Species distribution of encysted cyathostome larvae and attribution of cyathostome species to larval cyathostominosis in the Netherlands

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Abstract

Cyathostomes are the most common nematodes of the horse and are pathogenic parasites in horses. Over 50 species of cyathostomes are known. These parasites can cause a disease called larval cyathostominosis. This disease is caused by the en masse emergence of encysted larvae from the mucosa. Symptoms of this disease are, amongst other things, diarrhea, losing weight, subcutaneous edema, the presence of fourth stage larvae in the faeces and death. Treatment of larval cyathostominosis can consist of deworming with moxidectin and supportive therapy.

Fourth stage larvae expelled with the faeces of horses with larval cyathostominosis were supposed to be isolated with the Baermann method and differentiated with reverse line blot (RLB). Also, third and fourth stage larvae from the (sub)mucosa of slaughtered horses and L3s cultured from the faeces of these horses were differentiated. The hypothesis is that these species match, since only species that can inhibit development are likely to be involved with larval cyathostominosis.

The Baermann method was tested for isolation of L4s from the faeces, but unfortunately no cases of larval cyathostominosis were used in this study. Numbers of larvae recovered after overnight storage at 4 °C were lower than samples that were used immediately. We sampled 11 slaughtered horses to count and differentiate mucosal larvae. Of these horses, mucosal samples were taken and faecal cultures were made to differentiate larvae that represent the adult cyathostomes in the lumen. In 27.3 percent of the horses, no cyathostomes were found in the (sub)mucosa, nor in the faecal cultures. Additionally, in 36.3 percent of the horses only cultured L3s were found and in 36.3 percent of the horses, cyathostomes were found in the (sub)mucosa and in the faecal cultures. The age of horses in which cyathostomes were found in the mucosa was significantly lower than the age of horses where no cyathostomes were found. The 5 most prevalent species in the faeces were *Cylicocyclus (Cyc.) nassatus, Coronocyclus (Cor.) coronatus, Cyc. ashworthi, Cyathostomum (Cya.) catinatum* and *Cyc. leptostomum.* They comprised 74 percent of the total amount of larvae recovered. The 5 most prevalent species in the mucosa were *Cyc. insigne, Cyc. nassatus, Cylicostephanus (Cys.) longibursatus, Cor. coronatus* and *Cya. catinatum*. They comprised 86.9 percent of the total amount of larvae recovered.

In conclusion, the Baermann method is useful for recovering L4s of the faeces when the faeces is fresh. Larvae from faeces that has been cooled or stored for more than a day should be recovered using direct microscopic examination of the faeces. As a conclusion for species distribution, there was a prominent difference in the finding of *Cyc. insigne* in the faeces and the mucosa, but more slaughtered horses should be sampled.

Introduction

Cyathostomes are pathogenic parasitic nematodes in horses and are the most common nematodes in equids, found all over the world (*Herd*, 1990).

Etiology

Cyathostomes, or small strongyles, belong to the family of the Strongylidae, subfamily Cyathostominae (*Lichtenfels et al*, 2008). Their size varies from 4 mm to 25 mm (*Taylor et al*, 2013), and they are about 1 mm in diameter (*Sellon and Long*, 2013). The adult worms are white, but the fourth stage larvae are red in color. This is why the cyathostomes are also called small bloodworms. Over 50 species are known, divided by *Lichtenfels et al* (2008) in 14 genera: *Cyathostomum, Coronocyclus, Cylicocyclus, Cylicodontophorus, Tridentoinfundibulum, Cylicostephanus, Skrjabinodentus, Petrovinema, Parapoteriostomum, Poteriostomum, Gyalocephalus, Hsiungia, Caballonema* and *Cylindropharynx*.

Another subfamily of the Strongylidae are the strongylinae or large strongyles (*Lichtenfels et al*, 2008). The large strongyles are somewhat bigger in size (1.5-4.5 cm) (*Reinemeyer and Nielsen,* 2013) and have deeper and more round-shaped buccal capsules. Therefore, the cyathostomes attach more weakly to the gut mucosa than the large strongyles do (*Sellon and Long*, 2013).

Epidemiology and pathology

A large part of the equine population is infected with cyathostomes (*Kornás et al*, 2010). Horses develop an incomplete immune response to cyathostomes (*Klei and Chapman*, 1999). This immunity develops slowly, so older horses are more immune to cyathostomes than younger horses. As a consequence, older horses are mostly still infected with cyathostomes, but they are less likely to develop clinical symptoms and their average faecal egg shedding is lower (*Kornás et al*, 2010).

Not much is known about the pathogenicity of individual cyathostome species. In general, cyathostome infections are associated with a rough coat, weight loss (*Smith*, 1976) and various kinds of colic (*Love et al*, 1999), like caecocaecal and caecocolic intussusception (*Mair et al*, 2000) and caecal tympany (*Murphy and Love*, 1997). Also a decrease in motility and intermitted diarrhea after experimental infection with cyathostomes is described (*Smith*, 1976, *Bueno et al*, 1979, *Murphy and Love*, 1997). It can also lead to a growth reduction in foals (*Murphy and Love*, 1997). There is no evidence that adult worms damage the colon and caecal mucosa. Lesions from feeding of the mucosa are not visible macroscopically (*Ogbourne*, 1978) and adult horses with high worm burdens can appear completely healthy. Encysted larval stages can lead to black or red nodules of a few millimeters in the mucosa (fig. 1), but clinical disease only occurs when a large number of larvae penetrate the mucosa simultaneously (see ‘larval cyathostominosis’).

In contrast to large strongyles, the cyathostomes do not migrate through the body. This means that no symptoms caused by migration are present with cyathostome infections.

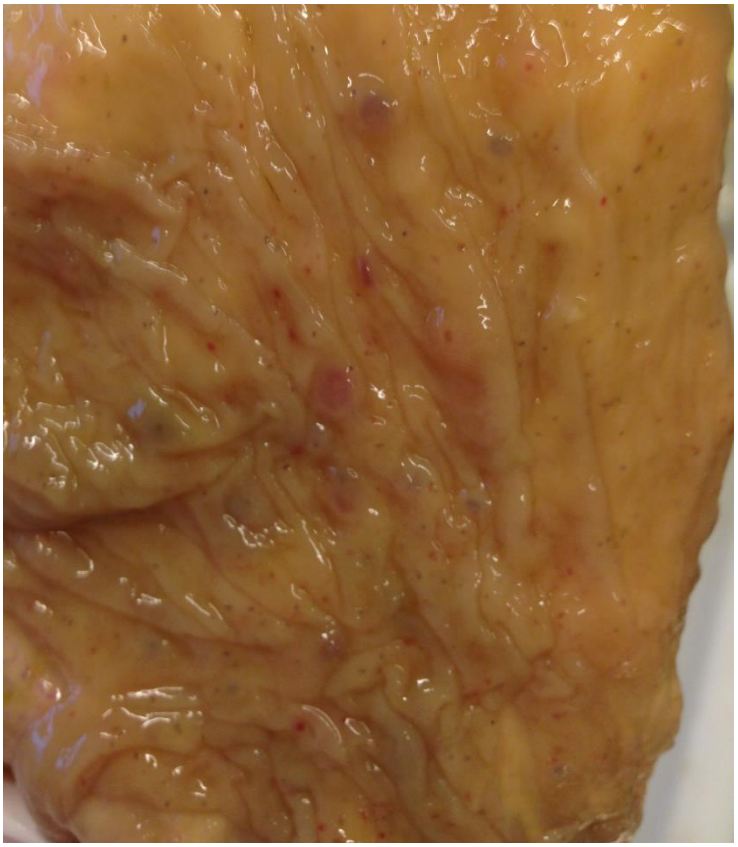

Figure 1: Nodules in the intestinal mucosa, caused by encysted larvae

Life cycle

Cyathostomes have a direct life cycle. The life cycles of all cyathostomes are comparable to each other (fig. 2).

The cycle begins with the ingestion of the infective third stage larvae (L3). After ingestion, the L3s exsheath in the small intestine and are then called early L3s (EL3). The EL3s invade the mucosa or submucosa (depending on the species) of the colon or caecum. The EL3 becomes encysted when a fibrous capsule of host tissue forms around the invaded larva (*Reinemeyer and Nielsen*, 2013).

The encysted EL3s can either develop further immediately or inhibit their development for over 2 years (*Eysker et al*, 1984, *Love et al*, 1999). Larvae taken up in late summer and autumn are most likely to inhibit their development (*Ogbourne*, 1978). The EL3 develops to a late L3 (LL3) and then an early L4 (EL4) stage. The EL4s break out of their cysts (excystment) and develop further to an adult stage in the gut lumen. Female adults produce eggs, which are shed with the faeces. The pre-patent period can be as short as 5-6 weeks (*Round*, 1969) but in larger species development can take 2 to 3 months (*Eysker et al*, 2008).

First stage larva (L1) hatch from eggs excreted with the faeces. The L1s develop to a second stage and later to the infective third stage. The first two stages feed on bacteria, but the cyathostome L3s are sheathed and thus live from food reserves (*Ogbourne*, 1978). The L3s migrate out of the faeces onto the grass. This is only possible in a layer of moisture. The process from egg to L3s migrated onto the grass can take 3 days under ideal circumstances

(*Reinemeyer and Nielsen*, 2013), but low temperatures can slow down or even arrest the development of egg to L3. Drought can also lengthen the process, since L3s cannot migrate without moisture (*Eysker et al*, 2008).

On the pasture, L3s can survive for several months in summer and even longer when it is cold. After surviving the winter, the L3s regain their activity and use up their food reserves until they die. Even though the number of L3s will diminish, this can still be a source of new infections in spring. A source of reinfection is the resumed development of inhibited larvae (*Eysker et al*, 2008).

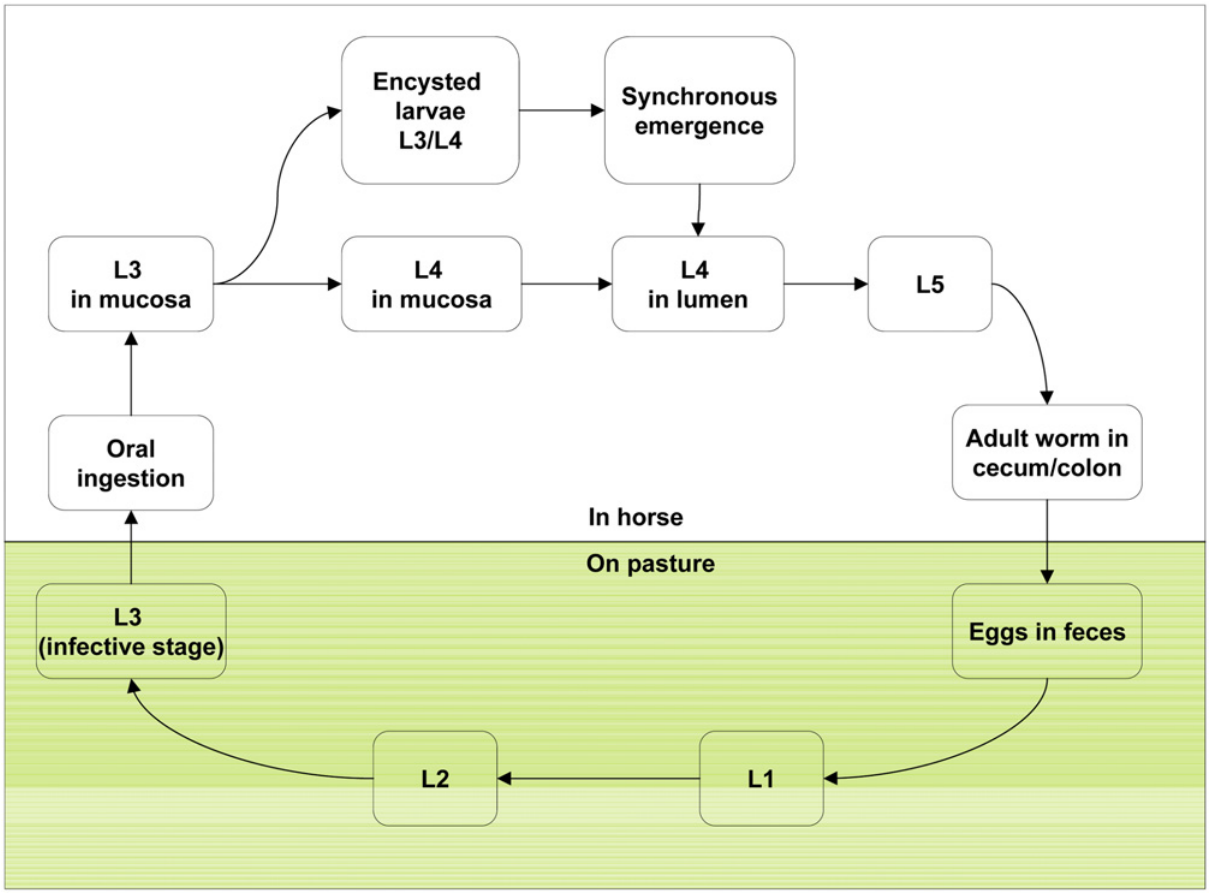

Figure 2: Life cycle of cyathostomes (*Corning*, 2009)

Diagnosis

It is not possible to determine the worm burden in a horse based on faecal egg counts (FEC) or faecal larval counts, since this only represents the luminal fraction of the worm burden (*Andersen et al*, 2013) and the encysted stages can represent up to 90 percent of the total worm burden (*Eysker et al*, 1984, *Eysker and Mirck*, 1986). Additionally, *Nielsen et al* (2010) stated that there is no direct linear correlation between the FEC or faecal larval count numbers and the luminal worm burden, although there was a correlation in lower EPGs: an EPG below 500 corresponded with significantly lower luminal worm counts than higher EPGs.

It is not yet possible to determine the encysted worm burden. However, a specific antigen for larval cyathostome stages is found: cyathostomin gut-associated larval antigen-1 (cy-GALA-1) (*Andersen et al*, 2013). This cy-GALA-1 can be bound by a specific serum IgG(T) in the

horse and this anti-rCy-GALA-1 serum IgG(T) has a significant positive correlation with the cyathostome burden in naturally infected horses. If a diagnostic ELISA for anti-rCy-GALA-1 can be developed, it may be possible to quantify the encysted cyathostome burden in the future.

Therapy and prevention

Most horses will be treated with anthelmintics to reduce faecal egg shedding and thus reduce the infection pressure of the pasture. There are multiple ways to determine whether a horse should be dewormed or not. The first is to treat based on egg reappearance period (ERP) (*Matthews,* 2014). This is the period after deworming with a certain anthelminthic until new eggs are found in the faeces. This was a successful way of controlling infection and disease, until cyathostomes developed resistance to multiple anthelmintics (*Kaplan,* 2004). To reduce the development of resistance, a different approach was used: selective therapy. A FEC is performed to determine the eggs per gram (EPG). Mostly, horses will be dewormed when the EPG is higher than 200 (*Nielsen et al,* 2014), but this so-called cut-off value can also be higher or lower, dependent on the country and the herd. Using this method, it has been discovered that the amount of eggs shed by an adult horse is consistent over time (*Gomez and Giorgi,* 1991), and that approximately 20 percent of the horses in a herd are high shedders (*Nielsen et al,* 2014). This means that a large amount of the adult horses can be left untreated.

In the Netherlands, there are three groups of anthelmintics available and effective against cyathostomes: the benzimidazoles, the tetrahydropyrimidines and the macrocyclic lactones. In a large part of the world, including the Netherlands, resistance against benzimidazoles and tetrahydropyrimidines has been reported (*Matthews,* 2014). The first signs of resistance against the macrocyclic lactones are also present: a reduced ERP (*Matthews*, 2014). There are two macrocyclic lactones registered for horses in the Netherlands. Ivermectin is effective against luminal stages and moxidectin is effective against luminal and mucosal L4 stages (*Eysker et al,* 2008).

Apart from anthelmintic treatment, there are other control measures. The herd is the main factor in the spread of infectious larvae, so herd management is an important aspect of these control measures. It is important to remove faeces from the pasture regularly to prevent infective larvae to develop. Once or twice a week is recommended, since L3s can develop in 3 days (*Reinemeyer and Nielsen,* 2013). It is also important to prevent overgrazing, since overgrazing can greatly increase the infective pressure of larvae on the pasture (*Reinemeyer and Nielsen,* 2013). Pasture rotation can be performed to reduce the amount of L3s on the pasture (*Reinemeyer and Nielsen,* 2013). Another method to reduce the infectivity of the pasture with cyathostome larvae is alternated grazing with ruminants (*Matthews,* 2014). These ruminants ingest the L3s, but they are not suitable as a host for cyathostomes and thus these L3s will not develop further to produce new eggs. Care should be taken for helminths of these ruminants that can be infective for horses, like *Fasciola hepatica.* Lastly, when introducing a new horse to the herd, a FEC should be performed. If necessary, treat the horse with ivermectin or moxidectin and keep the horse separated. At 0 and 14 days after treatment a FEC should be performed to check if the treatment has worked (*Reinemeyer and Nielsen,* 2013). The reduction in FEC at 14 days should be 95 percent or more.

Larval cyathostominosis

Larval cyathostominosis is a disease caused by either the grazing of the mucosa by adult worms, the mucosal penetration of ingested L3s or the excystment of L4s. Excystment of these L4s can occur en masse in winter or spring, resulting in a seriously damaged gut wall (fig. 3). This disease mainly occurs in horses under the age of 5 (*Love et al*, 1999) and has often been reported in the Netherlands and the rest of Europe (*Ogbourne*, 1976, *Mirck*, 1977, *Giles et al*, 1985, *Love et al*, 1992, *Van Loon et al*, 1995, *Murphy et al*, 1997, *Smets et al*, 1999, *Roumen et al*, 2004).

Inflammation of the gut wall, caused by excystment of L4s, is mainly seen at necropsy as edema and hemorrhage of the gut wall, congestion, ulceration and necrosis. This inflammation and the damage due to excystment results in a protein losing enteropathy. Microscopically, an infiltration of lymphocytes, plasma cells, eosinophils and macrophages can be seen (*Love et al*, 1999, *Peregrine et al*, 2005). This cellular response can be either diffuse or focused around the encapsulated larvae. Furthermore, a fibrous capsule around encysted larvae, focal areas in the submucosa of hemorrhage with infiltrated eosinophils, dilated submucosal lymphatics and variable epithelial shedding can be present (*Love et al*, 1999, *Corning*, 2009).

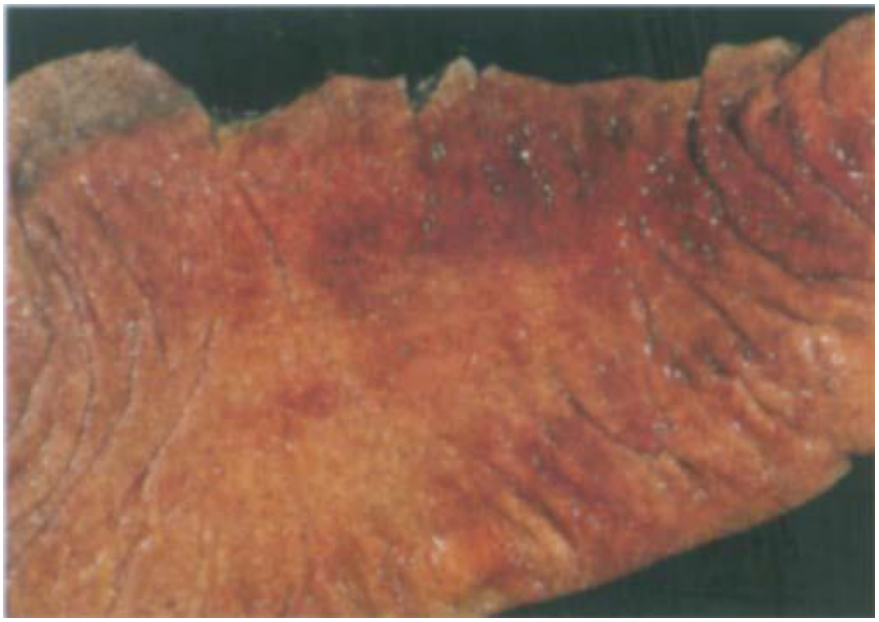

Figure 3: Colitis associated with larval cyathostominosis (*Murphy et al*, 1997)


Figure 4: Weight loss in a horse with larval cyathostominosis (*Murphy et al*, 1997)

The diagnosis of larval cyathostominosis in a horse should not be based on faecal examination alone. L4s can be present in the faeces of healthy horses, and FECs can be very low when a horse is suffering from this disease, because the adult cyathostomes are washed out of the gut lumen with the diarrhea (*Murphy et al,* 1997). *Smets et al* (1999) has proven that the combination of weight loss, diarrhea and the presence of L4s in the faeces (fig. 5) are a good indication of the disease. Furthermore, blood analysis can be done. Horses with larval cyathostominosis can have a hypoalbuminemia (less than 20 g/L) and a hyperglobulinemia, with a beta-globulin concentration of 21 per cent or higher and an albumin:globulin ratio of less than 0.7 (*Smets et al,* 1999). Total serum protein can be either low due to the protein losing enteropathy or high, possibly due to dehydration (*Corning,* 2009). Furthermore, a neutrophilia, eosinophilia, lymphocytosis and anemia can be present (*Love et al,* 1999, *Corning,* 2009). It must be noted that these blood findings are not pathognomonic.

To reduce the larval burden, an anthelmintic treatment is indicated. There are two anthelmintics that in general are effective against encysted larvae: fenbendazole and moxidectin (*Peregrine et al*, 2006, *Corning,* 2009, *Reinemeyer and Nielsen,* 2013). As mentioned earlier, fenbendazole (a benzimidazole) cannot be used because of the widespread resistance against this anthelmintic. Another advantage of moxidectin above fenbendazole is that there is only minimal inflammation of the gut after treatment with moxidectin (*Reinemeyer et al,* 2013). Moxidectin can be given as a single dose of 400 µg/kg (*Corning,* 2009, *Reinemeyer et al,* 2013). Nevertheless, it is advisable to administer anti-inflammatory drugs like corticosteroids in combination with moxidectin (*Roumen et al,* 2004).
Apart from anthelmintic treatment, supportive therapy is necessary in severe cases. Because of the protein losing enteropathy, intravenous fluid therapy with electrolytes is indicated (*Deprez and Vercruysse,* 2003, *Peregrine et al,* 2006) It may also be necessary to give nutritional support in the form of protein-rich food (*Peregrine et al,* 2006). To reduce the symptoms of diarrhea, codeine phosphate can be given orally (*Love,* 2003), although this is not registered for horses.

Larval cyathostominosis cannot be prevented by regular deworming. It is possible though to reduce the risk of this disease by controlling the infectivity of the pasture with cyathostome larvae and thus reduce the intake of infective larvae.

Figure 5: Cyathostome larvae (L4) in the faeces

Distribution of adult and larval cyathostomes

Of all cyathostome species, only 10 species are abundant (*Ogbourne,* 1978*). Cys. longibursatus, Cys. goldi, Cys. calicatus, Cya. catinatum, Cor. coronatus, Cys. nassatus* and *Cys. minutus* are the most frequently reported species (*Reinemeyer et al*, 1984, *Krecek et al*, 1989, *Mfitilodze et al*, 1990, *Gawor*, 1995, *Silva et al*, 1999, *Collobert-Laugier et al*, 2002, *Stancampiano et al*, 2010). A single horse can harbor many different species. Up to 29 species per horse have been reported (*Chapman et al*, 2003). The percentage of horses infected with cyathostomes ranged from 28% (*Stancampiano et al*, 2010) to 100% (*Reinemeyer et al*, 1984), and the total worm burden varied greatly, from a few hundreds to over 600,000 cyathostomes per horse (*Ogbourne*, 1976, *Krecek et al*, 1989, *Mfitilodze et al*, 1990, *Gawor*, 1995, *Collobert-Laugier et al*, 2002).

In the first part of the 20th century, the first association between cyathostome infection and clinical disease was made (*Ogbourne*, 1978). Since then, many studies on distribution and prevalence all over the world have been performed. With slight differences in species distribution, it has become clear that the predominant species per country are quite similar. Most luminal cyathostomes colonize the ventral colon, less colonize the caecum and the least cyathostomes colonize the dorsal colon (*Reinemeyer et al*, 1984, *Reinemeyer et al*, 1988, *Gawor*, 1995, *Collobert-Laugier et al*, 2002, *Stancampiano et al*, 2010). Some species have a preference for one of these intestinal parts. *Cys. nassatus, Cys. minutus, Cys. calicatus, Cya. catinatum* and *Cor. labratus* are mainly localized in the ventral colon, while *Cor. coronatus, Cyc. elongates* and *Petrovinema (Pet) poculatum* are mainly found in the caecum and *Cyc. insigne, Cys. goldi* and *Cys. longibursatus* in the dorsal colon (*Ogbourne et al*, 1976, *Krecek et al*, 1989, *Gawor*, 1995, *Collobert-Laugier et al*, 2002, *Stancampiano et al*, 2010).

Not much is known about the distribution of the larval stages, since the larvae cannot be differentiated morphologically into species. However, multiple larval counts have been performed and this has shown that most encysted larvae are located in the caecum, followed by the ventral colon and the dorsal colon (*Reinemeyer et al*, 1988, *Collobert-Laugier et al*, 2002, *Stancampiano et al*, 2010, *Scháňková et al*, 2014).

In 1997, *Hung et al* developed a different approach for differentiation. This method was based on earlier studies, that had proven that Strongylid species could be differentiated by differences in the sequence of their internal transcribed spacer (ITS)-1 and ITS-2 of ribosomal DNA (rDNA) (*Campbell et al*, 1995, *Chilton et al*, 1995). *Hung et al* (1997) compared DNA-sequences of ITS-1 and ITS-2 and of the 5.8S gene of three species (*Cys. nassatus, Cyc. ashworti* and *Cyc. insigne*) and discovered that each species had differences in the ITS sequence, so that differentiation was possible.
Kaye et al (1998) used the 26S-18S rDNA region, which is called the intergenic spacer region (IGS), for comparative sequencing. In this study, the IGS region from 16 cyathostome species were sequenced. These sequences can be used for the development of a PCR assay to differentiate cyathostomes.
A few years later, *Hodgkinson et al* (2003) developed 6 species-specific DNA probes based on the IGS regions given by *Kaye et al* (1998). These DNA probes could be used to detect IGS region sequences in a PCR-ELISA. *Traversa et al* (2007) developed a reverse line blot (RLB) assay to identify cyathostome species. This RLB assay is based on hybridization of an immobilized species specific probe with an amplified fragment of the IGS region. With this RLB, 40 larval samples can be used simultaneously when using a miniblotter, making this method faster and also cheaper than the PCR-ELISA used by *Hogdkinson et al* (2003). Initially, 13 cyathostome species could be identified with this RLB assay (*Traversa et al*,

2007). Nowadays, the differentiation of eggs and larvae for 21 species can be done by RLB assay (*Cwiklinski et al.*, 2012). The RLB assay has been used by *Čerňanská et al*, 2009, *Ionita et al*, 2010 and *Van Doorn et al* (2010), but has not yet been used for horses with larval cyathostominosis.

In 2009, *Kornás et al* developed a system for morphometric identification of cyathostome larvae. This method is based on a combination of measurements (body length with sheath, body width, distance to nerve ring, esophagus length, intestine length). However, with this method only 63 percent of the larvae were identified correctly. Since the RLB differentiates based on sequence differences and the probes have been validated with adult worms, the accuracy is higher. Therefore, the RLB is used in the present study.

The aims of this study are to determine how encysted cyathostome species are distributed in the colon and caecal mucosa and which cyathostome species are involved in larval cyathostominosis. We want to achieve this last aim by I) differentiation of the L4s expelled by horses with cyathostominosis and by II) differentiation of the inhibited L3s and encysted L4s from the (sub)mucosa of slaughtered horses at a commercial slaughterhouse. We want to compare the species found in horses with cyathostominosis with the species found in the (sub)mucosa of slaughtered horses.

The cause of larval cyathostominosis being the en masse emergence of L4 implies that species that do not inhibit their development are less likely to cause serious cyathostominosis. Thus, the hypothesis is that species involved in larval cyathostominosis are the same as the L3s found in the gut mucosa of slaughtered horses.

Materials & Methods

Collection of L4s from horses with larval cyathostominosis

The criteria for horses with larval cyathostominosis were: diarrhea, losing weight and the presence of L4s in the faeces. To include a horse with larval cyathostominosis in this study, the horse should not be treated with anthelmintics for at least 6 weeks prior to sampling.

L4s were collected from freshly dropped faeces of horses with larval cyathostominosis. Ten gram of faeces should then be sieved through a 63 µm sieve and the residue examined for L4s by using a dissecting microscope. L4s were collected using a small hook.

Another method to collect L4s is the Baermann technique. This method is based on *Olsen et al,* 2003. Freshly dropped faeces was collected of horses with larval cyathostominosis. Ten g faeces was weighed and a Baermann glass was filled with 0.9% NaCl. The faeces was placed in the center of a single gauze layer and the gauze was folded around the faeces. The free ends of the gauze were attached to a wooden spill with a paperclip and the spill was placed over the glass, so the faeces was immersed in saline (if not so, the glass was filled up until it was). This was maintained either at room temperature or 37 °C for 24 hours. After that, the sediment was aspirated with a pipette and transferred to a Petri dish. The larvae were counted with a microscope.

To validate this method, an experiment was performed in which the Bearmann technique and microscopic examination were compared. In this experiment, contents of the colon/caecum with living larvae were used, because we did not have faecal samples with living L4s. Since luminal contents also contain adult cyathostomes, the worms found in the sediment were divided in adults and larvae by looking at the buccal capsule and the tail. Only the larvae were counted. The experiment was repeated with samples that were maintained overnight at 4 °C to check if faecal samples could be preserved for 1 day after collection, and all samples were duplicated.

Population of the slaughtered horses

A commercial slaughterhouse was visited between January and March during 3 visits. In total, 11 horses were sampled. The first two visits were the beginning and the end of January (horses 1-8) and the last visit was in the beginning of March (horses 9-11). The horses were not selected based on age, gender, breed or any other feature. Of these horses, the only information available was age, breed and gender, thus nothing was known about deworming history or housing conditions.

Sampling the (sub)mucosa of the colon and caecum and the faeces of horses at the slaughter house

The caecum was located by finding the caecum point. The dorsal and ventral colon were located by finding the pelvic flexure and counting the taeniae. The caecum and colon were opened by a longitudinal incision and the contents were partially removed. Then, two 20x20cm samples of the caecum, dorsal colon and ventral colon were cut out. Faeces was collected from the rectal area for culturing of L3s.

Extraction of larvae from the (sub)mucosa of the colon and caecum

The mucosa and submucosa were washed with 0.9% NaCl and separated from the muscular layer and serosa by scraping with a knife. 100 ml of 0.9% NaCl with 2 mM EDTA was added to 50 gram of (sub)mucosa and processed in the Stomacher for 40 minutes (this is based on *Glover et al*. (2009)). The EDTA was added to the 0,9% NaCl to bind magnesium and thus prevent DNA to be degraded by DNAses. To fixate the tissue after processing in the Stomacher, 96% ethanol was added until a concentration of 70% ethanol was reached. The fixated tissue was stored until further use.

The fixated tissue was sieved through a 63µm sieve onto a 20µm sieve with 0,9% NaCl and larvae were collected from the residue on both sieves using a pipette for L3s and a small hook for L4s. The residues were fixated with 70% ethanol. Of each horse, 40 L4s and 40 L3s (or all larvae in 50 g mucosa if there were less than 40) were collected from the ventral colon, dorsal colon and caecum for RLB. Larvae were counted in either 10 or 50 g mucosa. Ten g mucosa was counted in horses with many larvae (more than 40 per 10 g) and in all the other horses, all 50 g that was processed in the Stomacher was counted. Larvae were stored at -80 ºC until further use.

Culturing of L3s from eggs

This method is based on *Van Doorn et al.* (2010). Rectally acquired faecal samples with added sawdust were cultured in glass jars with plastic lids for 10 days at 26 ºC. After culturing, the jars were filled with lukewarm tap water and turned upside down on a Petri dish (also filled with tap water). The following day, the water in the Petri dish with L3s in it was collected and stored at 4 ºC until further use. L3s were counted and the amount of larvae per gram faeces was calculated. Of each horse, 40 L3s (when available) were collected for RLB.

DNA extraction of the larvae

This method is based on *Van Doorn et al.* (2010). To each L3, 25 µl of Worm Lysis Buffer (WLB)/Proteinase K mixture was added. To each L4, 50 µl of this mixture was added. The composition of this mixture can be found in the Appendix.
Larvae with the added mixture were frozen for 15 minutes at -80 ºC. Then, the samples were thawed and spun and then incubated at 56 ºC overnight. The next morning, the lysed samples were heated at 95 ºC for 15 minutes to inactivate proteinase K.

Amplification of the inter genic spacer (IGS)

This method is based on *Traversa et al.* (2007) and *Van Doorn et al*. (2010). A polymerase chain reaction (PCR) was performed to amplify the IGS. The primers used were CY1 (5'-GGTCAAGGTGTTGTATCCAGTAGAG-3') and CY18 with biotin (5'-CTTAGACATGCATGGCTTAATC-3'). Precisely 22.5 µl of PCR mixture (see Appendix for substance composition) was added to 2.5 µl of lysed L3 or L4. A positive and negative control is included, with the positive control being a mixture of a large batch of L3s of many species and the negative control being the WLB/Proteinase K mixture.

The PCR was performed in a thermal cycler (model 2720, Applied Biosystems or model C1000, BioRad). The following cycle was used: 3 minutes at 95°C and 35 cycles at 95°C for 30 seconds, min, 60°C for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes.

To check for PCR-products, gel-electrophoresis was used. An agarose gel was used, stained with Midori green. The gel was run for 30-60 minutes at 7.5 volt per centimeter. The PCR products were then made visible by a molecular imager (BioRad Gel Doc XR+ System).

RLB

This method is based on *Traversa et al.* (2007) and *Van Doorn et al.* (2010).
RLB is performed to determine the species of collected cyathostome larvae. RLB-probes are made available for the following 21 cyathostome species: *Coronocyclus (Cor.) coronatus, Cor. labiatus, Cor. labratus, Cyathostomum (Cya.) catinatum, Cya. pateratum, Cya. tetracanthum, Cylicocyclus (Cyc.) ashworthi, Cyc. auriculatus, Cyc. bidentatus, Cyc. insigne, Cyc. leptostomum, Cyc. nassatus, Cyc. radiatus, Cyd. bicoronatus, Cylicostephanus (Cys.) calicatus, Cys. goldi, Cys. longibursatus, Cys. minutus A &B, Parapoteriostomum (Para.) mettami, Poteriostomum (Pot.) imparidentatum, Tridentoinfundibulum (Tri.) gobi*. The probe sequences are given by *Cwiklinski et al.* (2012).

A Biodyne© membrane, 0.45 µm with coupled probes was used (see Appendix for coupling probes) to hybridize the PCR products with the probes. First, 40 PCR-products were diluted: 10 µl PCR-product in 150 µl buffer (2x SPPE/0.1% SDS). The diluted PCR-products were denatured at 100 ºC for 10 minutes and then immediately cooled with a water-ice mixture to prevent renaturation of the DNA. The membrane was incubated for 5 minutes in 2x SSPE/0.1% SDS and then placed in the miniblotter (ImmunoBlot XL Hoefer). Residual fluid in the slots was removed by aspiration and the slots were filled with the diluted PCR-products. The first and the last slot were filled with buffer only and the 2nd, 43rd and 44th were filled with a positive control. This was hybridized at 42 ºC for 60 minutes and then the samples were removed by aspiration. The membrane was washed 3 times at 50 ºC with 2x SSPE/0.5% SDS and then incubated with 20 ml 2x SSPE/0.5% SDS + 5µl streptavidine-HRP (Roche diagnostics) at 42 ºC for 30 minutes. The membrane was washed 3 times with pre-heated 2x SSPE/0.5% SDS at 42 ºC, followed by 3 washes with 2x SSPE at room temperature. Then, 10 ml of ECL (GE Healthcare) was spread over the membrane and this was gently moved so that the membrane is fully covered. The membrane was sandwiched between two transparency sheets without excess fluid and air bubbles and placed in a cassette with an x-ray film. The film was exposed for 3-30 minutes (the exposure time lengthens when the membrane is used more often) and then developed. In figure 6, an example of a RLB result is shown. The probes in this figure lay horizontal and the samples vertical. First, it was checked if the positive controls were positive (the first vertical line on the left and the last two on the right). Then, each sample was checked. The catch-all probe is the lowest horizontal line. If there is a reaction to that probe, the reaction to a species-specific probe can be checked. To determine which dot belongs to which probe, the results are compared to a control model.

After use, the membrane was stripped (immediately, or after storage in 2x SSPE/0.1% SDS at 4 ºC): incubate twice with stripping fluid at 90 ºC for 20 minutes. Store in 2x SSPE/0.1% SDS at 4 ºC.

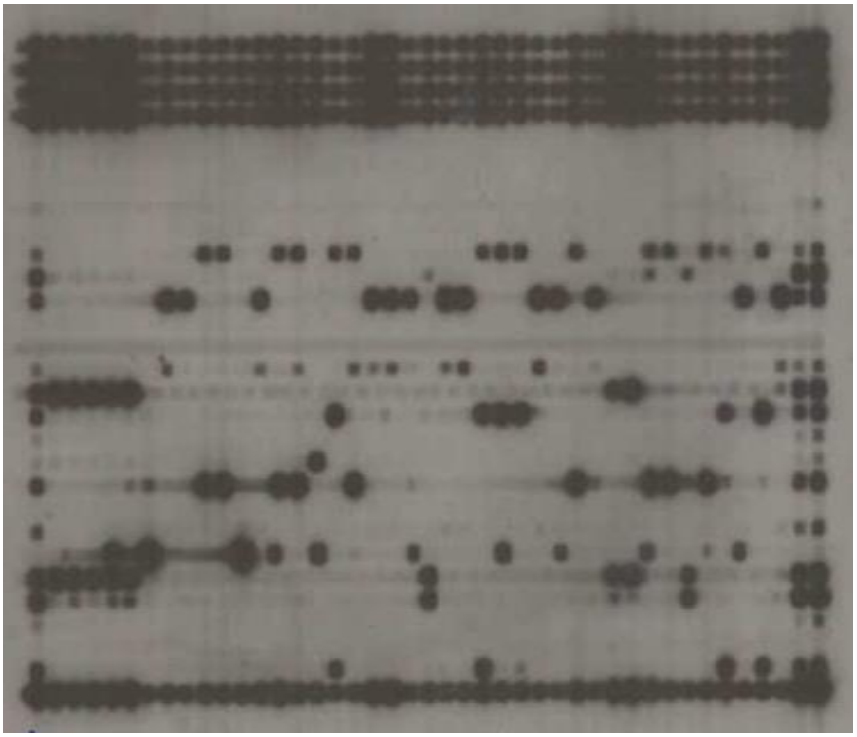

Figure 6: An example of a RLB result. The probes lay horizontal and the samples vertical. The first and the last two vertical lines are the positive controls and the lowest horizontal line is the catch-all probe.

Statistics

To determine whether age is significantly different in horses with or without larvae found in the mucosa, the non-parametrical Mann-Whitney U test was used, using SPSS 22 for Windows. P values of <0.05 were considered significant.

All other results were not statistically analysed, since too little data was acquired to perform a reliable statistical analysis. If this study is continued and more data is acquired, statistical analysis may be possible to compare the species found in the mucosa and the faeces, and to compare the species found in each intestinal part. The number of larvae found in each intestinal part can also be compared, although this has been researched multiple times already, if this study is continued and results need to be published, it may be useful to calculate how many horses with mucosal larvae are needed to perform a reliable statistical analysis.

Results

Larval cyathostominosis

Two horses with L4s in the faeces were sampled. Unfortunately, both horses did not meet the inclusion criteria for this study. The first horse had not lost weight and appeared healthy. The second horse had been treated with an anthelmintic a few days before the sampling.

Evaluation of the Baermann technique as a method to isolate L4s from the faeces

The larval counts in the experiment to evaluate the Baermann technique are given in table 1. The samples which were stored at 4 °C for one night had lower larval counts than the other samples.

Table 1: Experiment to compare the Baermann technique with microscopic evaluation as a method to isolate L4s from the faeces.

Experimental condition	Larval count
Room temperature, immediately used	24
	18
37 °C, immediately used	8
	20
Room temperature, maintained overnight at 4 °C before use	2
	0
37 °C, maintained overnight at 4 °C before use	2
	5
Direct microscopic examination	13
	19

Larval counts in the (sub)mucosa of slaughtered horses

The age, gender and breed of all slaughtered horses are given in table 2. No L3s were found in the (sub)mucosa of none of the slaughtered horses. All mucosal counts presented are L4s and the numbers given are larvae per 50 gram mucosa. In 3 (27.3 percent) horses, no cyathostomes were found in the (sub)mucosa, nor in the faecal cultures. Additionally, in 4 (36.3 percent) horses only faecally cultured larvae were found. In 4 horses (36.3 percent), cyathostomes were found in the (sub)mucosa and the faecal cultures. There were no horses that had (sub)mucosal larvae without faecally cultured larvae.

Age seemed to be lower in horses where mucosal larvae were found than in horses where no mucosal larvae were found. This was proven significant with the Mann-Whitney U test (P= 0.006).

Table 2: Age, gender and breed of the 11 slaughtered horses that were sampled

Horse	Age	Gender	Breed
1	23	Male	Icelandic horse
2	12	Male	Welsh
3	21	Female	KWPN
4	1.5	Female	KWPN
5	22	Male	KWPN
6	13	Female	KWPN
7	1.5	Female	Welsh
8	16	Male	KWPN
9	24	Female	KWPN
10	14	Male	KWPN
11	6	Male	KWPN

Table 3: Larval counts of the slaughtered horses. Data of the horses are given in table 1. The numbers given are L4s in 50 gram mucosa of each intestinal part and LPGs of the faecal cultures.

Horse	LPG faeces	Ventral colon	Caecum	Dorsal colon
1	0	0	0	0
2	712	5	0	0
3	0	0	0	0
4	712	1	35	0
5	10	0	0	0
6	0	0	0	0
7	788	170*	75*	10
8	1288	0	0	0
9	7.2	0	0	0
10	0.4	0	0	0
11	372	4	23	1

*: Only 10 gram mucosa was counted, the number given is calculated by multiplying the number of larvae found in 10 gram by 5.

Species differentiation

Differentiation was performed on all mucosal larvae found, with a maximum of 40 larvae per horse per intestinal part. Differentiation of the cultured L3s was performed only on the faeces from those horses in which mucosal larvae were found. In total, 276 larvae were differentiated with RLB and 13 cyathostome species were identified. The species with an RLB probe, but that were not identified are: *Cya. tetracanthum, Cyc. auriculatus, Cyc. bidentatus, Cyc. radiatus, Cyd. bicoronatus, Cys calicatus, Para. mettami* and *Tri. gobi*. In total, 11 larvae (4.0 percent) were not differentiated with RLB (cyathostome+/specific-). The differentiation of the L4 larvae per horse are given in figures 7-10.

The distribution of larvae in the faeces and mucosa is given in table 4 and figure 11. The distribution of mucosal L4s over the intestinal parts is given in table 5 and figure 12. In the faeces, the number of (known) species found per horse varies between 6 to 9 species. In the mucosa, The number of species found per horse varies between 1 to 9 species.

Table 4: Total of species distribution of cultured L3s and mucosal L4s. The numbers are given in percentages.

Larvae	*Cyc. insigne*	*Cys. longibursatus*	*Cor. coronatum*	*Cys. nassatus*	*Cya. catinatum*	*Pot. imparidentatum*	*Cys. minutus*	*Cyc. ashworthi*	*Cor. Labiatus*	*Cys. goldi*	*Cor. labratus*	*Cya. pateratum*	*Cyathostome+ /specific -*
L3 faeces	5.0	5.0	19.4	19.4	12.2	0	4.3	12.9	0.7	1.4	3.6	3.6	2.4
L4 mucosa	51.1	12.4	5.1	13.9	4.4	1.5	1.5	3.6	0.7	1.5	0	0	2.9

Table 5: Total of species distribution of mucosal L4s per intestinal part. The numbers are given in percentages.

Intestinal part	*Cyc. insigne*	*Cys. longibursatus*	*Cor. coronatum*	*Cys. nassatus*	*Cya. Catinatum*	*Pot. Imparidentatum*	*Cys. minutus*	*Cyc. ashworthi*	*Cor. Labiatus*	*Cys. goldi*	*Cyathostome+ /specific -*
Ventral colon	39.0	29.3	9.8	7.3	7.3	2.4	2.4	0	0	0	2.4
Caecum	56.5	5.9	2.4	18.8	3.5	0	1.2	5.9	1.2	0	2.4
Dorsal colon	54.5	0	9.1	0	0	9.1	9.1	0	0	18.2	0

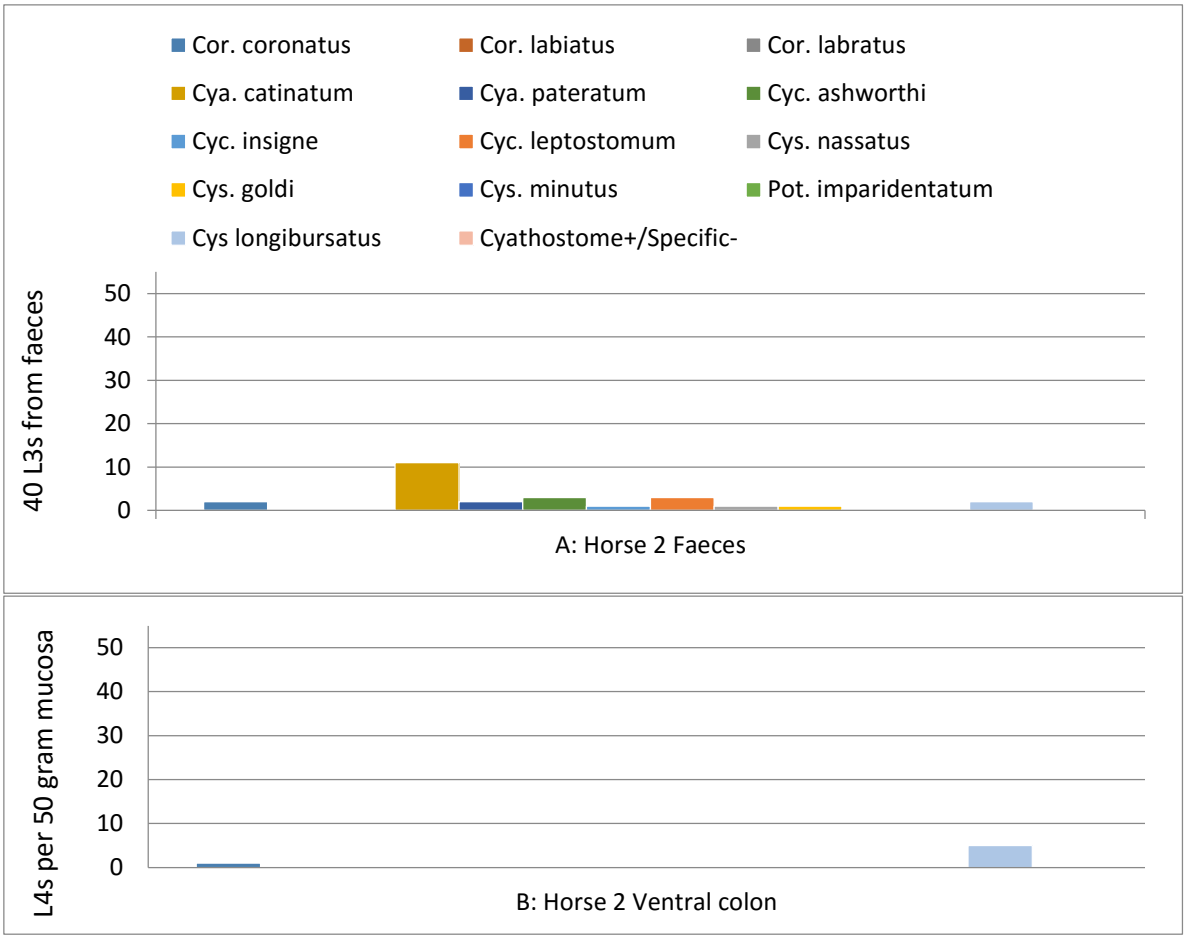

Figure 7: Differentiation of cyathostome larvae of horse 2. No larvae were found in the mucosa of the caecum and dorsal colon.
A: Differentiation of 40 L3 larvae from the faecal culture of horse 2
B: Differentiation of L4 larvae from 50 gram of the ventral colon of horse 2

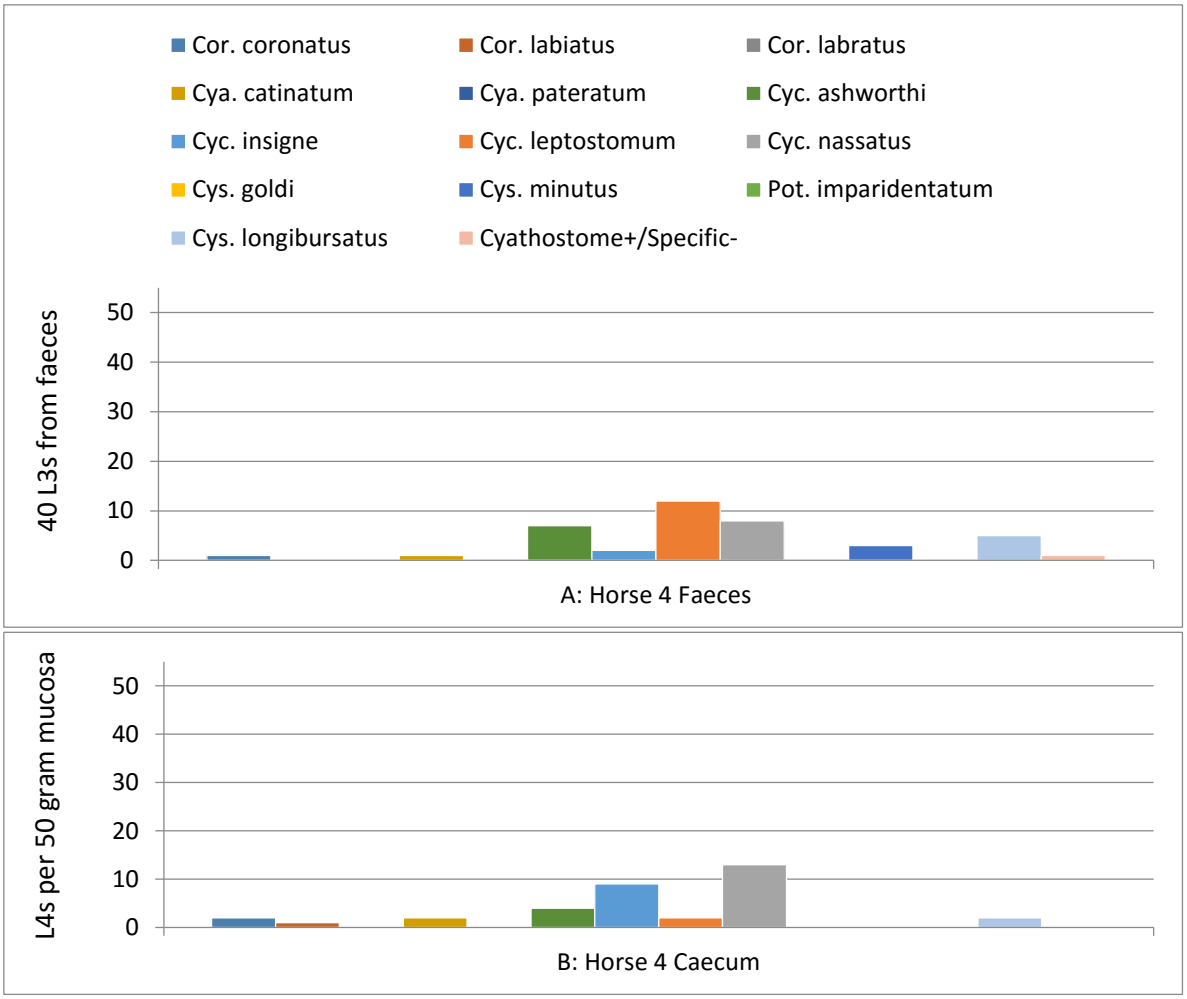

Figure 8: Differentiation of cyathostome larvae of horse 4. No larvae were found in the mucosa of the ventral colon and dorsal colon.
A: Differentiation of 40 L3 larvae from the faecal culture of horse 4
B: Differentiation of L4 larvae from 50 gram of the caecum of horse 4

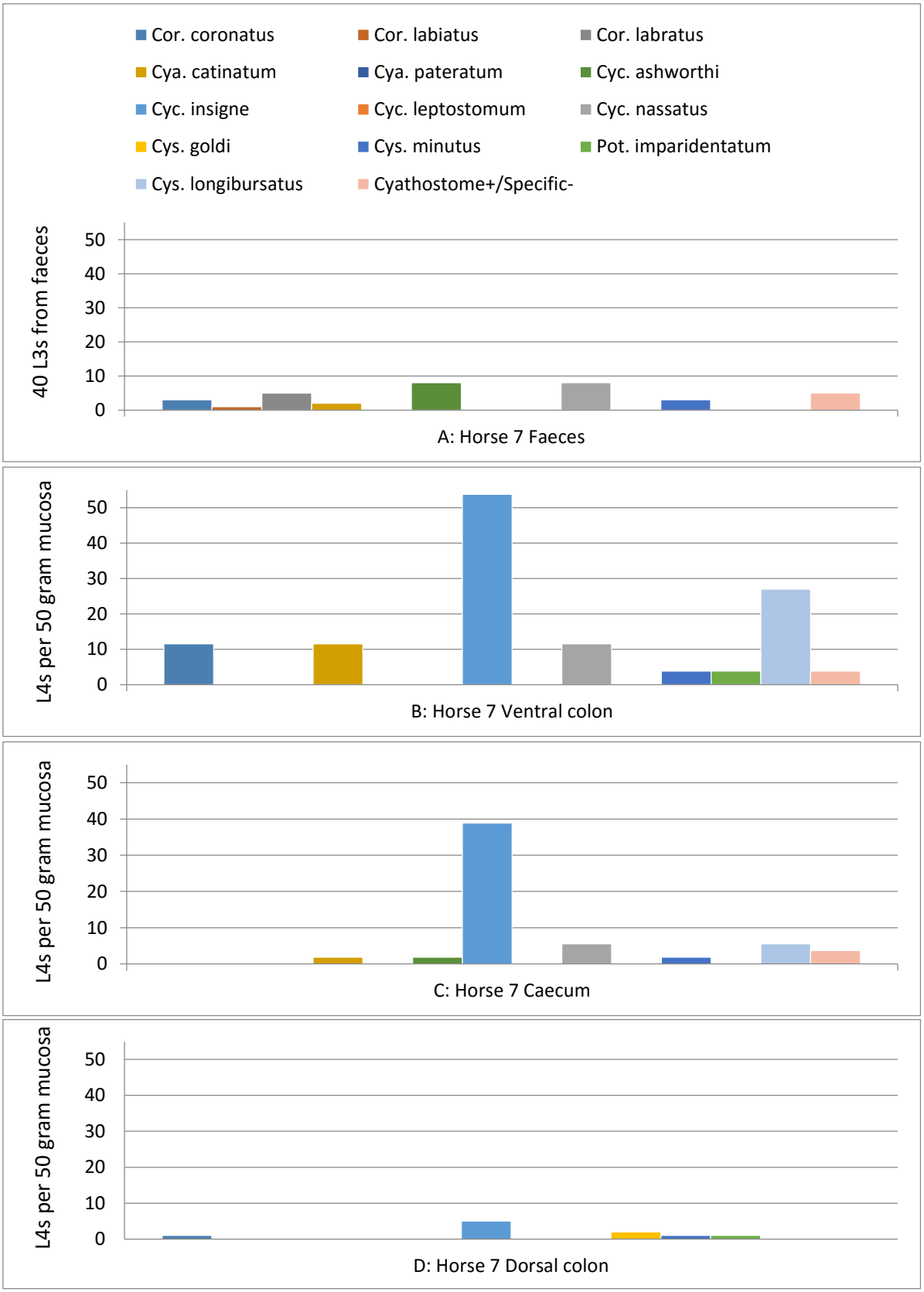

Figure 9: Differentiation of cyathostome larvae of horse 7
A: Differentiation of 40 L3 larvae from the faecal culture of horse 7
B: Differentiation of L4 larvae from 50 gram of the ventral colon of horse 7
C: Differentiation of L4 larvae from 50 gram of the caecum of horse 7
D: Differentiation of L4 larvae from 50 gram of the dorsal colon of horse 7

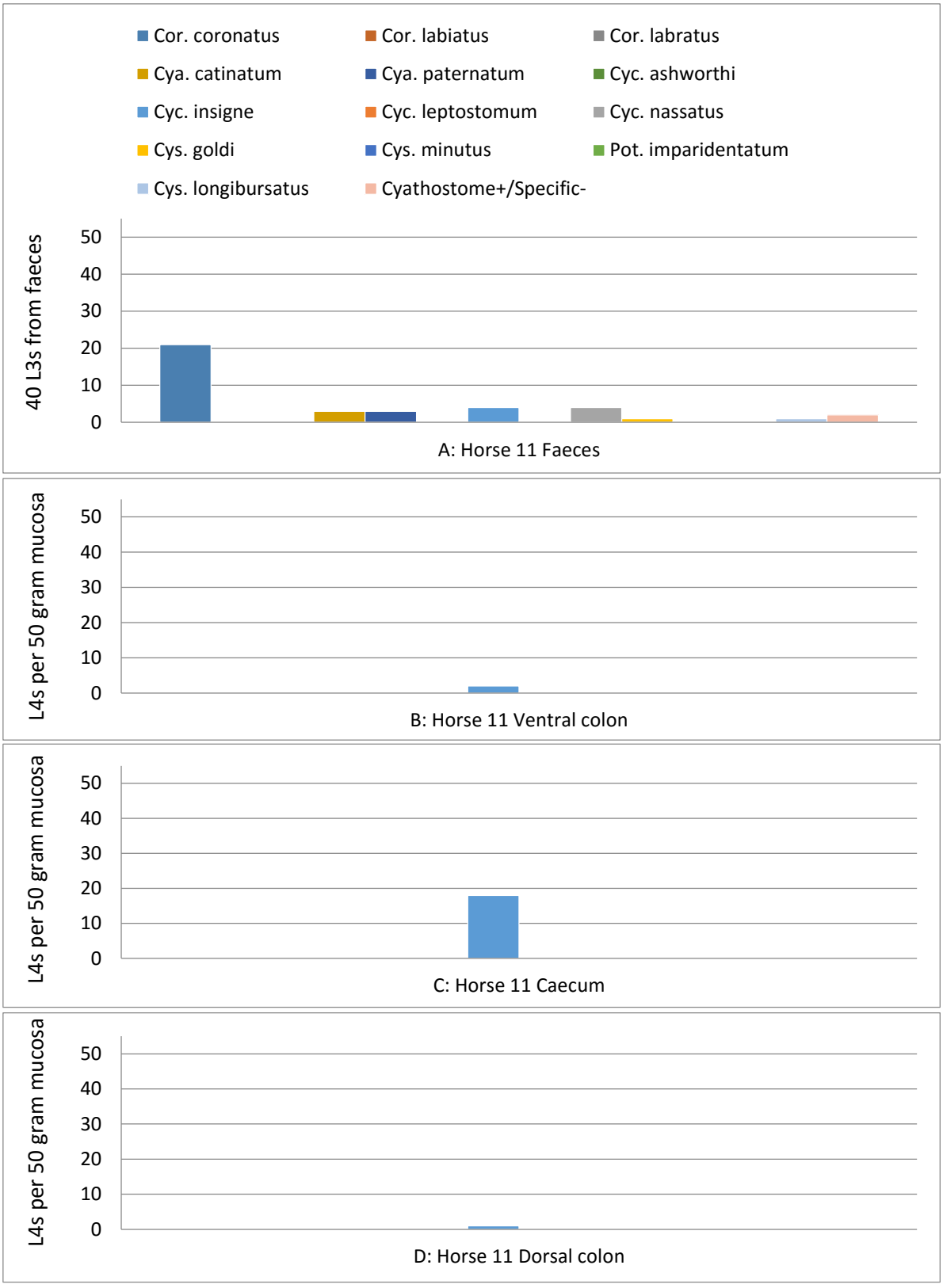

Figure 10: Differentiation of cyathostome larvae of horse 11
A: Differentiation of 40 L3 larvae from the faecal culture of horse 11
B: Differentiation of L4 larvae from 50 gram of the ventral colon of horse 11
C: Differentiation of L4 larvae from 50 gram of the caecum of horse 11
D: Differentiation of L4 larvae from 50 gram of the dorsal colon of horse 11

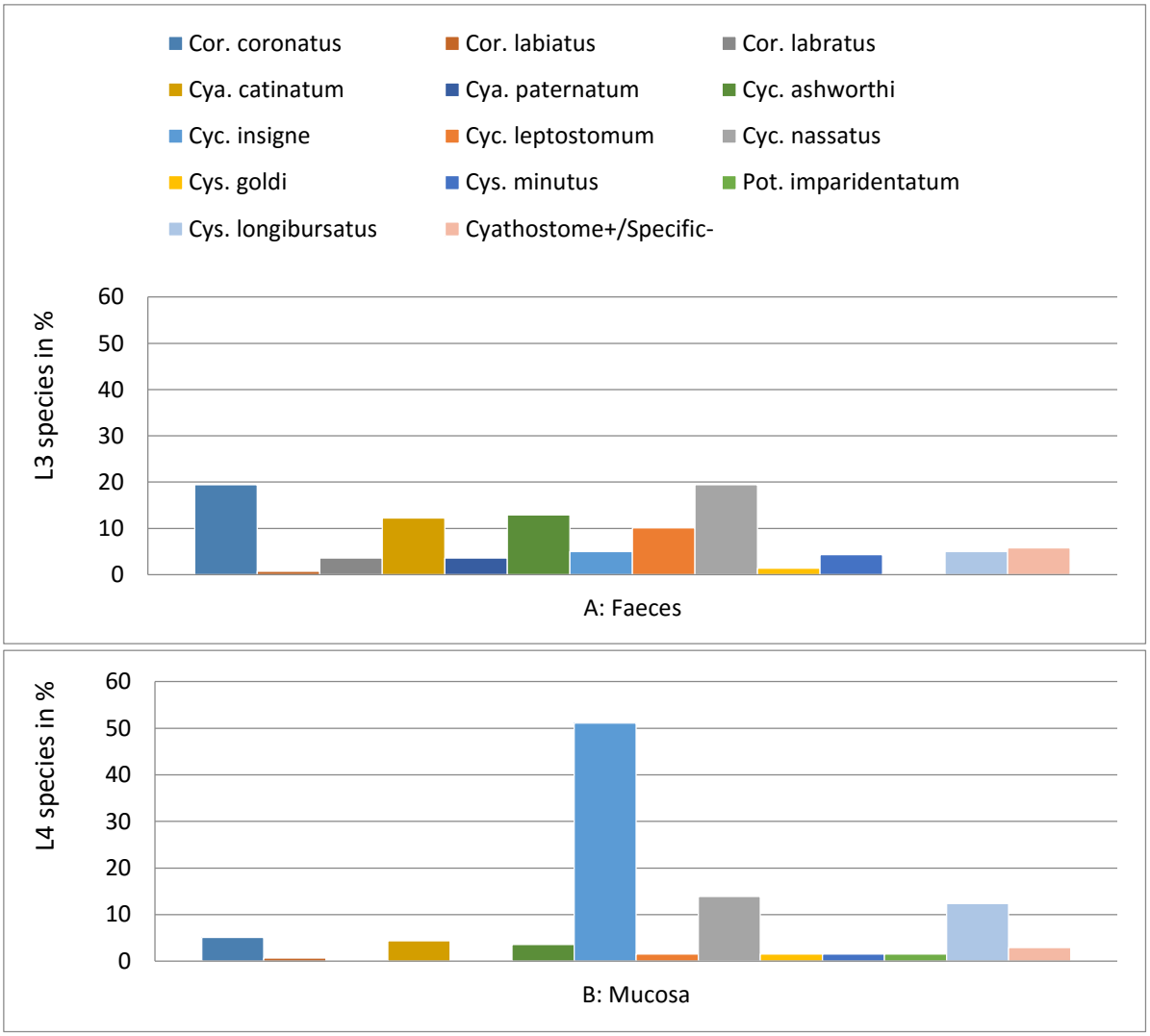

Figure 11:
A: Total of L3 species differentiation in the feces, given in percentages
B: Total of L4 species differentiation in the mucosa of all horses, given in percentage of total

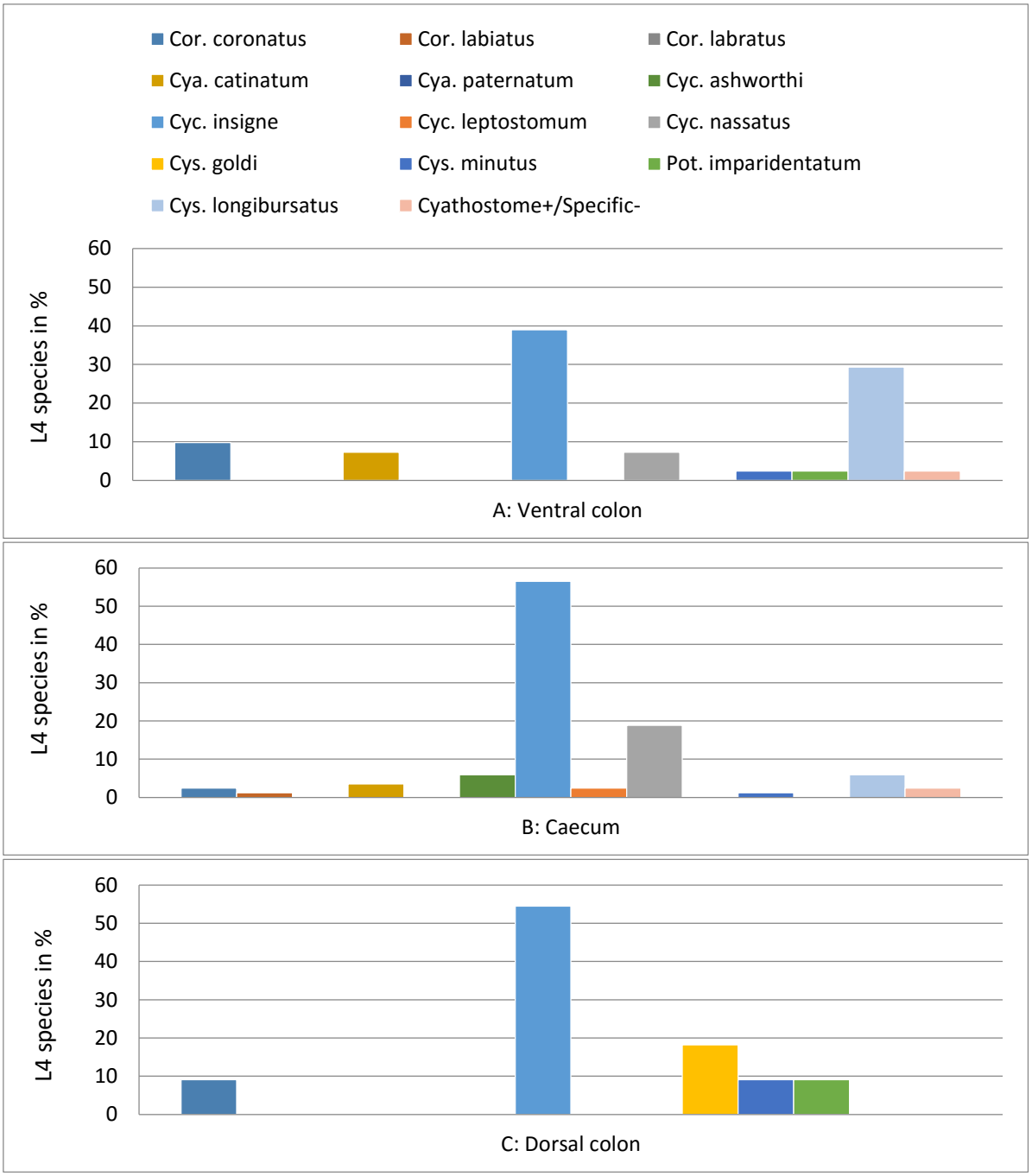

Figure 12:
A: Total of L4 species differentiation of the ventral colon, given in percentages
B: Total of L4 species differentiation of the caecum, given in percentages
C: Total of L4 species differentiation of the dorsal colon, given in percentages

Discussion

In this study, we tried to determine which cyathostome species are inhibited in the mucosa of slaughtered horses. These horses are thought to be healthy.

It is known that a large part of the total worm burden can consist of mucosal stages (*Eysker and Mirck,* 1986). In this study, however, in multiple horses no or little mucosal stages were found in 20x20 cm mucosal scrapings with in total 50 g, even though some of these horses did have a high LPG. Also, 100% of the mucosal stages found were L4s. An explanation may be that most of these horses are adults (which have developed some resistance against cyathostomes), while the horses used in the studies of *Eysker and Mirck* (1986) and *Eysker et al* (1984) were yearlings. *Love and Duncan* (1992) found varying amounts of mucosal larvae in young and adult horses, from 3% of the total worm burden to 51%. Other studies found larger numbers of mucosal larvae in young horses than in adult horses (*Klei and Chapman,* 1999, *Monahan et al,* 1998). Furthermore, it is known that FECs do not correlate with the actual total worm burden and not even with the luminal worm burden (*Nielsen et al,* 2010). It is not yet clear why no L3s were found in the mucosa of all horses. However, we went to the slaughterhouse once to test our methods. We did find L3s in one of those sampled horses, so not finding L3s is not because the method is not suitable for finding them. A study with more horses and for a longer period of time may give more information as to why no L3s were found.

Not much is known about species distribution of the mucosal stages. Studies have revealed that most encysted larvae are located in the caecum, followed by the ventral colon and the least amount of larvae are found in the dorsal colon (*Collobert-Laugier et al,* 2002, *Gawor,* 1995, *Reinemeyer et al,* 1984, *Reinemeyer et al,* 1988, *Stancampiano et al,* 2010). We also found the least amount of larvae in the dorsal colon, but the results of the ventral colon and caecum were not consistent. This perhaps can be explained by the relatively small samples that we took (50 gram of mucosa), since the larvae are not equally distributed through the intestinal parts. Another finding in our study is that the age of horses where mucosal larvae were found was lower than in the horses where no mucosal larvae were found. This age difference was significant. This poses an interesting hypothesis that can be tested in a larger study.

It is thought that the larvae move to their site of preference after they emerge from the (sub)mucosa in a more proximal intestinal region (*Eysker and Mirck,* 1986). This is supported by *Mathieson* (1964), who found that adult stages of *Cys. longibursatus* located in the caecum were smaller than those located in the colon. In the present study, it seems that mucosal cyathostomes do not have a strong preference for an intestinal part. For example, *Cyc. insigne* is almost equally distributed over the intestinal parts in horse 7 and horse 11. However, slight differences between the intestinal parts are found. For example, *Cys. longibursatus* seems to be found more in the ventral colon, while *Cyc. ashworthi* was only found in the caecum and *Cyc. goldi* was only found in the dorsal colon. For reliable results about larval counts and species distribution, this study should be continued until enough horses are sampled.

The 5 most prevalent species in the faeces were *Cyc. nassatus, Cor. coronatus, Cyc. ashworthi, Cya. Catinatum* and *Cyc. leptostomum.* They comprised 74 percent of the total amount of larvae recovered. The 5 most prevalent species in the mucosa were *Cyc. insigne, Cyc. nassatus, Cys. longibursatus, Cor. coronatus* and *Cya. catinatum*. They comprised 86.9

percent of the total amount of larvae recovered. The most prominent difference is found in the prevalence of *Cyc. insigne*. *Cyc. insigne* was by far the most prevalent species in the mucosa (51.1 percent), while it was only 5.0 percent in the faeces. It was apparent that the L4s of *Cyc. insigne* found were larger than most other L4s. It may be that these larvae are bigger, because they remain encysted for a longer time. This can be an explanation as to why this species was found more than in the faeces.
When comparing this to the existing literature, some differences are found. *Cys. goldi* and *Cys. minutus* were not found in large numbers in this study, while they belong to the most abundant species. Furthermore, *Cys. calicatus* was not found at all in this study. These differences may be explained by the low number of horses used in this study. This may be investigated further.

As mentioned before, *Hodgkinson et al* (2003) differentiated larvae from faeces of horses with larval cyathostominosis. When compared to the differentiation of L4s in this study, the most apparent difference is that no L4s in the study of *Hodgkinson et al* (2003) were identified as being *Cyc. insigne*, while *Cyc. insigne* comprised more than half of the encysted L4s in this study. To achieve the aim of this study, it is necessary to continue looking for horses with larval cyathostominosis and also to sample more slaughtered horses.

It may also be relevant to develop new probes for RLB. This way, more species can be recognized. This study has proven that it is necessary to develop more probes, since 4.0 percent of the larvae used for RLB did have a positive RLB reaction, but did not react to a specific probe. There can be two explanations for this. The first explanation is that these samples contained cyathostome species for which no probes were available. The other explanation is that these samples contained large strongyles, since large strongyles also react with the 'catch-all' probe of the RLB. In a different study, the PCR products of some of these larvae were sequenced and compared to sequence data of cyathostome species for which IGS sequences are available in the NCBI database. That particular study demonstrated that the sequences that were found were most likely of cyathostomes. One of these larvae was identified based on the sequenced as *Cyc. elongates*.

The second part of this study was to determine which species are involved in larval cyathostominosis, because not much is known about this. Also, little is known about the pathogenicity of individual cyathostome species.

Hodgkinson et al (2003) were the first to determine cyathostome species in horses with this disease. They sampled the faeces from 17 horses. In total, 712 L4s were isolated from the faeces of these horses and these were used in their PCR-ELISA. A PCR product in combination with hybridization with the positive control probe was obtained in 522 L4s. The results were as follows: 28.5% of the 522 L4 were *Cys. longibursatus*, 25.7% were *Cyc. nassatus*, 15.9% were *Cyc. ashworthi*, 7.3% were *Cys. goldi* and 1.7% were *Cya. catinatum*. No L4s were identified as being *Cyc. insigne* species. Faeces from each horse contained at least two different species. This study provides some information about the involvement of certain cyathostome species.

In this study, the Baermann technique was evaluated for the isolation of L4s from the faeces. It appeared that this method is as sensitive as direct microscopic examination of the faeces when the faeces was fresh. The samples cannot be stored in 4 ºC. In conclusion, this method

is suited for fresh faeces. If the faeces has been stored at 4 °C or the sample is taken more than 24 hours ago, the L4s should be isolated by direct microscopic examination. Unfortunately, no cases with larval cyathostominosis that fit our description were presented to us, although 2 cases were reported after treatment. The veterinarians that were contacted at the end of this study confirmed that no other cases of larval cyathostominosis were seen this season. It may be that the prevalence of this disease has lowered, possibly due to better awareness of the veterinarians and horse owners. Anthelmintics for horses are prescription only medication in the Netherlands since July 1st, 2009, that can be prescribed by a veterinarian based on faecal examination. This may have led to a better pasture management and therefore a lower cyathostome burden in horses. This theory is supported by the fact that in the majority of the horses (63.6 percent) in the present study no mucosal cyathostome stages were found. Another explanation for the low prevalence may be that the number of clinical cases of larval cyathostominosis differ per year. A multiyear study can demonstrate whether this is the case or not.

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Appendix: Protocols

Collection of L4s form horses with larval cyathostominosis

L4 larvae and eggs are collected from freshly dropped faeces of horses with larval cyathostominosis.
Mix collected faeces and weigh 10 gram.
Sieve the faeces through a 63 µm sieve with 0.9% NaCl and examine the residue for L4s with a dissecting microscope.
Collect L4s with a small hook and store at -80 ºC until further use.

Materials:

- Dissecting microscope
- 63 µm sieve
- Salt
- Hook
- Pump for sieving

Collection of L4s from the faeces of horses with larval cyathostominosis, using the Baermann technique
Method based on *Olsen et al.* (2003).

L4 larvae and eggs are collected from freshly dropped faeces of horses with larval cyathostominosis.
Mix collected faeces and weigh 10 gram.
Fill a Baermann glass with 0.9% NaCl, made with distilled water. The glass should be almost full. Place the faeces in the center of a single gauze layer (17 ply, 10x10 cm) and fold the gauze around the faeces. The free ends of the gauze are attached to a wooden spill with a paperclip and the wooden spill is placed over the edge of the glass. The faeces is now immersed in saline. If not so, fill up the glass until it does.
Maintain at room temperature or 37 ◦C for 24 hours.
Use a Pasteur pipette to aspirate the sediment and transfer it to a petri dish. Count larvae with a microscope at 16x or 20x.

The Baermann setting

Materials:

- Wine glass
- 10x10 cm gauze, 17 ply
- Wooden spill
- Paperclip
- Salt
- Distilled water
- Pasteur pipette
- Petri dish

Sampling the (sub)mucosa of the colon and caecum of horses at the slaughter house

Collect faeces from the rectum. Locate the caecum by finding the caecum point. Locate the ventral and caudal parts of the colon by finding the pelvic flexure and counting the taeniae. Open the caecum and colon by a longitudinal incision and remove the contents. Cut out two 20x20cm samples of the caecum, dorsal colon and ventral colon.

Materials:

- Ruler
- Sailcloth
- Aprons
- Boots and cap
- Refuse bags
- Lockable bags to sort samples per horse
- Knifes and scissors
- Hand gloves
- Stickers
- Pens/markers

Extraction of larvae from the (sub)mucosa of the colon and caecum
Method based on *Glover et al.* (2009).

Wash the mucosa with 0.9% NaCl to remove the contents of the intestines, which contains luminal stages. Separate the mucosa and submucosa from the muscular layer and serosa by scraping with a knife. Weigh 50 gram of (sub)mucosa.
Add 100 ml of 0.9% NaCl and 2 mM EDTA.
Double-bag this mixture with Stomacher bags.
Make sure there is no air in the bags to prevent breakage. Process the (sub)mucosa in the Stomacher Lab-Blender 400 for 40 minutes.

Fixating the processed tissue:
Add 96% ethanol until the concentration ethanol is 70%
Store until further use.

Isolation of larvae from the fixated tissue:
Sieve through a 63 µm sieve onto a 20 µm sieve with 0.9% NaCl and examine the residue on both sieves.
Use a dissecting microscope to frame the larvae and isolate them either by aspiration with a pipette (L3) or by using a small hook (L4). To ensure that no other material than the larvae is isolated, wash the L3s in 0.9% NaCl and isolate the individual L3s in 2 µl fluid.
Store at -80 ºC until further use.

Materials:

- Knife
- Spoon
- Scales
- Salt
- EDTA
- Distilled water
- Stomacher bags
- Stomacher Lab Blender 400
- 96% ethanol
- 63 µm sieve
- 20 µm sieve
- Pipette
- Hook
- Pump for sieving

Culturing L3s from eggs in faeces
Method based on *Van Doorn et al,* (2010).

Weigh 25 gram of faeces acquired from the rectum and 2.5 gram of sawdust. If the faeces is wet, use 5 gram of sawdust.
Put this in a glass jar with a plastic lid. Do not close the lid completely, so the larvae can use oxygen.
Culture at 26 ◦C for 10 days.
After 10 days, fill the jar with lukewarm tap water and turn upside down on a petri dish. Fill the petri dish with lukewarm tap water.
Maintain overnight at room temperature.
Pour water from the petri dish into a falcon tube, while pressing on the glass jar to prevent contamination of the water. Refill the petri dish and pour the water in the falcon tube.
Fill the tube with water until 50 ml is reached. Count larvae in 5 ml and convert to larvae per gram faeces.
If there are too much larvae to count 5 ml, a smaller amount of fluid can be counted. Count at least 100 larvae.

Materials:

- Glass jar with plastic lid
- Sawdust
- Water
- Falcon tube

Lysis L3s and L4s and use as template in PCR
Method based on *Van Doorn et al,* (2010).

Solutions:

Worm Lysis Buffer (WLB), (*Kwa et al.*, 1995)
For 10 ml:
All stock solutions are stored in -20 (drawer Mol Biol)

50 mM KCl	500 µl 1M KCl
10 mM Tris, pH 8.0	100 µl 1M Tris
2.5 mM MgCl2	1000 µl 25 mM MgCl2 (Fermentas)
0.45% NP-40	45 µl 10% NP-40
0.45% Tween-20	45 µl 10% Tween-20
0.01% gelatine	100 µl 1% gelatine
	1790 µl
Milli Q H2O	8210 µl
Total	10000 µl

Heat the solution slightly to dissolve the gelatine completely and store in 1 ml aliquots in WLB box in -20C

Before use: Thaw 1.2 ml WLB and add 6 µl proteinase K (stored -20 C, 20 mg/ml Fermentas) right before use (keep in the freezer as long as possible). The remaining WLB/Prot K will be used as a negative control and will be treated the same as the other samples.

Method:

Add to each L3: 25 µl WLB/Prot K. Add to each L4: 50 µl WLB/Prot K.
Do not vortex or mix end over end, because the L3s or L4s have to remain at the bottom of the vial.
Freeze the sample for 15 min at -80 °C
Thaw the sample and spin shortly.
Incubate overnight at 56 °C.
Next morning: Inactivate Prot K in the sample at 95 °C for 15 min.
After that, vortex thoroughly and spin shortly.

Materials:

- WLB/Prot K
- Centrifuge
- Incubator
- Pipette

CY1-CY18-biot PCR (for RLB)
Method is based on *Van Doorn et al*, 2010.

All pipetting before PCR is done with filter tips and clean hand gloves
Preparation of PCR reactions is done in PCR cabinet at lab W.347
Afterwards, clean cabinet with ethanol and 10% Bleach and switch on UV light
Everything that has been frozen should be mixed and spun, since ice crystals dehomogenize the mixture.

Thaw dNTP and Dreamtaq right before use. Dream green and the primers can be thawed a longer time before use.

PCR pipetting scheme

	met 2.5 ul templ		6 reacties
Scheme per reaction	ul		
Forw primer 10 uM	1		6
Rev primer 10 uM	1		6
10x dream green	2,5		15
dNTP 10 mM	0,5		3
DreamTaq (5 U/ul)	0,25		1,5
H2O	17,25		103,5
total	22,5		135
	(+2.5 ul template)		

Positive control: Large batch of mixture of many L3s of many species. Diluted to 1 L3 per 25µl. Storage in small vials (10 µl) for single use in -20 C.

Negative control: WLB + Proteinase K

Forward primer: 10 µM CY1 (5'-GGTCAAGGTGTTGTATCCAGTAGAG-3')
Reverse primer: 10 µM CY18 with biotin (5'-CTTAGACATGCATGGCTTAATC-3')
Dilute stock primer 10x to obtain a working solution of 10 µM.

PCR in BioRad model C1000 or Applied Biosystems model 2720 with the following cycle:

3 min 95°C	
30 sec 95°C	
30 sec Tm- 5°C	35
(60ºC)	x
1 min 72°C	
7 min 72°C	

PCR device:

- Before putting the samples in the PCR device: mix (by tapping to it) and centrifuge
- Power button is on the backside of the PCR device
- BioRadC1000: close the lid and turn the round button until you feel resistance, then make another quarter turn
- Program is called CY1-CY18D (BioRadC1000) or CY1-CY18 Dream (Abi2720)
- Always check the cycle!
- Make sure the lid is warmed

Materials:

- Pipette
- Centrifuge
- Primers
- dNTP
- Dreamtaq
- Dream green
- Distilled H2O

Checking of PCR product with agarose gel electrophoresis

NOTE: Midori green is mutagenic, use gloves!

Spin PCR products before use.

small gel:
weight 0.5 g agarose and add 50 ml TAE for a 1% agarose gel. Boil in microwave until all agarose is dissolved.
Let it cool to about 50 ˚C and add 4 µl Midori green.
Pour solution into gel casting device with 1 or 2 combs for 15 lanes each.
Let gel solidify for at least 30 min.

Samples: 5 µl PCR product, marker: 2ul 100 bp DNA ladder
Run in tray with TAE for 30-60 min at 7.5 V/ cm (=75V).
Make picture of the result

For medium gel: 1.25 g agarose and 125 ml TAE + 10 µl Midori green.
Combs with 30 lanes, running also at 75 V for 30-60 min.

Large gel: 2.5 g agarose and 250 ml TAE + 20 µl Midori green.
Combs with 26 or 30 lanes, running at 150 V for 30-60 min.

Make the PCR products visible by using a molecular imager.

Materials:

- Agarose
- TAE
- Green
- Microwave
- Gel casting device
- Power supply
- molecular imager (BioRad Gel Doc XR+ System).

RLB
Method based on *Traversa et al,* (2007) and *Van Doorn et al,* (2010). The RLB-probe sequences are given by *Cwiklinski et al,* (2012).

Protocol based on RLB protocol from ICTTD 2009
Changed: okt 2013

General remarks:
*Never let the membrane dry out; PCR products will remain on the membrane for ever
*Always use forceps to handle the membrane and keep the up-side up. Only hold the membrane on the "empty" places on the edge.
*Avoid contamination of all labs with the PCR products (otherwise future PCR-reactions can be contaminated) by working only in lab W.356 and W.344 with the PCR product.
* Membrane can easily be removed from a tray containing fluid, but is difficult to remove from an empty tray.
* Clean mini-blotter with warm tap water only. Do not use EtOH!

Coupling of probes to membrane

1. Dilute oligonucleotides with aminoC6 linker in 150µl 0.5M $NaHCO_3$, pH 8.4. 800 pmol/lanes is standard, but optimal concentration can be higher or lower .

2. Mark the membrane orientation by using ink (see below) in lane 1 only and write the number of the membrane were the 1st probe crosses the first PCR-product.

3. Activate membrane by 10 min incubation in 10 ml 16% EDAC at room temperature. Rinse twice with DI-water. **Discard EDAC in a bottle, not in sink!!**

4. Place membrane on support cushion in clean miniblotter. Turn screws hand-tight. Remove residual water by aspiration (vacuum)

5. Fill the first lane with diluted 100X ink in 2 X SSPE

6. Leave 2 or 3 lanes open, to ensure the probes are not too close to the edge

7. Fill other slots with 150µl diluted probes.

8. Incubate for at least 1 min at room temperature

9. Remove probe solutions by aspiration, in the same order as they were applied

10. Remove membrane from blotter with forceps and place in washing tray with 100ml 100mM freshly diluted from 5M NaOH, for 8 min. Time is critical! (maximum 10min)

11. Wash membrane in 100ml 2X SSPE/0.1% SDS at 60°C for 5 min.

12. Membrane is ready for use or can be stored at 4°C (for storage see membrane stripping section)

Hybridization with PCR product

Temperature and salt concentration have strong influence on hybridization efficiency. Keep them as constant as possible.

1. Preheat water bath at 100°C, incubator at 42°C and water bath at 50°C.
2. Spin PCR products before use.
3. Dilute PCR products: 10 µl in 150µl 2x SSPE/0.1%SDS in 1.5ml tubes, use non-filtered tips. Stick the tip of the pipette in the liquid to ensure all 10 µl is used. Include pos PCR control.
4. Denature diluted PCR products for 10 min at 100°C in water bath and cool on water-ice mixture immediately. Centrifuge after samples have cooled down.
5. Incubate membrane for 5 min in ~20 ml 2 X SSPE/0.1% SDS at room temperature
6. Place membrane in miniblotter, with slots perpendicular to line pattern of applied probes.
7. Remove residual fluid by aspiration.
8. Number of membrane must be where 1st probe is going to cross the first PCR product.
9. **Position of samples and controls:**
   Lane 1 buffer only
   Lane 2 pos PCR control
   Lane 3-42 samples (40x)
   Lane 43-44 pos PCR control
   Lane 45 buffer only
10. Fill slots with diluted PCR product (150µl). AVOID AIRBUBBLES. When air bubbles are formed, remove those by pipetting up and down.
11. Hybridize at 42°C for 60 min on a level surface in incubator.
12. Prepare developer on the 5th floor for use
13. Remove samples by aspiration. Put membrane in curver tray
14. Wash membrane **3x** in pre-heated **2xSSPE/0.5% SDS** at **50°C** in water bath. The 1st wash is short (removing excess of unbound material) followed by 2 washes of 5 min each.
15. Incubate membrane with **20 ml** 2xSSPE/0.5% SDS + 5µl streptavidine-HRP for 30 min at 42°C in incubator
16. Wash membrane **3x** with pre-heated **2xSSPE/0.5% SDS** at **42°C** in incubator. First wash short, followed by 2 washes of 5 min.
17. Wash 3x with 2x SSPE at RT (1x short, 2x 5 min)
18. Spread 10ml ECL (5ml ECL1+ 5 ml ECL2, in refrigerator) over membrane, move gently for few minutes so that membrane is fully covered
19. Sandwich the membrane between 2 transparency sheets without excess fluid ONLY USE BLANC SHEET/NO SHEETS FOR PHOTOCOPY PURPOSES
20. Expose x-ray film for 3-30 min (exposure depends strongly on the number of times that a membrane is used. For a new membrane starts with 3 min)
21. Develop film on 5th floor.
22. After exposure you can strip membrane directly (see below), otherwise: Wash membrane once with 2xSSPE/0.1% SDS and store in 2xSSPE/0.1% SDS at 4 ˚C. Make sure the whole membrane is submerged!
    Indicate on top of the tray whether membrane is stripped or not.

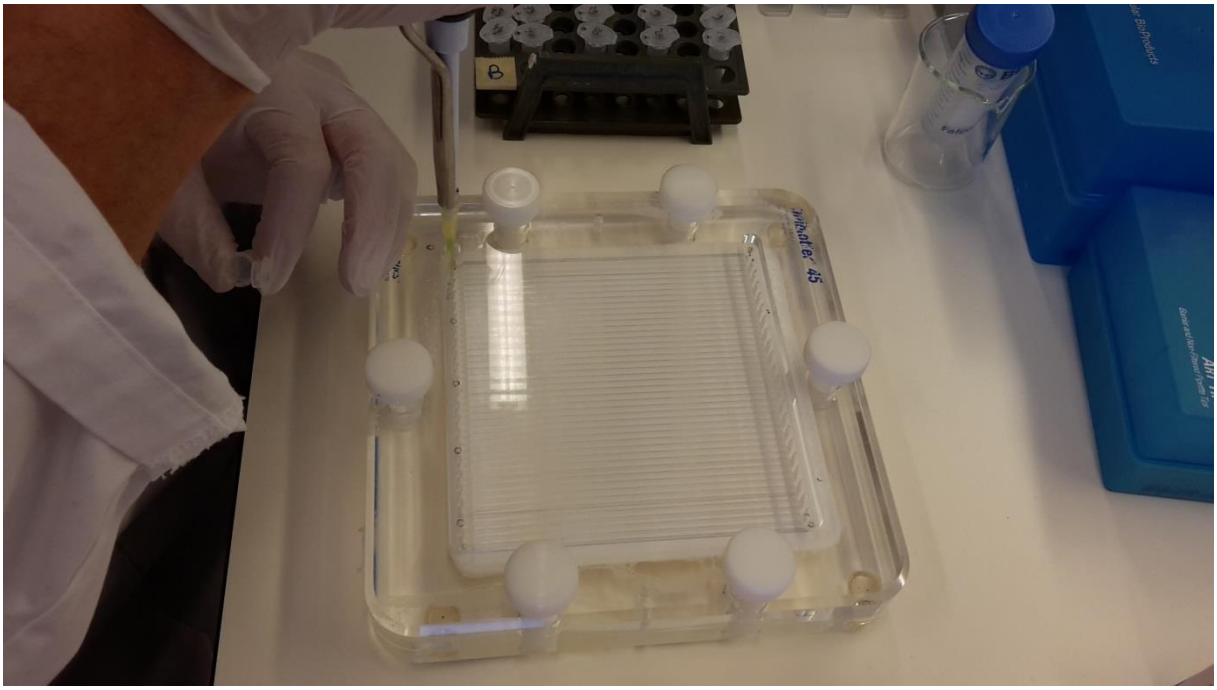
TIP: 2 BLOTS CAN BE PROCESSED SIMULTANEOUSLY IN 2 TRAYS
SAME BATCH OF ECL CAN BE USED, ONE AFTER THE OTHER.

Membrane stripping

Membranes can be re-used many times (up to 20x).
For that you have to remove the hybridized PCR-product (stripping)

500ml 1% SDS (50ml 10% SDS + 450 ml DI-water) preheated at 90°C

1. Place membrane in stripping tray with stripping fluid (1 % SDS) and incubate 2x for 20 min in a water bath at 90°C.
2. Store membrane at 4 ˚C in 2xSSPE/ 0.1 % SDS. Make sure the whole membrane is submerged!
   Indicate on top of the tray whether membrane is stripped or not.



Filling slots in miniblotter

Solutions and materials

- Miniblotter 45 (Immunetics, Cambridge, MA 02139)
- Plastic cushions PC 200 (Immunetics)
- Biodyne C Membrane (negatively-charged Nylon 6.6), 0,45µ pore size 60320 (Pall Corporation, Gelman laboratory)
- Streptavidin-POD Conjugate 1 089 153 (500U in 1 ml)(Roche)
- ECL detection reagents RPN 3004 (Amersham pharmacia biotech)
- Pipette
- PCR products
- Positive control
- Curver tray
- Incubator
- Ice
- Film
- Cassette
- Sheets

Reverse line blot solutions UU version adjusted 12 july 2006

- **5M NaOH (mw=40.00) (100ml)**
  Dissolve 20g NaOH in 50 ml DI-water
  Dilute NaOH in small portions till final volume of 100ml (EXOTHERM REACTION)
  Do not store in glass bottles (glass dissolves in 5M NaOH)
  2ml 5M NaOH + 98 ml DI- water = 100ml 100mM NaOH.

- **16% EDAC [1-ethyl-3(3-dimethylaminopropyl)carbodiimine (10mL) – Always prepare fresh]**
  Take EDAC out of -20 C and let it adjust to RT.
  Weight 1.6g EDAC and dissolves in 10ml DI-water
  Do not heat!

- **0.5M NaHCO3, pH 8.4 (250ml)**
  Dissolve 10,5g $NaHCO_3$ in 240 ml DI-water
  Adjust to pH 8,4 with NaOH
  Add DI-water till final volume is 250ml
  Filter sterilize (0.22µm), do NOT autoclaving (CO_2-escapes)
  Store at room temperature for no longer than 6 months?

  10% SDS
  Weight 40 g SDS (SDS is irritating powder ; do not shake bottle with SDS powder).
  Dissolve in DI-water with end volume of 400 ml.

  Stripping buffer
  Mix 40 ml of 10 % SDS with 360 ml of DI-water.

- **Prepare buffers, store at RT (almost indefenitly)**

1. 400ml 2XSSPE/ 0.1% SDS (40 ml 20X SSPE + 4 ml 10%SDS + 356 ml DI-water)
2. 400ml 2XSSPE/ 0.5% SDS (40 ml 20X SSPE + 20 ml 10% SDS+340 ml DI-water)
3. 400ml 2XSSPE (40ml 20X SSPE + 360ml DI-water)