findings, and the lack of regular deworming we paid a particular attention on the presence of *Strongylus vulgaris, Stongylus equinus* and *Strongylus edentatus*.

The results of the EPG obtained are not accurate because a simple microscope slide was used while the use of a McMaster cell is recommended. Morphologically we have succeeded in differentiating them at the interspecific level. The one found in the colon of A19-40 seems to belong to *S. vulgaris* but a molecular test should be carried out to confirm. For the others parasites it was impossible with our level of knowledge to differentiate them at the intraspecific level. The PCRs confirmed to us that we were in the presence of strongyles species since we had positive bands for the NC1-NC2 primers, which are the primers that will amplify fragments common to large strongyles and small strongyles (figure 38). The results of the nested PCR for *S. vulgaris, S. edentatus* and *S. equinus* (figure 39) showed many positive bands that can be explained by the fact that the primers differ only by a few nucleotides. Interpretation of these results is therefore almost impossible. Only one result (A19-40colon) present a band that can be consider as positive so it was sent for DNA sequencing (along with the PCR product, the nested PCR and the gel). A more sensitive test such as a PCR-ELISA or RLB assay methods would be more suitable and efficient for testing large numbers of samples. The DNA sequencing and the phylogenic trees allowed us to confirm that the most of the parasites were cyathostomins and one sample (A19-40 colon) was more close to the strongyles. Even if cyathostomins appear to be more prevalent in this two cases, this results have to be taken cautiously because no PCR was performed on actual feces (only on adult parasites), and consequently no test was made on the eggs which should have given us more information. Indeed, a larva of *Strongylus spp*. was found in the cranial mesenteric artery and regarding the histopathologic findings we have to keep in mind that the presence of *Strongylus spp*. is

possible. The absence of adult parasites does not mean that the horse is not infected. The larvae and their migration should be taken in consideration. Moreover, it is important to notice that these test does not reflect the parasite burden.

Conclusion:

In conclusion, GIN's are a worldwide concern and are responsible for many pathologies, 75% of which are caused by cyathostomins and can lead to up to 50% of mortality. This work allowed a brief review of the main GINs and the most used diagnostic techniques. Given the lack of any new anthelmintic classes for the equine industry on the horizon, it is necessary to thoroughly review horse management practices (Andersen U.V. *et al*., 2012). This study of two wild horses found dead in Japan (A19-40 and A19-41) showed the difficulties encountered in the field and in terms of diagnosis techniques. In the case of wild horses, regular clinical monitoring and the implementation of frequent coprology tests is almost impossible. The only opportunities to diagnose the parasitic species are in dead horses. Unfortunately, this implies that there is no access to fresh samples as the corpses are rarely found immediately. In the case of A19-40 and A19-41, the autopsies revealed the presence of parasites and parasitic pathognomonic lesions of *Srongylus spp*. Collection of tissue samples, adult parasites and of the contents of various intestinal segments was performed. Histopathology of the slides of individual A19-41 revealed gastroenteritis with thromboembolic due to parasitic infection. The FEC and identification of the eggs was not conclusive. The PCR and the DNA sequencing tests allow us to observe that the parasites present in greater numbers are from the cyathostominae family and one sample could belong to the Strongylinae family. The results should be taken cautiously for these tests were conducted on intestinal contents and therefore only detects the patent stage on infection, while the pathogenic migration or encysted larvae will go undetected.

According Andersen UV *et al*., (2012) the ideal requirement for diagnostic tools in equine parasitology can be summarized as follow: assays should be able to reliably detect and differentiate between the major pathogenic parasites, parasites should be detected at their pathogenic stage (the migrating and encysted larvae), assays should allow interpretation on at least a semi-quantitative level, as disease is often link to the abundance of parasites, assays should become quickly negative after treatment to allow evaluation of treatment efficacy and techniques applied should be practical in the field and affordable.

6. Annexes related to the bibliographic review:

Addendum 1: Non strongylid parasites:

Stomach worms:

There are 4 species of nematodes that can be found in the stomachs of horses; *Draschia megastoma, Habronema muscae, Habronema majus* and *Trichostrongylus axei. Draschia megastoma* and *Habronema spp.* belong to the superfamily of Spiuroidea (Saeed MA, *et al*., 2019). They share the same morphological characteristics and present a biological cycle requiring an intermediate host; Musca domestica (housefly) or Stomoxys calcitrans (stable fly). Spirurid eggs containing the L1 are expelled in the feces and will subsequently be ingested by the maggots. The development of the larva will take place in parallel with the development of the fly larva until stage L3, which is the infectious stage (2 weeks). The infected fly will then come and lay its eggs around the horse's mouth. They will be swallowed and will develop into adult worms in the stomach (2 months) (Saeed MA, *et al*., 2019; Morsky K, *et al*., 2016).
Draschia megastoma is the most pathogenic species of spiruid. According to Dunsmore *et al.* (1985), it has been associated with multiple nodular granuloma (1-7 cm) in the stomach of the horses from western Australia. These granulomas are usually located near the *margo plicatus* and can form fibrous masses. There would be no consequences if the nodules are small, but if the nodules are large, it can interfere with gastric motility and lead to gastric rupture.
Habronema spp causes a parasitic disease in horses called Habronemosis which is relatively important. They can cause pathologies in horse especially of the stomach wall where they can stimulate a large amount of tick glandular secretions, in particular near the *margo plicatus* (Saeed MA, *et al*., 2019, Morsy K, et al ., 2016, Nielsen MK, et al., 2016). Both *Habronema spp.* and *D. megastoma* can infect the skin. If the larva is deposited near a wounded skin it can lead to a local inflammation and the development of granulation tissue which is a typical lesion of cutaneous habronemiasis (Reinemeyer CR, *et al*., 2009).
Trichostrongylus axei usually only causes subclinical infections. It is a parasite that has a significant economic impact on domestic livestock and therefore on horses when co-grazing (Saeed MA, *et al*., 2019).

Ascarids:

The Ascarididae family is composed with two important species, *Parascaris equorum* and *Parascaris univalens. Parascaris equorum* is a large parasite which measures 28 cm in length for the male and 50 cm for the female. The eggs of these parasites have the particularity of being extremely resistant, up to several years unless they are exposed to more than 39 °C (Reinemeyer CR, *et al*., 2009, Monica A, *et al*., 2018). The development of the larva to L3 takes place in the egg in 9 to 14 days if conditions are optimal (25 °C - 35 °C) but stops if the temperature drops below 10 °C (Saeed MA, *et al*., 2019). Once ingested the parasite eggs end up in the small intestine and follow a direct life cycle. They will hatch, pass through the mucosa and migrate to the liver and lungs. They will then go up to the pharynx where they will be

swallowed and develop into an adult in the duodenum and jejunum. The pre-patent period is 75 to 115 days. Clinical signs are lethargy, inappetence, reduced weigh gain, nasal discharge, edema and colic in young horse. Ascarid impaction of the small intestine can occur and will require intestinal surgery (Clayton HM, *et al*., 1977).

Oxuirids:

Oxyuris equi has worldwide distribution. The eggs are deposited on the perianal border. They have a gelatinous matrix allowing them to stick to the hairs and can cause irritation and pruritus. Adults reside mainly in the dorsal colon and the cycle follows a direct pattern. The lesions are caused by the larval stages which feed in the intestinal crypts and thus causing erosion and influx of inflammatory cells (Nielsen MK *et al*., 2016).

Threadworms:

According to Miller F, *et al.* (2017), *Strongyloides westeri* can be found in young foals up to approximately 16 weeks of age. Parasites can be transmitted through breast milk or through percutaneous invasion. They have a direct life cycle and the adults reside in the small intestine where, only the females, can induce enteritis with consequences such as malabsorption and diarrhea (Saeed MA, *et al*., 2019).

Intestinal parasite: Tapeworm:

Anoplocephala perfoliata has for many years been considered a minor problem and was rarely associated with a clinical disease. For the past fifteen years, this parasite has become an emerging problem for breeders and veterinarians (Tomczuk K *et al*., 2014 , 2010; Rehbein *et al*. 2013). *Anoplocephala perfoliata* belongs to the family of Anoplocephalidae (Cyclophyllidea, Cestoda) and has an indirect life cycle via pasture dwelling oribatid mites from *Oribatidae spp* (Tomczuk K, *et al*., 2014). When the eggs are eliminated in the feces they will be ingested by the moths and will develop into larvae into their host. Once the mites containing infective cysticercoid are ingested, they will attach to the intestinal mucosa at the ileocaecal junction using their scolex. They will mature in 6 to 10 weeks and will reach a size of 5 to 8 cm. Proglottids containing eggs will break off and be removed with the faeces (Tomczuk K, *et al*., 2014). According to Pavone *et al*. published in 2011 (and De Almeida *et al.* 2008), mucosal ulceration, submucosal oedema, hypertrophy of the distal ileum, and decreased ileocaecal valve distensibility have all been reported to occur at the site of parasite attachment, and the severity of pathology is directly proportional to parasite infection intensity.

Addendum 2, 3, 4 : Fecal worm egg count analysis:

Addendum 2 :the FECPAK methodology:

The FECPAK and the McMaster test has the same technique, that of flotation-dilution. This is a commercial test that differs from the McMaster technique by using a larger microscope slide to increase the detection limit (Cringoli G, *et al*., 2004). This technique was initially used for cattle in the 1990s but has been adapted for horses from 2004 (Tyson F, *et al*., 2020). This

test no longer requires centrifugation and is available as a kit. It can therefore be easily used by veterinarians and horse owners. For this technique, 15 to 20 g of feces will be mixed with 4 volumes of water in a sealed plastic bag. Then 45 ml will be taken and mixed with 185 ml of saturated salt solution. This solution will be passed through a 1mm opening filter to remove organic debris. An aliquot will be taken from the filtered solution and put into a custom-made acrylic chamber with 1ml under the grids (a McMaster slide contains only 0.3 ml). After 30 seconds the nematode eggs will rise to the surface and can be counted under a microscope. The multiplication factor used will be chosen according to the dilution. In this example each egg counted represents 20 eggs per gram of faeces (EPG). It is really important to choose the right dilution. While too high dilution will decrease sensitivity, too low dilution will prevent egg flotation and reduce visibility leading to underestimation of egg density (Pereckiene A, *et al*., 2007). According to Preslan SL. *et al*., (2005) the FECPAK method is more sensitive than the McMaster method at standard dilutions.

Addendum 3: the FLOTAC methodology:

The development of the FLOTAC method was inspired by other flotation techniques. Therefore, it includes a step of centrifugal flotation of a fecal sample suspension and the translation of the apical portion of the floating suspension. An advantage of the FLOTAC technique is that it allows parasite eggs to be counted in large fecal aliquots (over 1g or more). One of the disadvantages of this technique is that it requires specific equipment (large volume centrifuge or benchtop centrifuge with rotor for micro-titer plates), which is not always available in current practice. The installation and use of the device requires some training. Moreover, the FLOTAC apparatus (manuals, component and accessories) is not commercially available (Cringoli G, *et al*., 2010). It is particularly important, as for all techniques based on flotation, to make the right choice in terms of flotation and preservation medium and specific gravity solution (Table V) because it could influence the performance of the FLOTAC test. There are two versions of FLOTAC apparatus: FLOTAC-100 which allows a magnification of x100 and FLOTAC-400 which allows a magnification of x400. FLOTAC-400 therefore has a more advanced technology which allows the detection of intestinal protozoa (Cringoli G, *et al*., 2010). FLOTAC-100, however, remains recommended for the detection of eggs and helminth larvae. The reason is the reading disc is more ticker and hence more robust than the one used in FLOTAC-400, and because it is more easy to fill the flotation chambers. There are three FLOTAC techniques; a basic, a dual and a double. The basic FLOTAC technique requires two flotation chambers for a volume of 10ml corresponding to 1g of feces. The analytical sensitivity of this technique is 1 egg per gram (EPG), 1 larva per gram (LPG), 1 oocyst per gram (OPG), or 1 cyst per gram (CPG). The FLOTAC dual technique analyzes in parallel the same sample using two different floating solutions but which have the same specific gravity. This technique is recommended for performing a wide-ranged parasitological screening (Cringoli G, *et al*., 2010). The analytic sensitivity of the FLOTAC dual technique is 2 EPG, 2 LPG, 2 OPG or 2 CPG. With the FLOTAC double technique it is possible to simultaneously examine 2 samples from 2 different horses using a single FLOTAC apparatus. Each sample has its own single flotation chamber with the same flotation solution (Cringoli G, *et al*., 2010). The analytical sensitivity of the FLOTAC double technique is 2 EPG, 2 LPG,

2 OPG or 2 CPG. Each of the three FLOTAC methods can be used on fresh faeces, stored at 4 °C 1 to 3 days or preserved in a 5 or 10% formalin or sodium acetate-acetic acid-formalin (SAF) solution for several weeks or even months (Cringoli G, *et al*., 2010). The conservation ratio should be 1: 4. The 11 operation steps of the three techniques are summarize in the Figure 5.

Addendum 4: the Ovatec methodology:

The OvatecTM (Zoetis UK Ltd) is a method which provides a rapid response in the form of positive or negative results depending on the presence or absence of nematode eggs. Therefore, this is not a quantitative method and if the system does not detect any eggs, it is 95% certain that the FWEC of the sample is <150 EPG and 100% that it is <250 EPG (Ronsyn R, *et al*., 2012). This test is therefore useful for identifying medium to high shedders, but given its lack of sensitivity it is not recommended for evaluating the effectiveness of anthelmintics.

Addendum 5: wet mount techniques:

Methylene Blue-Glycerol Wet Mount: A thick smear of faecal sample was prepared by adding a greater volume of faeces to a drop of methylene blue-glycerol solution on a microscope glass slide and placing a coverslip over the smear. Methylene blue-glycerol solution was prepared by mixing methylene blue dye separately with increasing quantities of glycerol in concentrations of 10%, 12.5%, 16.6%, 25%, and 50%.

Saline wet mount and iodine wet mount: Saline wet mounts and iodine wet mount were prepared by separate a small volume of stool sample with a drop of physiological saline, methylene blue dye, and Lugol's iodine (diluted in 1: 5 distilled water), respectively, on a glass slide and placing a coverslip over the smear. "

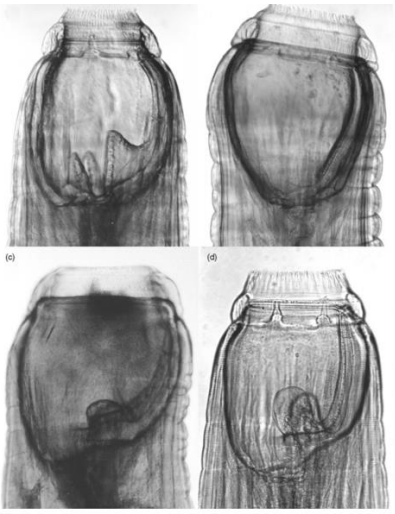


Figure 1: Heads of different *Strongylus* species, lateral view. (a) *S. equinus*. (b) *S.edentatus*. (c) *S.asini*. (d) *S.vulgaris*. from Lichtenfels, J. R, *et al*., 2008

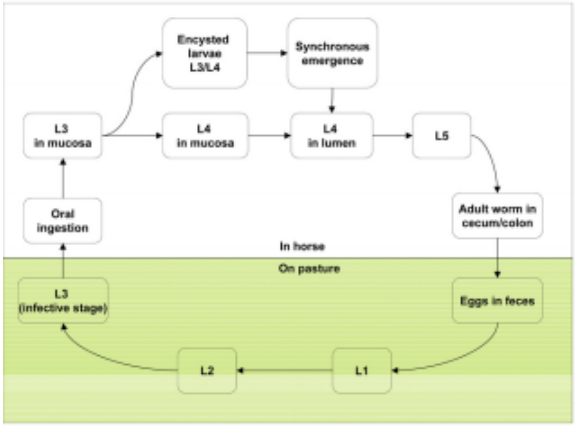


Figure 2: life cycle of cyathostomins. Corning, Susan, 2009.

Table I:

SOLUTION	IDENTIFIABLE PARASITES
Sodium chloride*†	Common helminths Protozoan ova and cysts
Sheather's*	Common helminths Protozoan ova and cysts (particularly Cryptosporidium oocysts)
Sodium nitrate*†	Common helminths Protozoan ova and cysts
Zinc sulfate*	Common helminths (particularly Giardia) Protozoan ova and cysts (particularly lungworm larvae)
Magnesium sulfate*†	Common helminths Protozoan ova and cysts

*Noneffective if flukes are suspected; use of fecal sedimentation is advised

†Distorts Giardia rapidly, subsequently impeding diagnosis

Quick Guide to Solutions & Target Parasites Oreta. M, 2013.

Table II:

SOLUTION	SPECIFIC GRAVITY	FORMULATION
Sodium chloride*	1.2	Add table salt to boiling water until the salt no longer dissolves
Sheather's†	1.2–1.25	454 g (1 lb) sugar + 355 mL water + 6 mL 40% formaldehyde (or 1 g crystalline phenol)
Sodium nitrate	1.2–1.33	315 g sodium nitrate: 1 L water
Zinc sulfate	1.18	386 g zinc sulfate: 1 L water
Magnesium sulfate	1.32	350 g Epson salts: 1 L water

*Sodium chloride solution, although widely used due to its low cost, is highly corrosive and will distort eggs

†Due to high sugar content, Sheather's solution is sticky and will attract insects if not kept sealed and the surrounding area kept clean.

Fecal Flotation Solutions. Oreta, 2013.

Table III:

FWEC method	Detection limit of test*	Special equipment needed	Cost of equipment	Time taken from weighing out subsample to FWEC result	Relative ease of method (based on number of steps, expertise required)	Number of steps in protocol from weighing subsample to result
Centrifugal-flotation[a]	1–9 EPG	Miller eyepiece graticule, centrifuge, cuvettes, plastic tubes	Approximately £500	~10–15 min	Complex	7
FECPAK[b]	20 EPG	FECPAK system	£760	~5–10 min	Moderate	4
FLOTAC[c]	1 EPG	FLOTAC device and centrifuge	NA	12–15 min	Complex	11
McMaster[d]	15–100 EPG	McMaster slides	£15–£150 per slide	~5–10 min	Easy	4
Ovatec[e]	Only recorded as + or -	Ovatec devices	£95 for 50 tests	~10 min	Easy	6

Abbreviations: EPG = eggs per gram; FWEC = faecal worm egg count; NA = not assessed.

[a]Bartley and Elsheikha (2011) [44].

[b]Innovis Ltd.

[c]Cringoli *et al.* (2010) [33,46].

[d]MAFF (1986) [37].

[e]Zoetis UK Ltd.

*Faecal worm egg count methods a–d are quantitative and give and EPG estimate, whereas method e is qualitative and gives a positive or negative result.

The detection limit, equipment, approximate cost of equipement, time taken per sample, ease of method and number of steps taken for each faecal worm egg count method Lester H.E *et al*., 2013.

Table IV:

Study	Details	Methods investigated	Volume of faeces (g)	Volume of water (ml)	Centrifugation (min:r.p.m.)	Flotation solution (specific gravity)	Flotation time (min)	Multiplication factor	Detection limit (EPG)	Major findings
1 [39]	Comparison of modifications of the McMaster methods for counting *Ascaris suum* eggs in pig faeces	1. Henriksen and Aagaard (1976) [59]	4	56	7:1200	NaCl + sugar (1.27)	2–3	20	20	When counting all chambers, Methods 1, 2, 5 and 6 showed 100% sensitivity. Method 7 showed the least sensitivity (83.3%). Method 7 was the easiest and quickest to perform but the least sensitive. Method 1 was the most complex and most sensitive. Counting eggs in the chambers increased sensitivity of all methods
		2. Kassai (1999) [60]	3	42	3:1500	NaCl (1.2)	3	50	50	
		3. Urquhart *et al.* (1996) [61]	3	42	2:2000	NaCl (1.2)	2–3	50	50	
		4. Urquhart *et al.* (1996) [61]	3	42	None	NaCl (1.2)	2–3	50	50	
		5. Gronvold (1991) [62]	4	56	None	NaCl (1.2)	2–3	50	50	
		6. Gronvold (1991) [62]	4	56	None	NaCl + sugar (1.27)	2–3	50	50	
		7. Thienpont *et al.* (1986) [63]	2	60	None	NaCl (1.2)	2–3	100	100	
2 [40]	Investigation of sensitivity and reliability of three different McMaster techniques for counting *Teladorsagia circumcincta* eggs	8. Wetzel (1951) [64]	2	60	None	NaCl (1.2)	2–3	67	67	Method 10 demonstrated the greatest sensitivity and reliability compared with the other methods.
		9. Zajicek (1978) [65]	1	15	2:2000	$MgSO_4 + Na_2S_2O_3$ (1.28) [67]	5	33	33	
		10. Roepstorff and Nansen (1998) [66]	4	56	5:1200	NaCl + glucose (1.3)	3–5	20	20	

Results drom 2 studies that investigated different modifications of the McMatser method; study 1 (Pereckienė A, *et al*., 2007) compared 7 published modifications, and study 2 (Vadlejch J, *et al*., 2011) evaluated which of 3 McMaster modifications was the most reliable. Lester H.E *et al*., 2013.

Table V:

Host	Fecal preser-vation	Parasite	Parasitic element (PE)	Flotation solution (FS) and specific gravity (s.g.)								
				FS1 1.20	FS2 1.20	FS3 1.20	FS4 1.20	FS5 1.25	FS6 1.28	FS7 1.35	FS8 1.44	FS9 1.45
Cattle, buffalo, sheep and goat	Fresh	Lungworm	Larvae (L_1)	#	#	++	#	#	+	++	+	+++
Horse	Fresh	*Parascaris equorum*	Eggs	+	++	+	+++	+++	+	+	+	+
Horse	Fresh	*Anoplocephala* spp.	Eggs	+++	+	+	+	+	+	+	#	+
Horse	Fresh	Strongyles	Eggs	+	+++	+	+	+++	+	+	+	+
Cat and dog	Fresh	*Toxocara* spp.	Eggs	+++	+	++	++	+	+++	+++	+	++
Cat and dog	Fresh	*Toxascaris leonina*	Eggs	++	++	++	++	++	+++	+	+	+
Cat and dog	Fresh	*Trichuris* spp.	Eggs	+++	++	++	+++	++	++	++	++	+
Cat and dog	Fresh	Hookworm	Eggs	++	+++	++	++	++	++	+	+	+
Dog	Fresh	*Angyostrongylus* spp.	Larvae (L_1)	+	#	+++	+	+	++	++	+	+++
Dog	Fresh	*Crenosoma* spp.	Larvae (L_1)	++	#	+++	#	++	+	+	#	+
Human (*)	SAF-pre-served	*Ascaris lumbri-coides* (**)	Eggs	+++	+	+	++	+++	++	+	+	+
Human (*)	SAF-pre-served	Hookworm (**)	Eggs	+	+++	+	+++	++	+	+	+	+
Human (*)	SAF-pre-served	*Trichuris trichiura*	Eggs	+++	+	+	++	+++	+	+	+	+
Human (*)	SAF-pre-served	*Schistosoma mansoni*	Eggs	#	#	#	#	#	#	+++	+	++

+++ most efficient; ++ efficient; + less efficient; # not suggested; (*) work in progress. (**) do not use ether.

Experiences obtained thus far using FLOTAC techniques for diagnosis of parasites harbored in different animal host species (cattle, buffalo, sheep, goat, cat, dog, and horse) and humans, depending on the fecal preservation, parasitic elements and flotation solution. Cringoli G, *et al*., 2010

Figure 3: The 11 operating steps of the FLOTAC techniques. Cringoli G, *et al*., 2010.

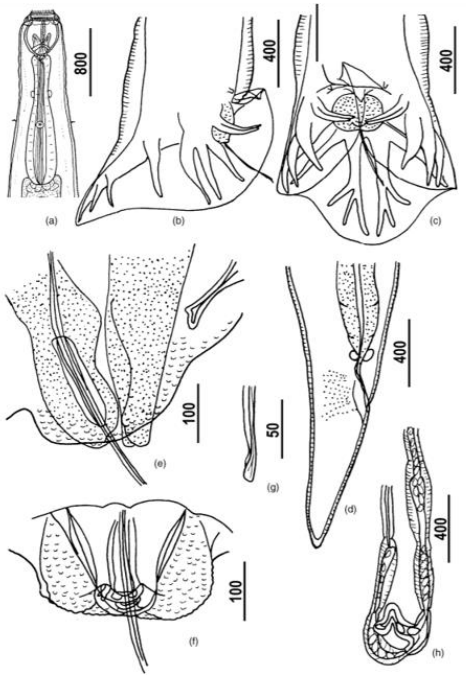


Figure 4: Strongylus vulgaris. (a) Esophageal region, ventral view. (b) Male tail, lateral view. (c) Male tail, dorsoventral view. (d) Tail of female. (e) genital cone, lateral view. (f) tip of genital cone, ventral view. (g) fudes spicule tips of male. (h) ovojectors and vagina, lateral view.

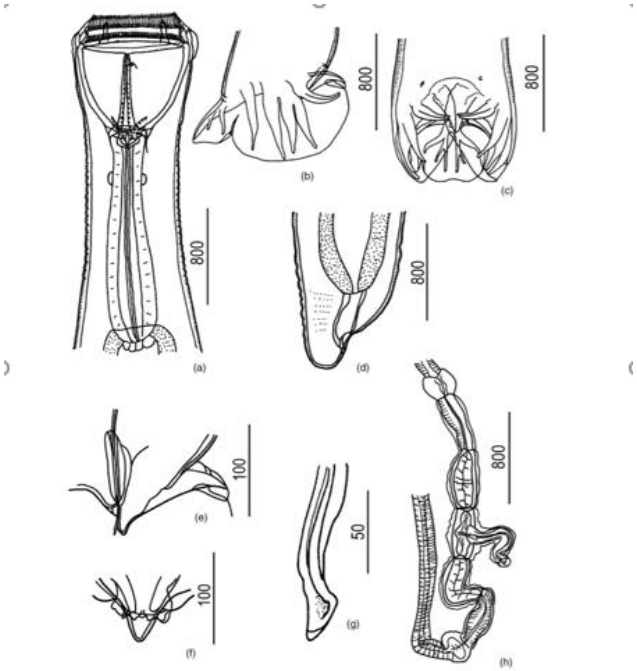


Figure 5: *S. edentatus*. (a) Esophageal region, ventral view. (b) Male tail, lateral view. (c) Male tail, dorsoventral view. (d) Tail of female. (e)Genital cone, lateral view. (f) Tip of genital cone, ventral view. (g) Fused spicule tips of male. (h) Oveiectors and vagina, lateral view

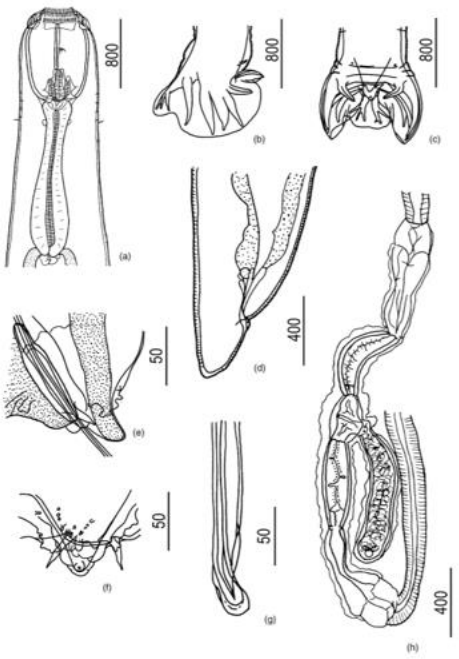


Figure 6: *S. equinus.* (a) Esophageal region, ventral view. (b) Male tail, lateral view. (c) Male tail, dorsoventral view. (d) Tail of female. (e) Genital cone, lateral view. (f) Tip of genital cone, ventral view. (g) Fused spicule tips of male. (h) Ovejectors and vagina, lateral view

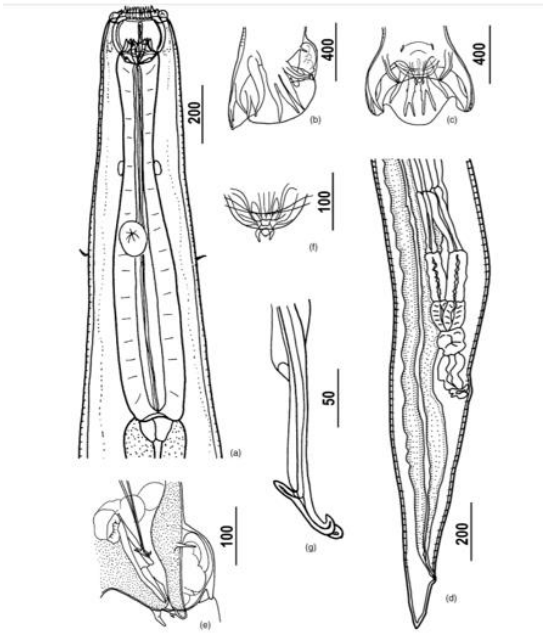


Figure 7: *Triodontophorus serratus*. (a) Esophageal region, ventral view. (b) Male tail, lateral view. (c) Male tail, dorsoventral view. (d) Tail of female. (e) Genital cone, lateral view. (f) Tip of genital cone, ventral view. (g) Fused spicule tips of male (modified from

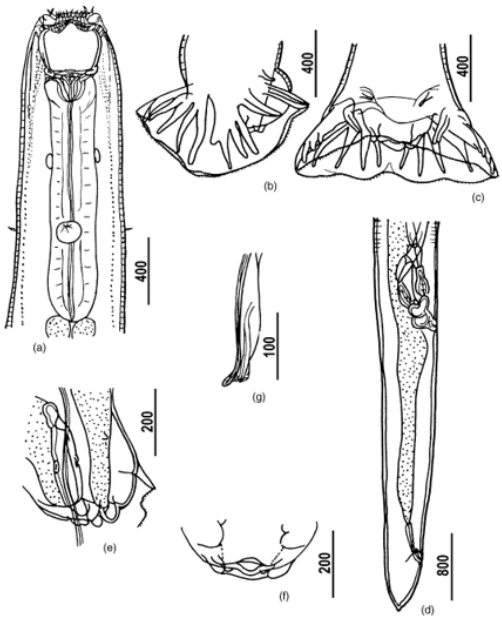


Figure 8: *Oesophagodontus robustus*. (a) Esophageal region, ventral view. (b) Male tail, lateral view. (c) Male tail, dorsoventral view. (d) Tail of
female. (e) Genital cone, lateral view. (f) Tip of genital cone, ventral view. (g) Fused spicule tips of male

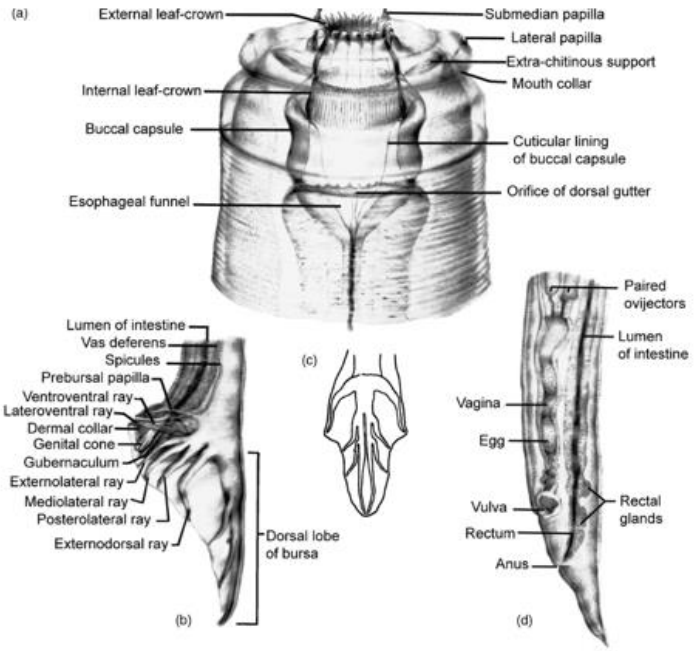


Figure 9: *Coronocyclus coronatus*, labeled drawings, showing characteristics of Strongylidae. (a) Head, ventral view. (b) Male tail, lateral view. (c) Dorsal lobe of bursa of male tail, dorsal view. (d) Female, lateral view of posterior end.

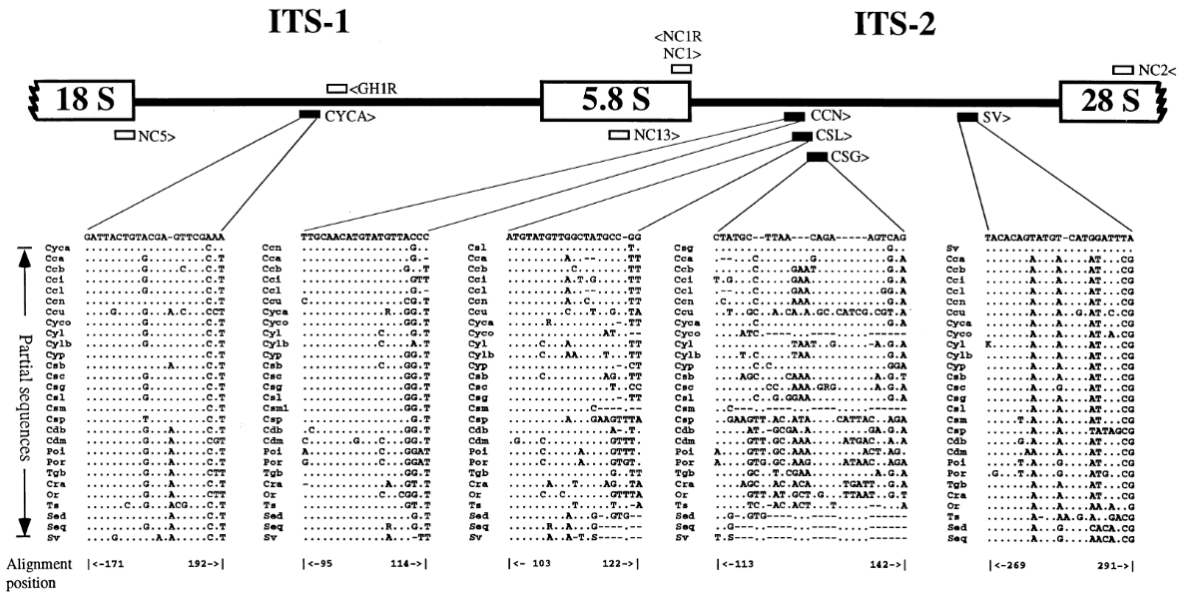


Figure 10: Schematic representation of rDNA transcriptional unit including the 18S, 5.8S and 28S genes, the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) showing the positions of the forward (>) and reverse (<) primers (open boxes) used for PCR and sequencing. Hung, G *et al*., 1999.

Table VI :

Anthelmintic agent	Dosage (mg/kg)[a]	Efficacy (FECR %)	Days post-treatment	Parasite	Reference
Abamectin + praziquantel	0.2, 2.3	≥ 90	28	Strongyles	[15]
Ivermectin	0.2	≥ 90	42	Cyathostomins	[78]
	0.2	≥ 90	42	Cyathostomins, *Gyalocephalus* spp., *Triodontophorus* spp.	[17]
	0.2	≥ 90	42	Cyathostomins	[23]
	0.2	na	42	Strongyles	[75]
Ivermectin + praziquantel	0.2, 1.5	≥ 90	42	Cyathostomins, *Gyalocephalus* spp., *Triodontophorus* spp.	[17]
Moxidectin	0.4	≥ 90	84	Cyathostomins	[78]
	0.4	≥ 90	42	Cyathostomins, *Gyalocephalus* spp., *Triodontophorus* spp.	[17]
	0.4	≥ 90	84	Cyathostomins	[23]
Moxidectin + praziquantel	0.4, 2.5	≥ 90	84	Strongyles	[15]
Fenbendazole	10	100	49	Strongylins	[18]
Oxibendazole	10	≥ 90	14	Cyathostomins	[23]
	10	100	49	Strongylins	[18]
Thiabendazole + piperazine + trichlorphon	44, 125, 40	100	20	Cyathostomins	[80]
Morantel	9.4	≥ 90	14	Cyathostomins	[23]
	10	≥ 90	27	Cyathostomins	[18]
	10	100	49	Strongylins	[18]
	12.5	99	20	Cyathostomins	[80]
	10	100	7	Cyathostomins	[79]
Morantel + abamectin	9, 0.2	100	56	*Parascaris equorum*	[24]
Morantel + oxibendazole + dichlorvos	10 each	99	27	Cyathostomins	[18]
	10 each	100	49	*P. equorum*, Strongylins	[18]

[a] Oral route

Abbreviations: FECR, fecal egg count reduction; GINs, gastrointestinal nematodes; na, not applicable/available

Anthelmintics used against gastrointestinal nematodes of horses in Australia. Saeed, M *et al*., 2019.

7. Annexes related to the case report:

Figure 11: Misaki feral horses, Cape Toi Japan. Kobayashi, I, et al., 2019.

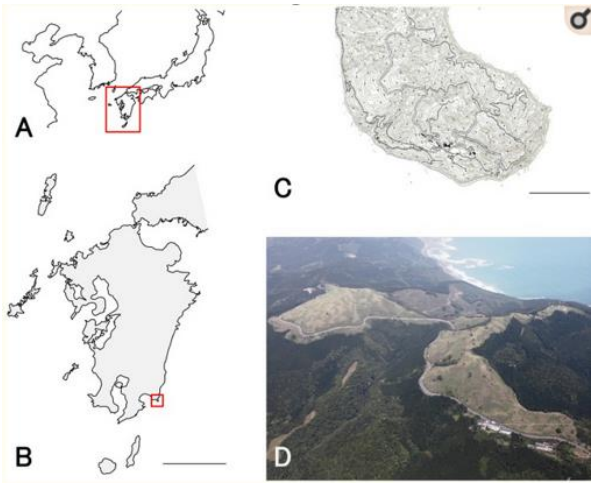


Figure 12 : Cape Toi. A wide area map (A), a map og Kyushu: southernmost of the four main islands of Japan, bar=100km (B), Digital map 25000 (Map image) of the cape, published by Geospatial Information Authority of Japan, bar=1km (C), and an aerial photograph of the cape (D). Kobayashi, I, et al., 2019.

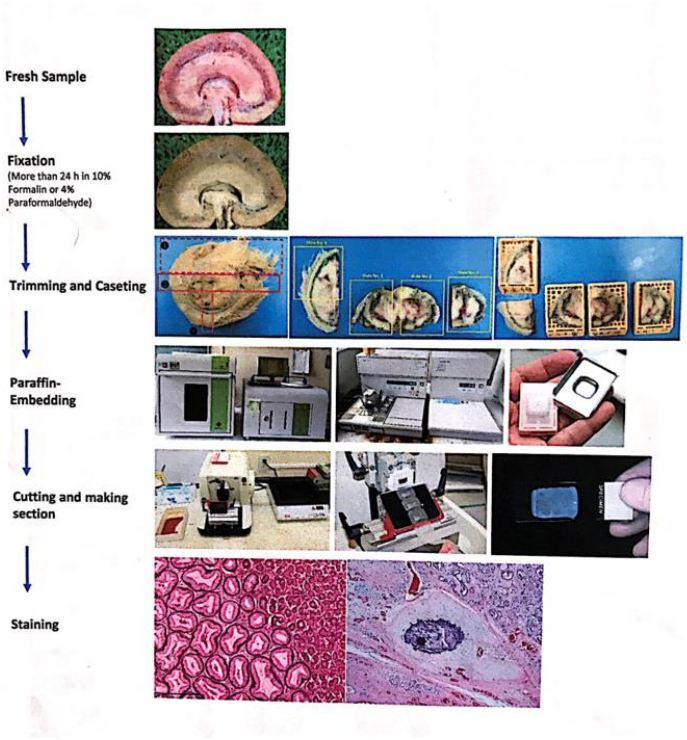


Figure 13: protocol used to make HE slides from fresh samples

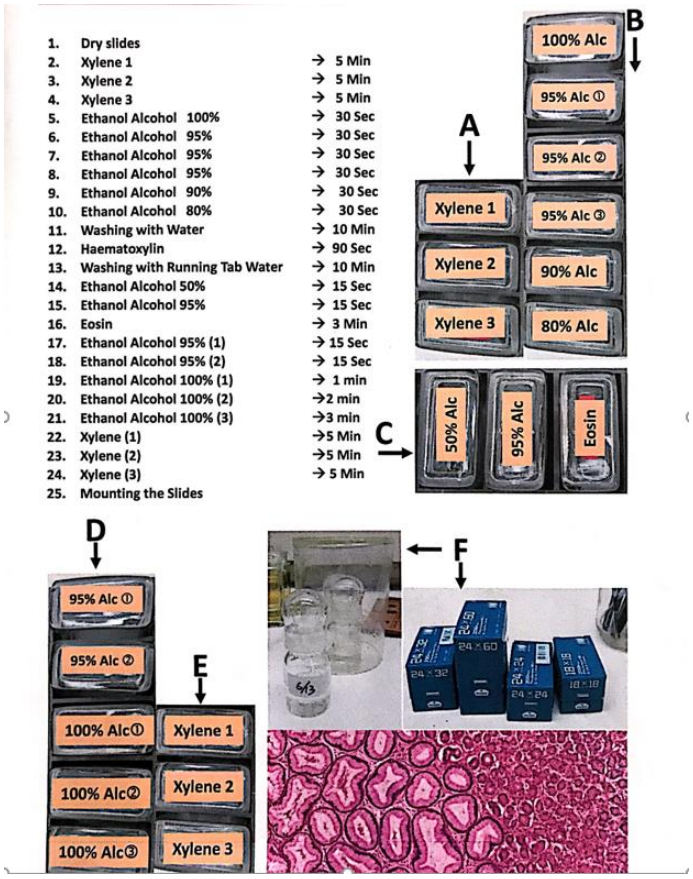


Figure 14: protocol used for HES staining.

	DNA extraction from gel	Purification of PCR products
Sample preparation	up to 300mg of gel 500µl of GP1 Vortexing 55℃ ; 10 - 15min Invert the tube	PCR products : Buffer GP1 = 1 : 5 (e.g. 40µl : 200µl) Vortexing
Sample loading	Load the sample onto the column 13,000rpm ; 30sec	Load the sample onto the column 13,000rpm ; 30sec
Membrane washing	600µl of GP2 13,000rpm ; 30sec] * * For TBE gels this wash step should be repeated.	600µl of GP2 13,000rpm ; 30sec
Membrane drying	13,000rpm ; 2min	13,000rpm ; 2min
Elution	20 - 50µl of GP3 2min at room temperature 13,000rpm ; 2min	20 - 50µl of GP3 2min at room temperature 13,000rpm ; 2min

Figure 15: protocol for DNA purification from PCR samples or Electrophorese gel samples before sending them for DNA sequencing.

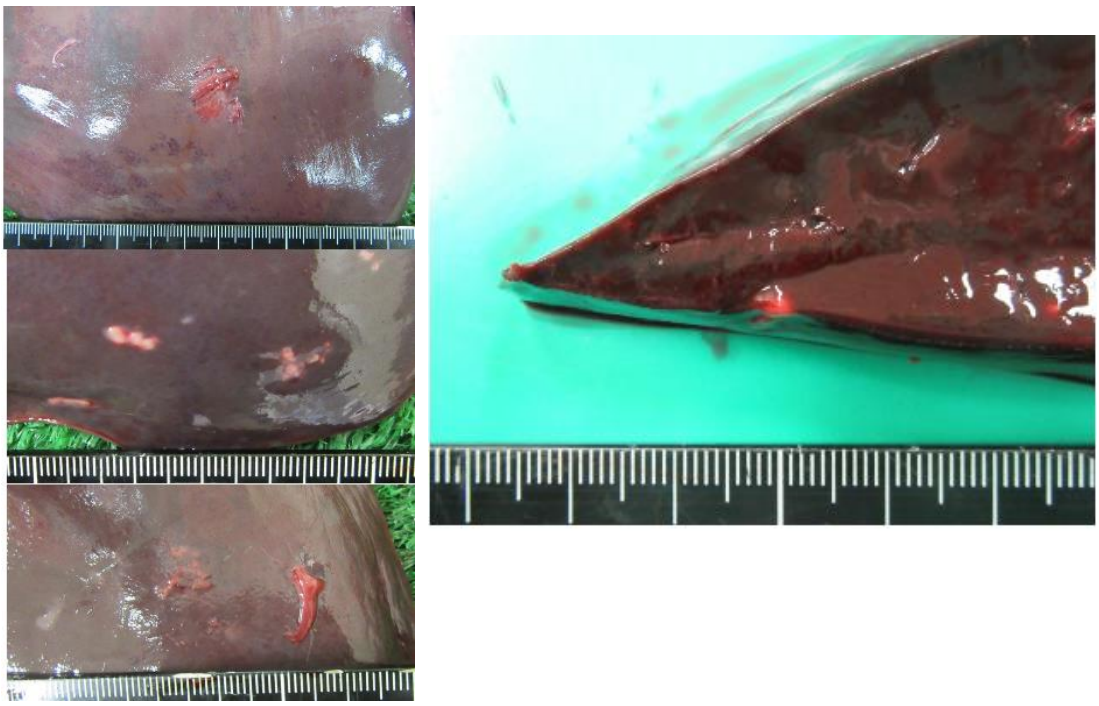


Figure 16: A19-40; Adhesions on the liver and white spots of 2-3 mm.

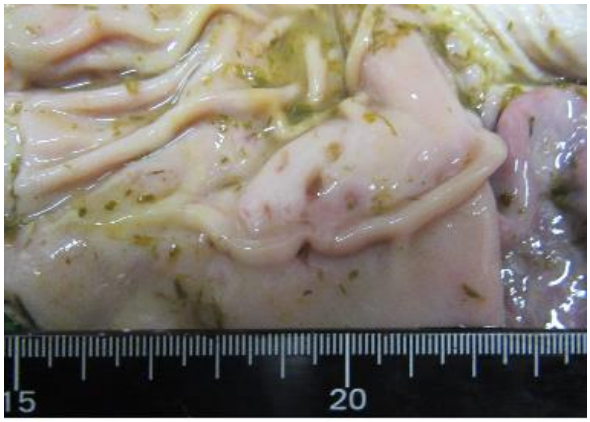


Figure 17: A19-40; Ulcers in the pyloric area of the stomach.

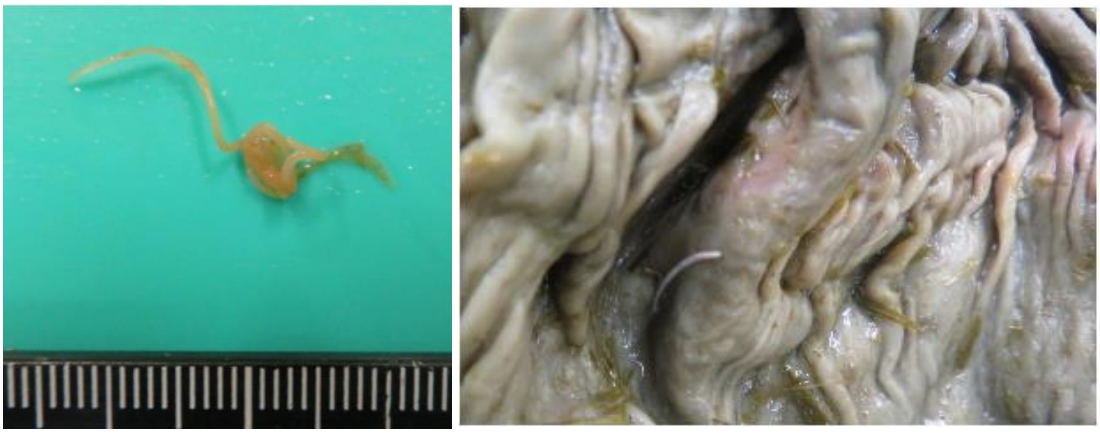


Figure 18: A19-40; Parasites found in the colon.

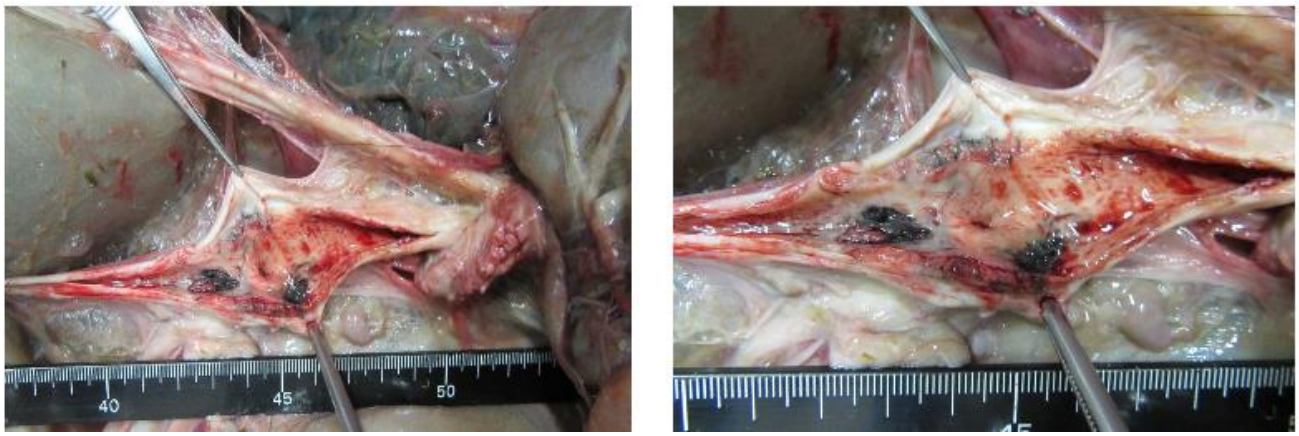


Figure 19: A19-40: Tickeness of the craial mesenteric artery and clot adherent to his lumen.

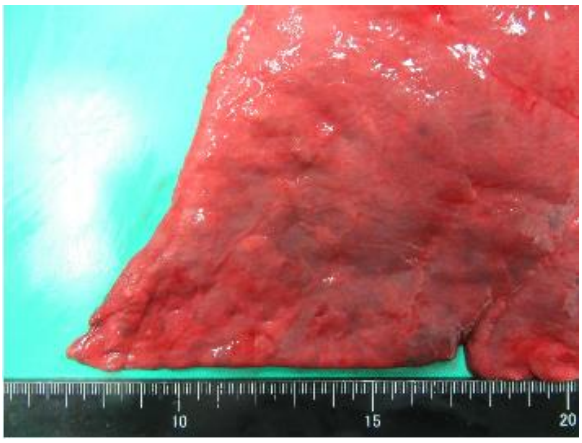


Figure 20: A19-40: Parasite-like path on the edge of a pulmonary lobe.

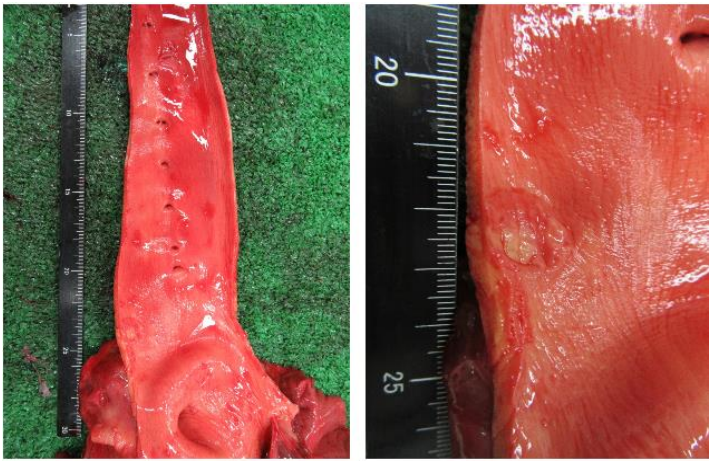


Figure 21: A19-40 Lesion of 1.5 cm in the aorta.

Figure 22: A19-41; Brown spots and fibrin on the serosa of the liver.

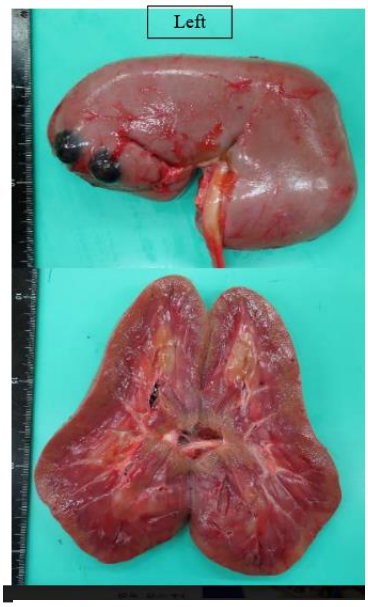


Figure 23: A19-41; Black cystic structure in the serosa of the left kidney

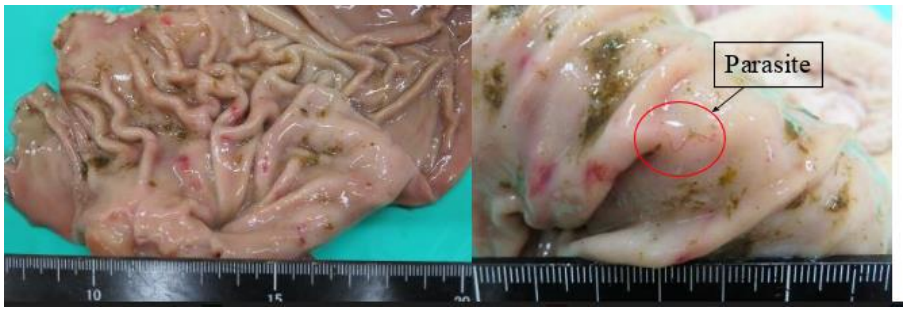


Figure 24: A19-41; petechial hemorrhages in the mucosa and presence of small red worms.

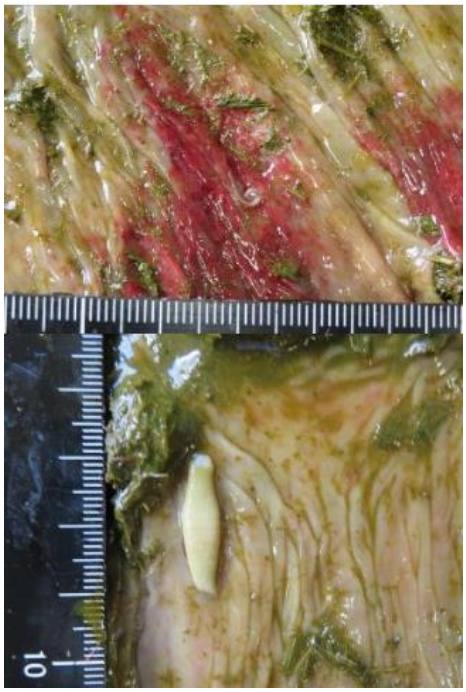


Figure 25: A19-41; White intestinal parasites were found in the colon.

Figure 26: A19-41: Pleural adhesion to the thoracic wall.

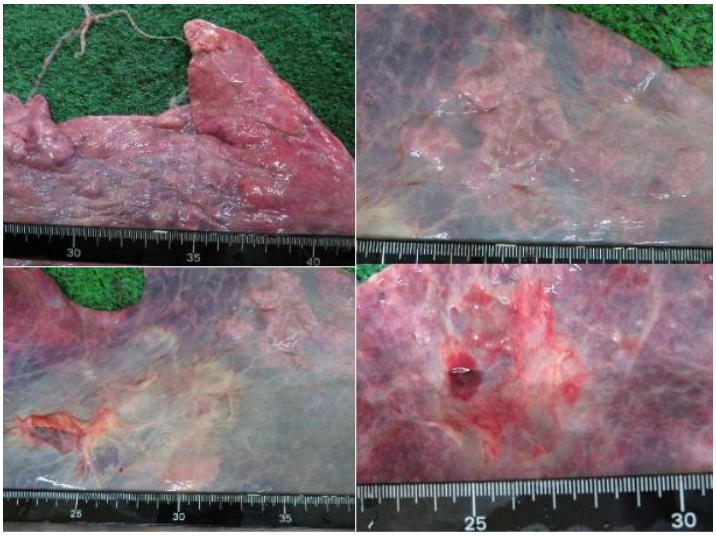


Figure 27: A19-41: fibrin covering the pulmonary serosa and multifocal white spots were found.

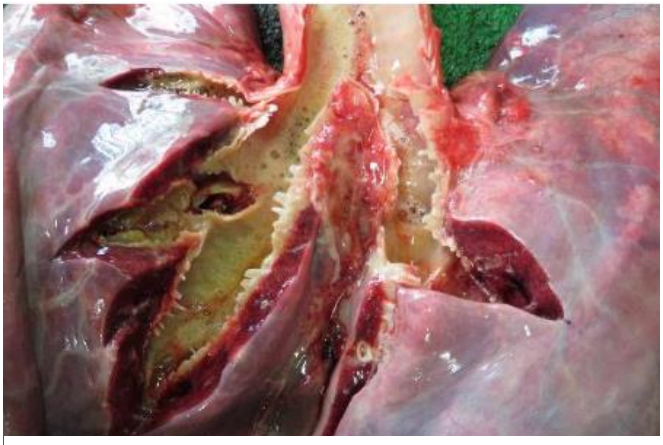


Figure 28: A19-41: Foamy content in the trachea.

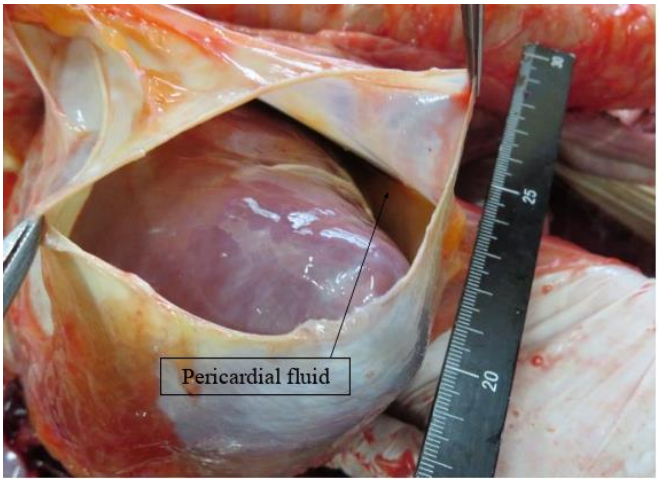


Figure 29: A19-41; pericardial effusion with a yellowish fluid.

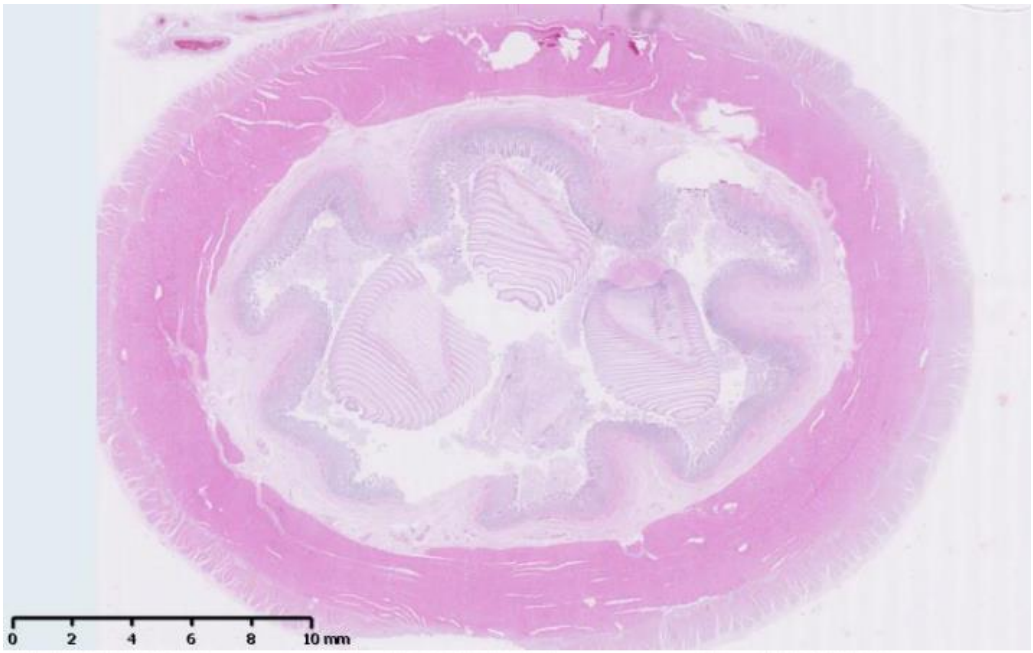


Figure 30: A19-41; Segments of proglottids of tapeworn in the ileum.

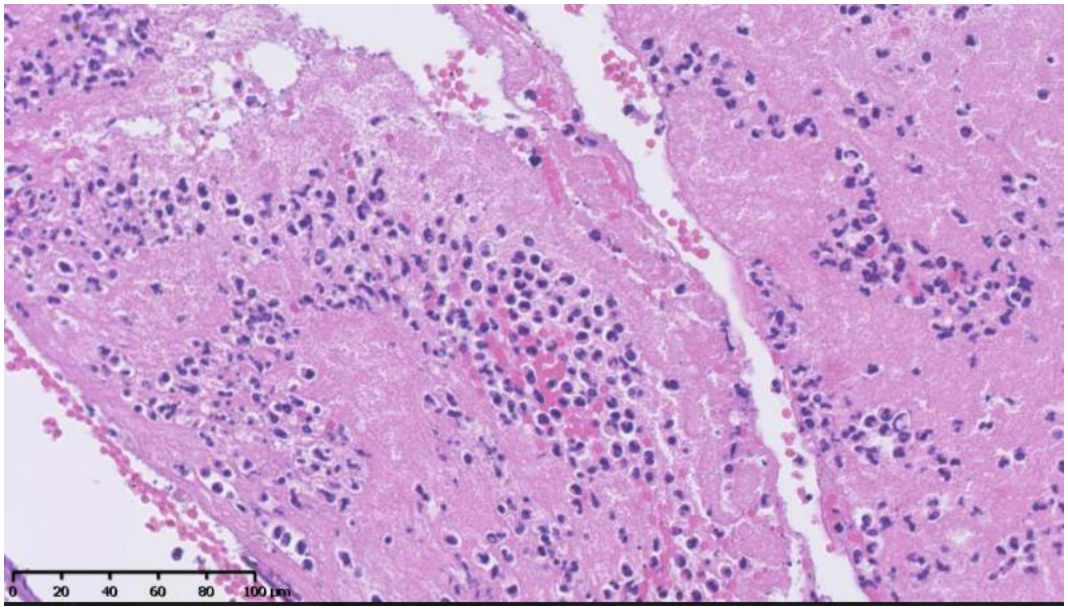


Figure 31: A19-41; Eosinophils, lymphocytes, neutrophils, and macrophages were found in the thrombus.

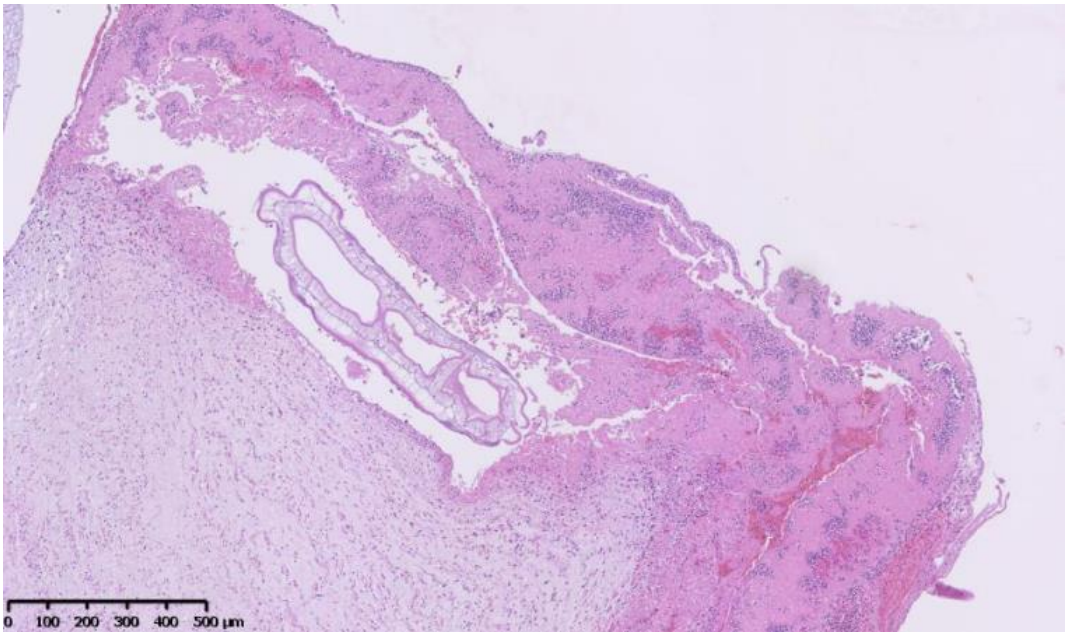


Figure 32: A19-41: presence of a Strongylus spp larva in the thrombus of the cranial mesenteric artery

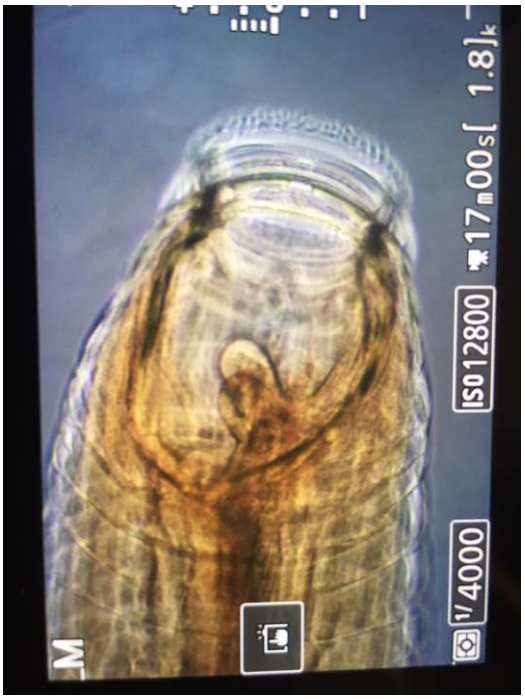
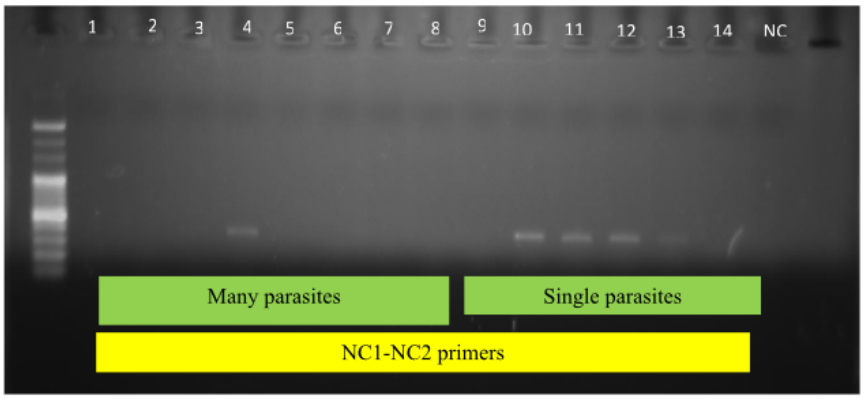


Figure 33: A19-40 : Parasites extracted from the colon with bilobed thoot and external leaf-crowns numerous and prominent. This parasite had the length (< 2cm) and the morphological characteristics of a *Strongylus vulgaris.*

Figure 34: A19-41: parasite extracted from the cecum.

Figure 35: A19-41: parasites extracted from the colon.

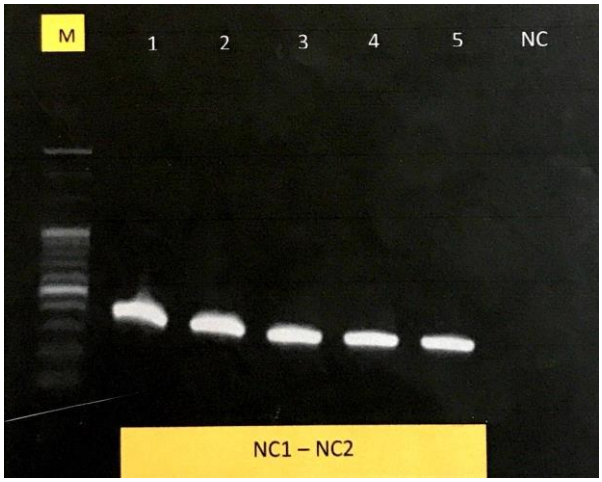
Figure 35: A19-41: parasites extracted from the colon.



Lane	Sample	Results	Remark
1	A19-40 colon	Negative	Extracted from many parasites
2	A19-40 colon formalin	Negative	
3	A19-40 cecum	Negative	
4	A19-41 colon 1	Positive	
5	A19-41 colon 2	Negative	
6	A19-41 cecum 1	Negative	
7	A19-41 cecum 2	Negative	
8	A19-41 Abdominal cavity	Negative	
9	A19-40 colon formalin	Negative	Extracted from single parasite
10	A19-40 colon	Positive	
11	A19-40 cecum	Positive	
12	A19-41 colon 1	Positive	
13	A19-41 cecum 2	Positive	
14	A19-41 Abdominal cavity	Negative	

Figure 37: PCR from the extraction of a single parasites or from many parasites from different samples of the cecum, colon and abdominal of A19-40 and A19-41 with Five positives results; A19-41 colon1, A19-40 colon,

.



Lanes	Samples	Results	Remark
1	A19-41 colon 1	Positive	Extracted from many parasites
2	A19-40 colon Real⊕?	Positive	Extracted from single parasite
3	A19-40 cecum	Positive	
4	A19-41 colon 1	Positive	
5	A19-41 colon 2	Positive	

Figure 38: The Five positives sample from A19-41 colon1, A19-40 colon, A19-40 cecum, A19-41 colon 1 and A19-41 cecum2 were used for the nested PCR

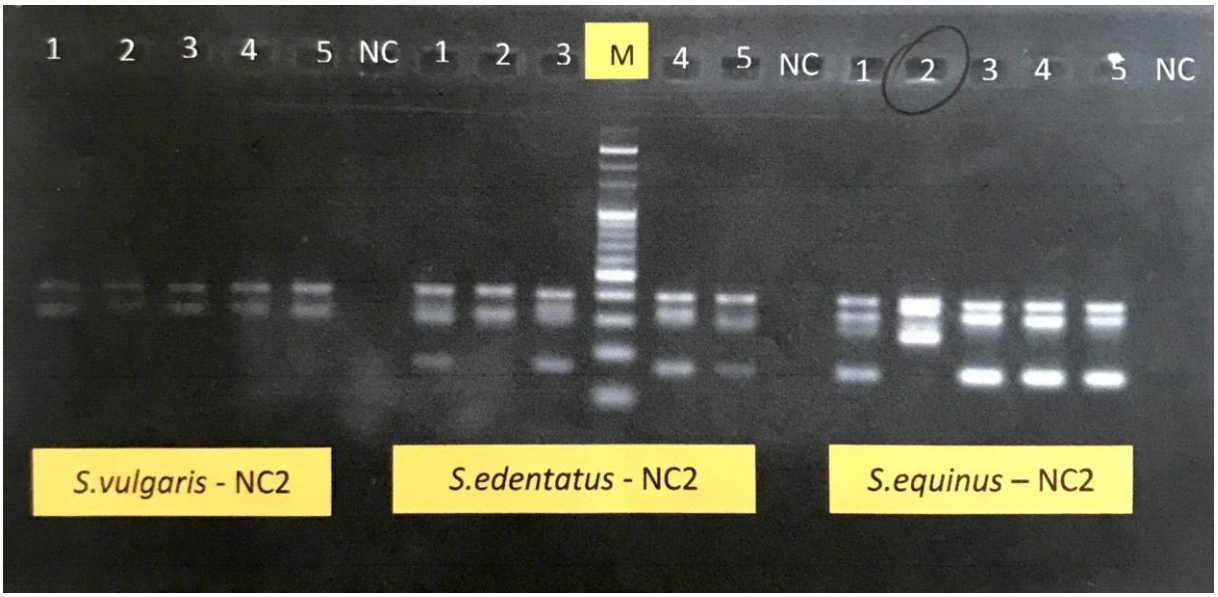


Figure 39: Each positive sample was amplified with the primers *S.vulgaris*-NC2, *S.edentatus*-NC2, *S.equinus*-NC2. The presence of many positives bands does not allow us to confirm or infirm the presence of *Strongylus spp.*

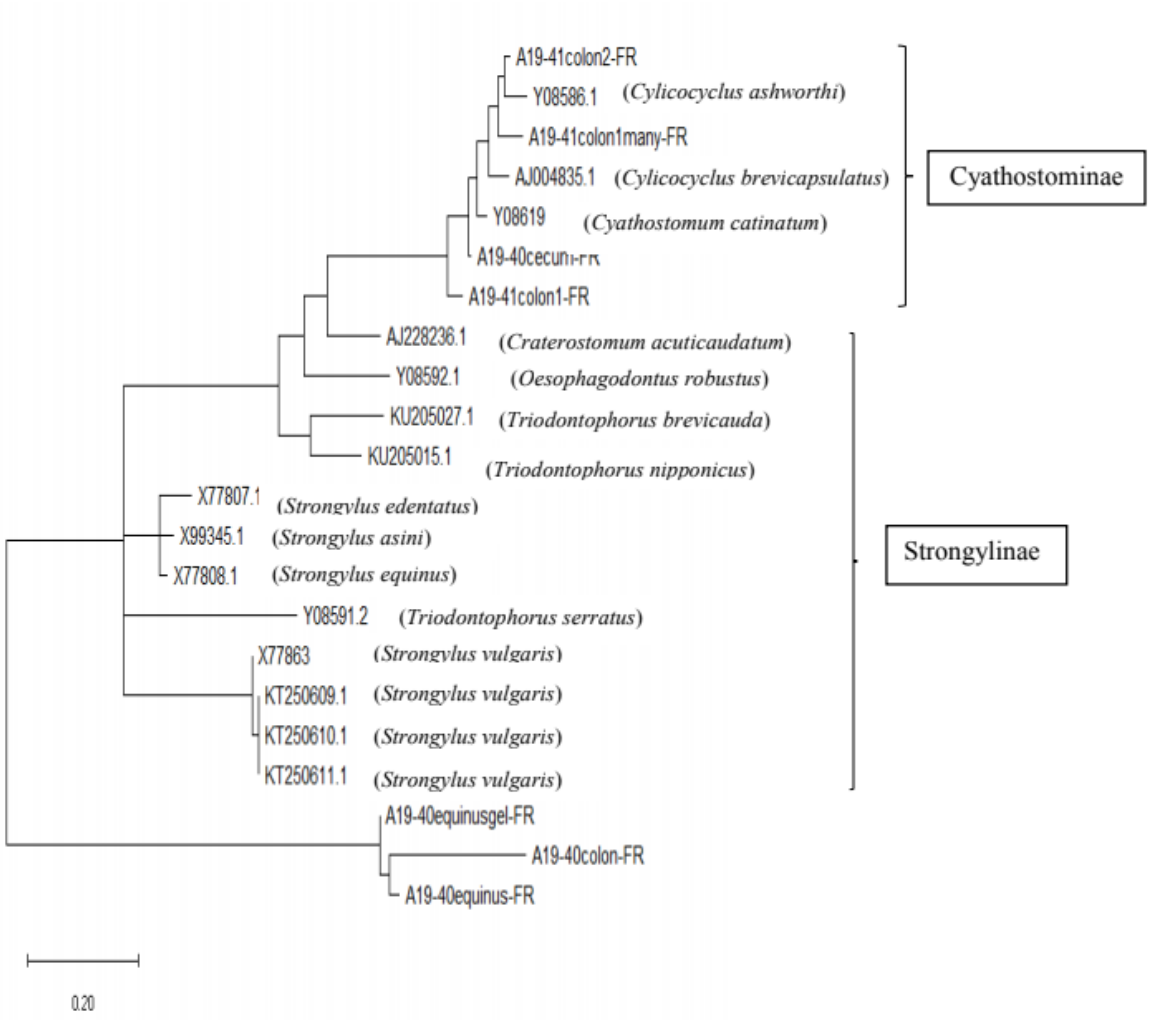


Figure 40: Phylogenetic tree of the five positives samples from the first PCR. A19-41colon2, A19-41colon1many, A19-40 cecum and A19-41 colon1 belong to the cyathostominae family. A19-40equinusgel, A19-40colon and A19-40equinus is closer to the Strongylinae family.

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