Serology as a diagnostic tool for *Parascaris* spp. in foals

- A pilot study

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Independent Project • 30 credits
Swedish University of Agricultural Sciences, SLU
Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Uppsala 2023

Serology as a diagnostic tool for *Parascaris* spp. in foals – A pilot study

Serologi som ett diagnostiskt verktyg för spolmaskinfektion hos föl – En pilotstudie

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Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Independent Project in Veterinary Medicine
Course code:	EX1003
Programme/education:	Veterinary Medicine Programme
Course coordinating dept:	Department of Clinical Sciences
Place of publication:	Uppsala
Year of publication:	2023
Cover picture:	Agnes Lind af Hageby
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Keywords:	Roundworm, Ascariasis, Equine, Serodiagnostics, Indirect ELISA, AsHb, L3-Lung, *Ascaris suum,* Anthelmintic resistance

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Abstract

Parascaris spp. are prevalent equine gastrointestinal nematodes that predominantly infect foals. The infection causes impaired welfare and can have a lethal outcome due to complications such as intestinal obstruction. Due to a prepatent period of 10-14 weeks, diagnosis with coprological techniques is limited and therefore most foals are routinely dewormed during their first year without knowledge of infectious status. The parasite is showing an alarming emerging resistance to numerous anthelmintic drug classes, limiting the possibilities of treatment. Diagnostics through serological techniques have been developed for the related pig roundworm, *Ascaris suum*, using an indirect ELISA targeting either *Ascaris suum* haemoglobin (AsHb) or L3-Lung antigens. This study investigated the possibility of using a modified version of the indirect ELISA protocol, targeting the same AsHb and L3-Lung antigens to identify *Parascaris* spp. infection in foals. Two farms were included with a total of 15 foals concluding the study. The foals were sampled for blood for serum extraction from two weeks of age and followed at four-week intervals. Faecal samples were examined from six weeks with about two-week intervals until positive for *Parascaris* spp. eggs to determine a patent infection. The results showed that antibodies for *Parascaris* spp. could be detected with both the AsHb ELISA and L3-Lung ELISA. Moreover, an initial increase in antibody levels were observed for both antigens. A significant correlation between antibody levels in two-week-old foals and their mother was found, indicating passive transfer of antibodies via colostrum. The antibody levels of the foals thereafter decreased from the first to the second and third sampling occasion and then significantly increased to the fourth occasion for both AsHb and L3-Lung ELISAs, indicating an immunological response to the *Parascaris* spp. infection. This study shows the potential of developing a clinically applicable serological technique for *Parascaris* spp. The possibility of a clinically applicable test could be used for targeted deworming and decrease the anthelmintic use, thereby limiting the emerging anthelmintic resistance in *Parascaris* spp. and reducing the environmental contamination with residues of anthelmintic compounds.

Keywords: Roundworm, Ascariasis, Equine, Serodiagnostic, Indirect ELISA, AsHb, L3-Lung, *Ascaris suum*, Anthelmintic resistance

Table of contents

List of figures

Abbreviations

AsHb	*Ascaris suum* Haemoglobin
ELISA	Enzyme-Linked Immunosorbent Assay
EPG	Eggs Per Gram
FBZ	Fenbendazole
FEC	Faecal Egg Count
FECR	Faecal Egg Count Reduction
FECRT	Faecal Egg Count Reduction Test
IVM	Ivermectin
ML	Macrocyclic Lactones
OD	Optical Density
ODR	Optical Density Ratio
PYR	Pyrantel

1. Introduction

Parascaris spp. infection of foals on stud farms has been widely known for hundreds of years within the equine community and is found in all parts of the world where equids are present (Nielsen & Reinemeyer 2018). The parasites infestation of the foal's small intestine and invasive hepato-pulmonary migration, leads to impaired growth, weight loss, and lethargy which diminishes welfare and contributes to economic losses. Additionally, in severe cases the parasite can cause obstruction and rupture of the small intestine, acute peritonitis, sepsis, and death (Clayton & Duncan 1978; Cribb *et al.* 2006).

Since *Parascaris* spp. has a long prepatent period of 10-14 weeks (Clayton & Duncan 1977, 1979b), the time between infection of the host and the possibility of detecting the infection through parasite eggs in faeces, current faecal diagnostics are limited as infected foals may go undetected with risks of complications. In Sweden this has subsequently led to the recommended routine use of anthelmintics for preventive measures (SVA 2022). However, anthelmintic resistance has been emerging to all available anthelmintic substances in several countries, beginning with macrocyclic lactones during the 21 century (Peregrine *et al.* 2014). This increasing occurrence of multi-resistance could be the result of a frequent and unnecessary use of anthelmintics on foals (Ahuir-Baraja *et al.* 2021). Therefore, it could be beneficial to develop alternative diagnostic methods and treatment approaches to limit the development of resistance.

A serodiagnostic test has been developed for detection of the pig roundworm, *Ascaris suum* (Vlaminck *et al.* 2012). The method has shown to have a high sensitivity in comparison to the standardised coprological technique at the farms and to examination of livers at slaughter (Joachim *et al.* 2021).

The main purpose of this study was to investigate if the serological technique used for detection of *A. suum* in pigs can be used to detect *Parascaris* spp. infected foals. This could be useful as a future tool for diagnosis of infection and indicator for selective treatment as well as further studies of prevalence and development of immunity to *Parascaris* spp.

2. Literature review

2.1 Parascaris spp.

Parascaris spp. are nematodes of the Ascarididae family, and their geographic distribution is worldwide (Nielsen & Reinemeyer 2018). They are host specific to equids and is primarily pathogenic in juveniles. In the adult form, *Parascaris* spp. are the largest of all equine gastrointestinal nematodes.

There are two known roundworm species within the *Parascaris* spp., namely *Parascaris equorum* and *Parascaris univalens*. Morphologically the two parasites are indistinguishable by ocular inspection, both as adult and larval stages and as eggs under the microscope. They can however be distinguished by karyotyping as *P. univalens* possesses only one chromosome pair, while *P. equorum* has two (Goday & Pimpinelli 1986). Previous, *P. equorum* was considered the dominant species infecting horses, however, studies of the parasite over the last 50 years indicate it might be the opposite. In a study conducted in Italy where over 2000 specimens were analysed, researchers found that the occurrence of *P. univalens* was 93% (Bullini *et al.* 1978 see Nielsen *et al.* 2014). In a large-scale study by Tydén *et al.* (2013) involving *Parascaris* spp. specimens collected from Sweden, Norway, Iceland, Germany, Brazil and the USA, the samples were found to be homogenous. Even though no karyotyping was performed to identify the species in the study by Tydén *et al.* (2013), recent studies have shown that, *P. univalens* is the dominant spices in several of the included countries (Nielsen *et al.* 2014; Martin *et al.* 2018, 2021b; von Samson-Himmelstjerna *et al.* 2021). The clinical difference between the two species is unclear and might be hard to unravel since *P. equorum* is currently rarely found and studies made previously with *P. equorum* might have been misidentified *P. univalens* if no karyotyping has been made (von Samson-Himmelstjerna *et al.* 2021).

2.2 Lifecycle

Infection with *Parascaris* spp. commonly occurs in the first days after birth by ingestion of infective eggs present in the environment or pasture (Nielsen &

Reinemeyer 2018). The parasite has a direct lifecycle with a hepato-pulmonary route starting in the small intestine where the larvae hatch from their protective protein coating (Figure 1). The larvae penetrate the mucosal wall of the small intestine and migrate to the liver through lymphatic vessels or veins within 24 hours (Clayton & Duncan 1979b). In the liver the larvae roam through the parenchyma for about a week. Thereafter they continue to the lungs where they reside for one to three weeks. The larval migration through the lung tissue induces respiratory inflammation, which stimulates coughing (Clayton & Duncan 1978). The coughing helps the larvae to move up through the bronchi and trachea to be swallowed back down into the digestive system again where they can be found already two weeks after initial infection. The larvae settle in the small intestine and increase in size as they mature into adult roundworms. The size of an adult *Parascaris* spp. depends on the roundworms gender and age, but a fully reproductive female can reach up to 50 cm in length, whilst the male counterpart is about a half or one third of the females' size (Nielsen & Reinemeyer 2018). As the parasites size increases over time, the number of parasites decrease, which is suggested to be due to space limitation or immunological response (Srihakim & Swerczek 1978; Clayton & Duncan 1979b). The prepatent period is 10-14 weeks (Clayton & Duncan 1977, 1979b) and the number of eggs shed by an adult female worm is believed to be similar to the human roundworm, *Ascaris lumbricoides*, which lay approximately 200,000 eggs per day (Sinniah 1982).

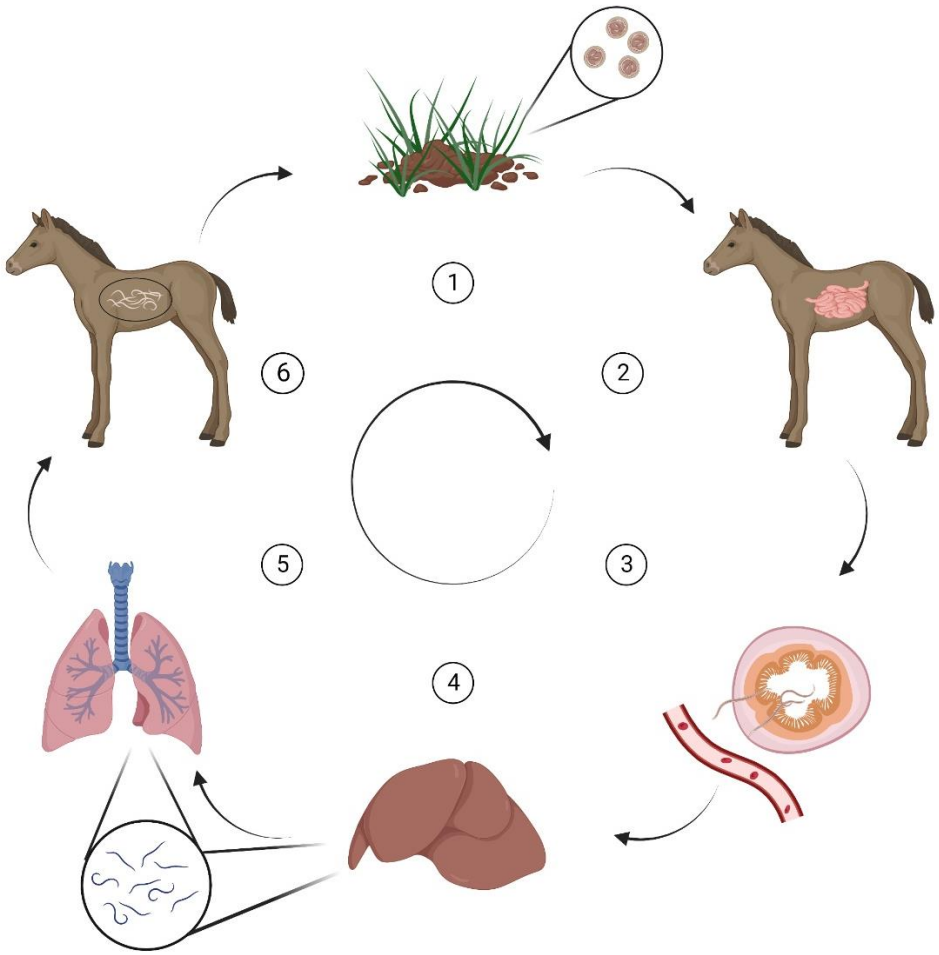

Figure 1. Lifecycle of Parascaris *spp. 1. Viable eggs contaminating the environment and becomes infective from 10 days after leaving previous host. 2: Foal ingest the infective eggs. 3. The eggs hatch into larvae and penetrate through the intestinal wall over to lymph vessels or veins. 4. The larvae pass over to the liver and migrates through the tissue. 5. The larvae reach the lungs and cause a respiratory inflammation response by their abrasive path and are coughed up and swallowed down again. 6. The larvae settle in the small intestine and mature into fully reproductive adult parasites and start shedding eggs in 10-14 weeks after infection. Created with BioRender.com*

Under optimal environmental conditions (25°C to 35°C) the immature eggs can become infective within 10 days after being excreted into the environment with the faeces (Nielsen & Reinemeyer 2018). The eggs are resilient to harsh climate due to their protective triple-layered protein coat. Thus, the eggs can remain viable for many years in suboptimal temperatures (Nielsen & Reinemeyer 2018). For how long these eggs can survive in different climates and surfaces is yet to be studied. However, according to an unpublished epidemiological study in Sweden, the *Parascaris* spp. eggs may survive in grass plots up to at least 1.5 years (Lindgren & Höglund 2010).

2.3 Impact on host

Infection with *Parascaris* spp. can vary from asymptomatic to severe colic with a lethal outcome. Symptoms associated with the infection are lethargy, distended abdomen, weight loss, impaired growth and poor hair coat (Slocombe 1985). Furthermore, respiratory symptoms such as coughing and nasal discharge can be observed as the larvae migrate through the lung tissue (Clayton & Duncan 1978). The larval migration through the liver parenchyma is considered to be asymptomatic. However characteristic focal inflammatory and haemorrhagic lesions, or at a later stage, white fibrotic foci commonly referred to as white spots, can be identified post-mortem, caused by the larvae's passage through the parenchyma (Brown & Clayton 1979).

The most severe complication can arise after the larvae are reintroduced to the small intestine, as they increase in size when they develop into adult roundworms. A massive parasitic burden has the potential to cause obstruction of the small intestine which can lead to rupture of the intestinal wall, volvulus or disturb the peristalsis and trigger intussusception (Clayton & Duncan 1978; Cribb *et al.* 2006). Surgery is often necessary, and a retrospective study by Cribb *et al.* (2006) found that both short- and long-term prognosis are guarded, as 64% survived the surgery and only 27% survived more than one year post surgery. On the other hand, another study by Tatz *et al.* (2012) found that if the impaction was resolved by manual manipulation to move the parasites to the cecum without having to put an incision into the intestine during operation, the prognosis improved.

2.4 Prevalence and immunity

A Swedish study including 146 foals found the prevalence of *Parascaris* spp. to be 48% (Lind & Christensson 2009). This is within the observed prevalence's of 31-58% found in other countries (Laugier *et al.* 2012; Relf *et al.* 2013; Armstrong *et al.* 2014; Alanazi *et al.* 2017). After a peak of increased faecal egg shedding at four months (Fabiani *et al.* 2016), a level of immunity to *Parascaris* spp. is developed (Clayton 1986). Therefore, foals older than six months and adults are rarely found to be infected with *Parascaris* spp. and the infection is thus predominantly considered a problem in foals and juveniles before the development of immunity (Clayton 1986). It has been shown that the prevalence of *Parascaris* spp. can be as low as 3-4% in horses between one- to three-year-olds (Fritzen *et al.* 2010; Relf *et al.* 2013). In contrast some studies have found higher prevalence in other countries. A study of working horses in Ethiopia with poor nutritional status, found that horses over the age of four years had a prevalence above 15% on coprological examination (Getachew *et al.* 2008). Similarly, a prevalence of 20% in two-year-olds and 34% in yearlings has also been reported in a study made in Finland where the majority

of the participants (83%) were Standardbred trotters (Hautala *et al.* 2019). The authors hypothesised that the high prevalence may be linked to a possible predisposing genetic factor for the breed or stress caused by intense training regime trotters undergo from an early age, which might suppress the young horses' immune capabilities.

The exact mechanism of development of immunity against *Parascaris* spp. has not yet been mapped. It has however been proposed to originate from an immunological response in the lungs or liver. A study by Clayton & Duncan (1979b) including eight unexposed two- to four-weeks-old ponies, experimentally infected with a single dose of 8000 infective *Parascaris* spp. eggs and autopsied at various stages, found that at 14 days post infection larvae could be found in the lungs and in the airways. However, eight days later, larvae were found in the lung parenchyma, but they were smaller and were not present in the upper respiratory airways. The authors speculated that an immunological response inhibited the larvae from migrating further. The same authors compared experimentally infected groups of unexposed foals of six to 12 months, and foals six to 12 months with previously exposure to *Parascaris* spp. to the previous results of the unexposed two- to four-weeks-old foals (Clayton & Duncan 1979a). The younger foals had a high intestinal burden, whilst both the older groups had a low infection burden. The author speculated that the larvae were probably destroyed in the liver or lungs by immunological response and the immunity is possibly connected to the immunocompetence derived with age rather than exposure to previous encountered antigen (Clayton & Duncan 1979a).

2.5 Diagnostic methods

2.5.1 Coprological techniques

The only diagnostic tool currently available for *Parascaris* spp. is coprological technique, which is identification of parasite eggs in the foal´s faeces. Commonly, faecal egg count (FEC) is used which is a procedure where the number of eggs per gram (EPG) in faeces is counted and used as a value to determine the infection burden of an individual (Nielsen 2021). Coprological techniques have some limitations, such as FEC values not always correlating with the worm burden (Andersen *et al.* 2013), which is often the case for ascarid and strongylid in horses (Nielsen *et al.* 2010). In addition, faecal examination does not account for prepatent stages such as larvae in migratory phase and immature parasites.

The FEC can be repeated 10-14 days after anthelmintic treatment to evaluate the efficacy of the anthelmintic treatment (Cabaret & Berrag 2004). This is called a faecal egg count reduction test (FECRT) and is calculated as the percentage difference of pre- and post-treatment FEC (Nielsen 2021).

2.5.2 Transabdominal ultrasound

A transabdominal ultrasound and scoring system was developed and evaluated by Nielsen *et al.* (2016) to determine the *Parascaris* spp. burden in the small intestine. The method might not be the most cost-efficient to be used on a large-scale control programme. However, it showed to have the potential to be implemented on a clinical level in critical situations such as suspicion of *Parascaris* spp. induced colic, intestine impaction, intussusception and more (Nielsen *et al.* 2016).

2.6 Anthelmintic drugs and resistance

Three anthelmintic substance groups are registered for treatment of *Parascaris* spp. infections in foals, namely macrocyclic lactones (ML), tetrahydropyrimidines, and benzimidazoles.

2.6.1 Macrocyclic lactones

Macrocyclic lactones (ML) block transmission of neuro signals of invertebrates by binding and opening glutamate-gated chloride channels in muscle and nerve cells in addition to acting as a gamma-aminobutyric acid (GABA) agonist. The resulting effect is paralysis, which kill the parasite through starvation or facilitates parasite expulsion from the host (Lanusse *et al.* 2018b). Ivermectin (IVM), an avermectin, and moxidectin, a milbemycin, are both registered for horses in Sweden.

Resistance to ML in *Parascaris* spp. has been reported for over 20 years across the world (Boersema *et al.* 2002; Hearn & Peregrine 2003; Lyons *et al.* 2006, 2008; Schougaard & Nielsen 2007; Slocombe *et al.* 2007; von Samson-Himmelstjerna *et al.* 2007; Molento *et al.* 2008; Veronesi *et al.* 2009). Even Iceland with its harsh regulations and ban on import of horses which has protected the country from many pathogens, has been found to harbour *P. univalens* populations with widespread resistance to ML (Martin *et al.* 2021b). Resistance to ML has also been a concern observed in Sweden since 2008 (Lindgren *et al.* 2008; Lind & Christensson 2009).

2.6.2 Tetrahydropyrimidines

Tetrahydropyrimidines have a spastic paralytic effect on parasites as it interferes with neuromuscular junctions as an agonist on nicotine acetylcholine gated ion channels (Lanusse *et al.* 2018a). In Sweden, Pyrantel (PYR) ebonate is the only tetrahydropyrimidine-derivative registered for horses (FASS 2022).

The first notion of resistance to PYR was in a study in Kentucky by Lyons *et al.* (2008), where in some farms 100% of the foals excreted *Parascaris* spp. eggs, even after treatment with double the recommended dose. In Sweden resistance to PYR was first detected in 2018 as four out of 11 investigated groups had an efficacy of

≤ 85% according to FECR (Martin *et al.* 2018). Correspondingly, lack of effect of PYR treatment has also been observed in a Finnish study of 26 horses where the mean FECRT was 68% (Hautala *et al.* 2019).

2.6.3 Benzimidazoles

Benzimidazoles (BZs) targets the parasites metabolism, constructional, and enzymatic reactions by binding to the parasites β-tubulin subunits, which subsequently leads to the parasite's death (Lanusse *et al.* 2018a). Fenbendazole (FBZ) is the only BZ registered for use in horses in Sweden (FASS 2022).

Fenbendazole is considered as one of the most effective anthelmintics against *Parascaris* spp. along with other BZs (Lyons *et al.* 2008). Nonetheless, recent studies have been showing some faltering in treatment in Sweden (Martin *et al.* 2018, 2021a). Similarly, a few other studies have reported resistance in Australia and Saudi Arabia (Armstrong *et al.* 2014; Alanazi *et al.* 2017). In Sweden FBZ is still the opted anthelmintic substance against *Parascaris* spp. along with PYR (SVA 2022).

2.7 Current Swedish treatment strategy

Following a new legislation of the European Union in 2001 (Directive 2001/82/EC of the European Parliament and of the Council), anthelmintic substances have been prescription-only in Sweden for livestock, including horses, since 2007 in accordance with legislation imposed by chapter 7 in The Swedish Medical Products Agency´s regulations about approval of medicines for sale, etc (LVFS 2006:11). Therefore, with reference to §24 in The Swedish Agricultural Agency's regulations on medicines and medicinal use (SJVFS 2009:84), a veterinarian is required to prescribe anthelmintic drugs only if required, and this decision should be based on faecal examination and dialogue with the owner.

A targeted strategy is often applied for treatment of gastrointestinal parasites in adult horses (SVA 2022). Individuals targeted for treatment are either those that have high egg counts of Cyathostominae, or horses with detected presence of *Strongylus vulgaris* or *Anoplocephala perfoliata*. However the treatment of *Parascaris* spp. does not follow the same principle as most foals are infected and the risk associated with the infection can be severe. Therefore, the recommendation is multiple treatment occasions without previous diagnosis for foals in Europe (ESCCAP 2019).

The current recommendation for *Parascaris* spp. in Sweden is routine treatment of all foals at the age of 8-10 weeks and 16-18 weeks with FBZ or possibly PYR (SVA 2022). It is also recommended to do a faecal examination of yearlings in January or February and treat only if infection is identified. It is also advised to

regularly follow up treated horses with an additional faecal examination 10-14 days post-treatment to detect early signs of anthelmintic resistance (SVA 2022).

2.8 Serology

2.8.1 Serological methods

Serology is primarily the study of immunological responses to a pathogen and is commonly implemented for diagnostic purposes. The term is mostly associated with the detection of antibodies in serum or plasma, although other bodily fluids can also be used for antibody detection, for example saliva, semen, or cerebrospinal fluid (Gurtler & Pavia 2020). There are many different techniques for detecting antibodies, for example enzyme-linked immunosorbent assay (ELISA) and western blot.

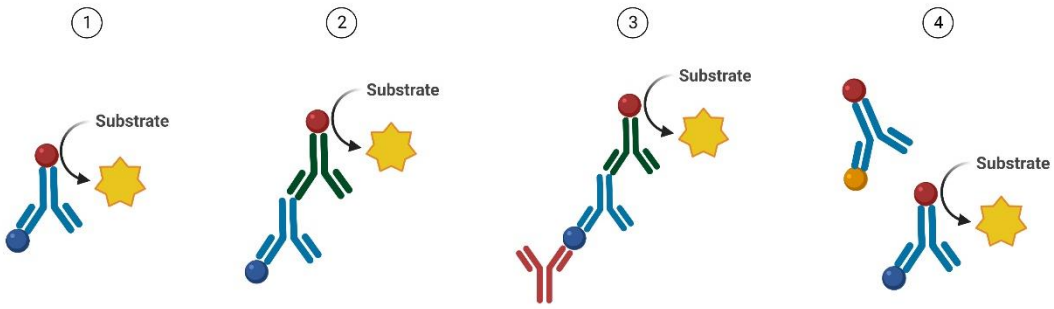

Figure 2. Enzyme-linked immunosorbent assay (ELISA) techniques. From left showing direct ELISA (1), indirect ELISA (2), sandwich ELISA (3), and competitive ELISA (4). Created with BioRender.com

The ELISA technique is based on enzyme labelled antibody binding to specific antigens or antibodies and is followed by a colour conversion when a substrate is added. Direct ELISA was the first technic developed by Engvall & Perlmann (1971) but more modified versions such as indirect, double, and competitive ELISA (Figure 2) has been developed. Indirect ELISA involves adding a serum sample with antibodies linking to a specific antigen coating the bottom of a well (Aydin 2015). Thereafter, a conjugate containing an enzyme-labelled secondary anti-antibody targeting the specific antibody in the antigen-antibody complex is added to the well. Finally, a substrate is added which will be colour converted by the enzyme on the secondary anti-antibody (Figure 3). The colour conversion can be measured by its optical density (OD). For evaluation, optical density ratio (ODR) can be applied by comparing the sample with positive and negative controls.

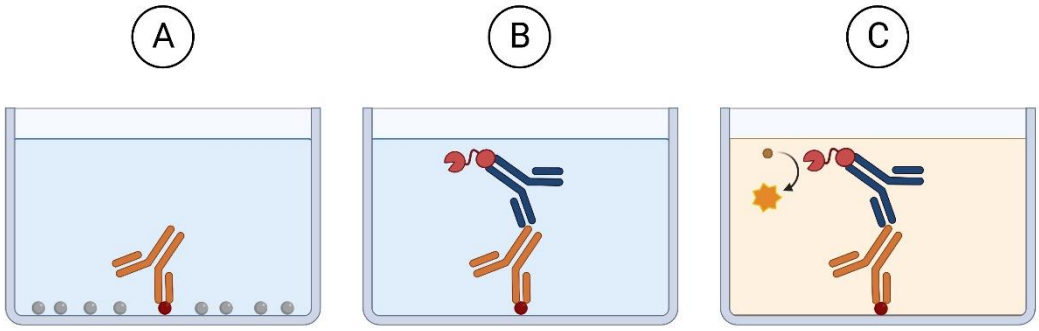

Figure 3. Indirect ELISA illustration. Antibody added to antigen coated well (A), conjugate including enzyme-labelled secondary antibody added (B), and substrate added for colour conversion by the conjugated enzyme (C). Created with BioRender.com

Western blot is another method that can be used to identify antibodies in serum (Hirano 2012). The technique separate proteins, including antibodies, by their molecular weight by gel electrophoresis. The proteins of interest can then be identified by specific antibodies.

2.8.2 Serological advancements of *Parascaris* spp.

A previous attempt to identify antibody response to infection with *Parascaris* spp. by serological techniques has been made by Burk *et al.* (2016). Western blot was used to analyse excretory-secretory products from *Parascaris* larvae. The study observed that pre-suckling foals had no detectable antibodies. However, 24h postpartum and after they received colostrum, the foals had detectable antibodies against the parasite´s excretory-secretory products examined. The authors identified this as passive transfer of maternal antibodies via the colostrum and argued that the maternal antibody interference might be a limitation in a diagnostic tool.

2.8.3 Serological advancements of *A. suum*

In the last decade, a diagnostic technique using serology was developed for *A. suum*, at the University of Ghent. The technique was developed and refined by Vlaminck *et al.* (2012) after a vaccination experiment with an antigen purified from adult worms (Vlaminck *et al.* 2011) which also revealed its potential as a diagnostic antigenic marker for ELISA. The antigen, *A. suum* haemoglobin antigen (AsHb), is derived from adult worms collected from the small intestine of infected pigs and thereafter purified from the pseudocoel of the roundworm (Vlaminck *et al.* 2012).

The AsHb was then further developed as a diagnostic tool in trials of experimentally infected 10-week-old fattening pigs. The IgG antibodies against AsHb was detected from six to eight weeks post-infection and the experiment showed that the

ELISA had a high sensitivity (99,5%) and specificity (100%). In comparison with faecal examination and liver examination at slaughter, which is typically used for detection of *A. suum*, the ELISA demonstrated a significantly higher sensitivity (Vlaminck *et al.* 2012).

Another ELISA, L3-Lung, has been developed based on antigens from migrating third stage larvae, which are isolated from lung tissue of infected piglets. The larvae are homogenisation and refined to the L3-Lung antigen for use in the ELISA (Vandekerckhove *et al.* 2017).

In a study examining piglets continuously infected with *A. suum* eggs daily for 7 weeks, Vandekerckhove *et al.* (2017) found that almost no seroconversion was observed on the AsHb ELISA. However, there was a notable seroconversion on the L3-Lung ELISA, which increased with time and was found to be dose-dependent as groups given a higher daily infection dose had a higher seroconversion. The authors calculated a specificity and sensitivity of 99% and at least 90% respectively when given an infection dose of a minimum 20 eggs per day for the L3-Lung ELISA. On the other hand, the sensitivity dropped to 40% if the infection dose was reduced to 10 eggs per day. The authors concluded that a minimum dosage of infection was necessary for detection of antibodies with the L3-Lung ELISA. In addition, it was found that the AsHb ELISA was not as effective to identify infection at an earlier age in pigs.

A cut-off value for differentiating between positive and negative infection were derived by comparing samples to negative and positive controls. For the AsHb ELISA the threshold for positive was set to be 0.5 OD mm (Vlaminck *et al.* 2012) and the L3-Lung ELISA value was set to be 0.25 OD mm (Vandekerckhove *et al.* 2017).

Considering the target molecule for the AsHb and L3-Lung ELISA have been used to identify the closely related human roundworm, *Ascaris lumbricoides* (Vlaminck *et al.* 2016; Dana *et al.* 2020), the test might be compatible for detection of *Parascaris* spp. in foals.

2.9 Aim of the study

The purpose of this study was to investigate if *Parascaris* spp. infection of foals can be detected with the AsHb and L3-Lung ELISAs developed for *A. suum*. A serodiagnostic test for *Parascaris* spp. could be advantageous for early detection of infection in foals considering that foals are routinely dewormed without being tested with coprological techniques. It can also contribute to further studies of the prevalence and geographic distribution of *Parascaris* spp.

3. Materials and methods

3.1 Ethical statement

This study has been approved by Uppsala Ethics Committee for animal experiments at the Swedish University of Agricultural Sciences (Dnr:5.8.18-15533/2018).

Written consent was given for all included animals from the stable supervisors.

3.2 Farms and horses

This project was carried out on two large trotting stud farms in middle of Sweden with previous occurrence of *Parascaris* spp. infection. One farm contributed with eight foals, the second farm with 10 foals. The foals were of similar age and divided into four sample groups for simplicity in carrying out sample collection at similar age. Partum of the foals were in April and May of 2021. The foals were kept on pastures in herds with other foals and mares.

3.3 Collection of serum samples

The first blood sample was collected when the foals were approximately two weeks old. Thereafter three additional samples were collected from each foal at approximately four-week intervals until the foals were approximately 14 weeks old (Figure 4).

One hour prior to sampling, a local anaesthetic, Emla® (dermal cream, 25 mg/g lidocaine + 25 mg/g prilocaine) was applied on the jugular vein. The blood sample was collected in two BD Vacutainer® Clot Activator Tubes with a BD Vacutainer® PrecisionGlideTM needle 20G x 1,5” (0,9 x 3,8mm). The tubes were inverted 8-10 times after sample collection and placed in a vertical position until centrifuged at 1500 x G for 10 min back at the lab within approximately 2 hours. The serum was separated and transferred to 1.5 ml Sarstedt Micro Tubes and stored at -20 °C for one to five months and then sent on dry ice to the University of Ghent for analysis.

During the first serum sample collection from the foals, a sample was also collected from each corresponding mare to identify presence of antibodies against *Parascaris* spp. The procedure was carried out the same way as the foals, though without the local anaesthic.

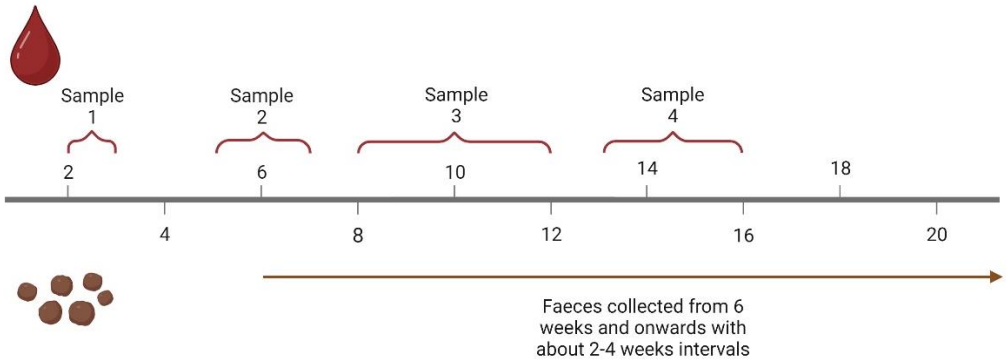

Figure 4. Timeline of collection of blood and faecal samples in weeks from each foal's birth. Created with BioRender.com

3.4 Serology

The serum samples were analysed with a modified version of the indirect ELISA protocols describe for the AsHb ELISA and for the L3-Lung ELISA (Vlaminck *et al.* 2012; Vandekerckhove *et al.* 2017) and performed with two different conjugates. The ELISA procedures were carried out by experienced personnel at the University of Ghent as briefly described below.

3.4.1 Indirect Enzyme-linked Immunosorbent Assay

Coating plates with antigen

Nunc Maxisorp flat-bottomed 96 well plates were coated with 100 µl antigen solution, AsHb 1 µg/ml or L3-Lung 5µg/ml in 0.05 M coating buffer (12.3 mM Na_2CO_3; 37.7 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Thereafter the plates were washed three times with wash buffer PBST (0.05% PBS/tween 20, pH 7.2).

Blocking of nonspecific binding sites

For blocking of nonspecific binding sites, 100 µl blocking buffer (5% fat free milk powder in PBS), were dispensed in each well and then incubated for one hour at room temperature. Afterwards all plates were washed as previously described.

Serum samples

Serum samples were diluted to 1:250 in PBST and added into their assigned wells. For conjugate, substrate, and negative controls only PBST were added in the wells. All plates were incubated at room temperature for one hour for the antibody-antigen binding to occur, and then washed as previously described.

Conjugate

Two different conjugated secondary antibodies were used in the ELISA. Conjugate one was Anti-Horse IgG (Fc)-HRP derived from goats (Sigma SAB3700145) and conjugate two was Anti-Horse IgG (whole molecule)-Peroxidase Antibody derived from rabbits (Sigma A6917). The conjugates were diluted in blocking buffer to a concentration of 1:5000. 100 µl were added to each well except for the substrate control where only blocking buffer was added. The plates were incubated one hour at room temperature and then washed as previously described.

Substrate

Substrate solution was prepared with one o-Phenylenediamine tablet (Sigma P-5412) dissolved in 20 ml citric acid/phosphate buffer (38 mM citric acid; 83.5 mM $Na_2HPO_42H_2O$) in a dark bottle. Prior to use 10 µl of hydrogen peroxide was added to activate the substrate solution. Finally, 100 µl of the substrate solution was added to each well and after exactly 10 minutes the reactions were stopped by addition of 50 µl stop solution (2.5M HCl) to each well.

Reading

The optical densities in the wells were read by an absorbance microplate reader for ELISA (TECAN Infinite® F50 Plus) at 492 nm.

3.5 Faecal examination

Faecal samples were collected from foals beginning from approximately six weeks of age with around two-week intervals (Figure 4) until presence of *Parascaris* spp. eggs were confirmed. The samples were mainly collected freshly after defecating in pasture or stall, but on few occasions rectally by stud personal. The samples were analysed within one to three days, by a modified version of the McMaster technique according to Coles *et al.* (1992) with a sensitivity of 25 EPG. Briefly, six grams of faeces were combined with 42 ml of tap water in suitable containers. The sample was left to soften for a few minutes and then stirred for homogenisation. The mixture was passed through a 150 µm mesh sieve into a bowl. The liquid was then poured into a Clayton Lane test tube and centrifuged at 1500 rmp for three minutes. After removal of the supernatant, the sediment at the bottom was mixed with a

saturated sodium chloride solution. The solution was then transferred to a McMaster chamber for parasite egg enumeration. Foals with a positive faecal sample (≥ 25 EPG) were treated orally by stud personnel with 7.5 mg/kg bodyweight FBZ paste (Axilur® Intervet).

3.6 Data analysis

The data was collected on spreadsheets (Microsoft Excel®) and was processed and analysed with the computer programme GraphPad Prism version 9.4.1.

The difference in antibody levels between the sampling occasions of the foals for the two different conjugates and AsHb and L3-Lung ELISAs were analysed with a nonparametric one-way ANOVA and Dunn´s multiple comparisons test.

The correlation between antibody levels in the mares and the corresponding two/three-week-old foals were investigated by a Pearson r correlation test.

4. Results

4.1 Farms and horses

A total of 18 foals were included in the beginning of this study. However, three foals were excluded due to impeding circumstances. On one of the farms, a foal left the farm before the end of study. On the other farm two foals were excluded because they were treated with Ivomec® vet. (ML) before the end of the study. This was due to an onset of diarrhoea and a high FEC of *Strongyloides westeri*. Ultimately, a total number of 15 foals completed the study (Table 1). In addition, on the second sampling occasion one foal was absent from the farm, resulting in no sampling of this individual.

*Table 1. Blood and faeces collection occasions in weeks from each foal's birth. Faeces collection is represented by "X" and a positive faecal sample is symbolized as "O". Blood sample colour coder as shown in the box bellow the table." *" Indicates a sample not collected due to the foal not being present at the farm. Identification of foal with the first number indicating farm, letter specifying if foal "F" and the last letter indicating what number given at the farm.*

Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Foal																					
1F2									X		X		O								
1F3									X		X		O								
1F4							*		X		X		X	X			X		O		
1F5									X		X		X		X		O				
1F6										X		X	X			X		O			
1F7										X*		X	O								
1F8								X		X		X	O								
2F1							X				X		O								
2F2							X				X		X		O						
2F3							X				X		X		O						
2F4							X					X		O							
2F5							X				X		O								
2F6						X			X		X		X		O						
2F7						X				X		X		O							
2F8									X		X		O								

Blood sample 1	
Blood sample 2	
Blood sample 3	
Blood sample 4	

4.2 ELISA

4.2.1 Antibody levels over time

To examine the optimal conditions for diagnosis of *Parascaris* spp. antibodies, two different antigens (AsHb and L3-Lung) as well as two different conjugated secondary antibodies (Anti-Horse IgG (Fc)-HRP and Anti-Horse (whole molecule)-Peroxidase Antibody) were evaluated. Both the AsHb and L3-Lung ELISAs with both the Anti-Horse IgG (Fc)-HRP and the Anti-Horse (whole molecule)-Peroxidase Antibody conjugates showed positive results. The individual foals displayed an overall relatively high antibody level in the first sample, followed by a slight drop between the first and second sample. Thereafter, a sharp increase in antibody levels were observed between the third and fourth sample (Figure 5). Significant differences were found between the second and fourth, and the third and fourth sampling occasions for both antigens and both conjugates (Figure 6).

For the AsHb ELISA with conjugate one (Anti-Horse IgG (Fc)-HRP) the P value between the second and fourth sample was 0.0004 and between the third and fourth sample 0.0006. Similarly, the AsHb ELISA with conjugate two (Anti-Horse (whole molecule)-Peroxidase Antibody) had a P value of <0.0001 between the second and fourth sample, and a P value of 0.0003 between the third and fourth sample. A significant P value of 0.0028 could also be found between the first and fourth sampling occasion in the AsHb ELISA using conjugate two.

For the L3-Lung ELISA with conjugate one, the P value was found to be 0.0067 between the second to fourth sampling and 0.0277 between the third and fourth. For L3-Lung ELISA with the conjugate two the P value was 0.0012 and 0.0458 between the second to fourth and third to fourth sampling respectively.

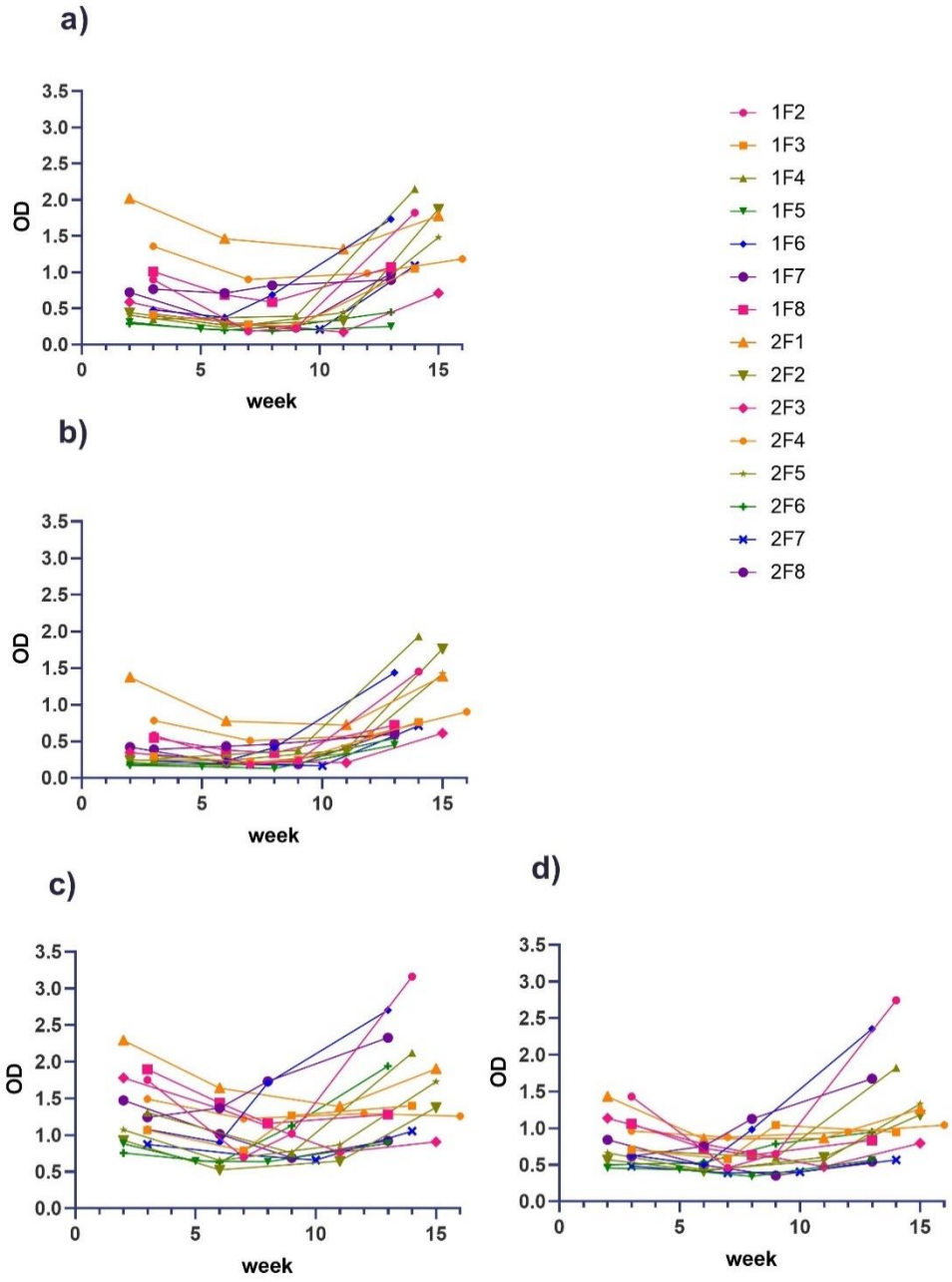

Figure 5. Individual OD levels for the individual foals over time. Analysis of the: a) AsHb antigen and conjugate one, Anti-Horse IgG (Fc)-HRP. b) AsHb antigen and conjugate two, Anti-Horse (whole molecule)-Peroxidase Antibody. c) L3-Lung antigen and conjugate one Anti-Horse IgG (Fc)-HRP. d) L3-Lung antigen and conjugate two, Anti-Horse (whole molecule)-Peroxidase Antibody. Antibody levels are shown as OD on the y-axis vs time of serum collection in weeks from birth of all foals on the x-axis.

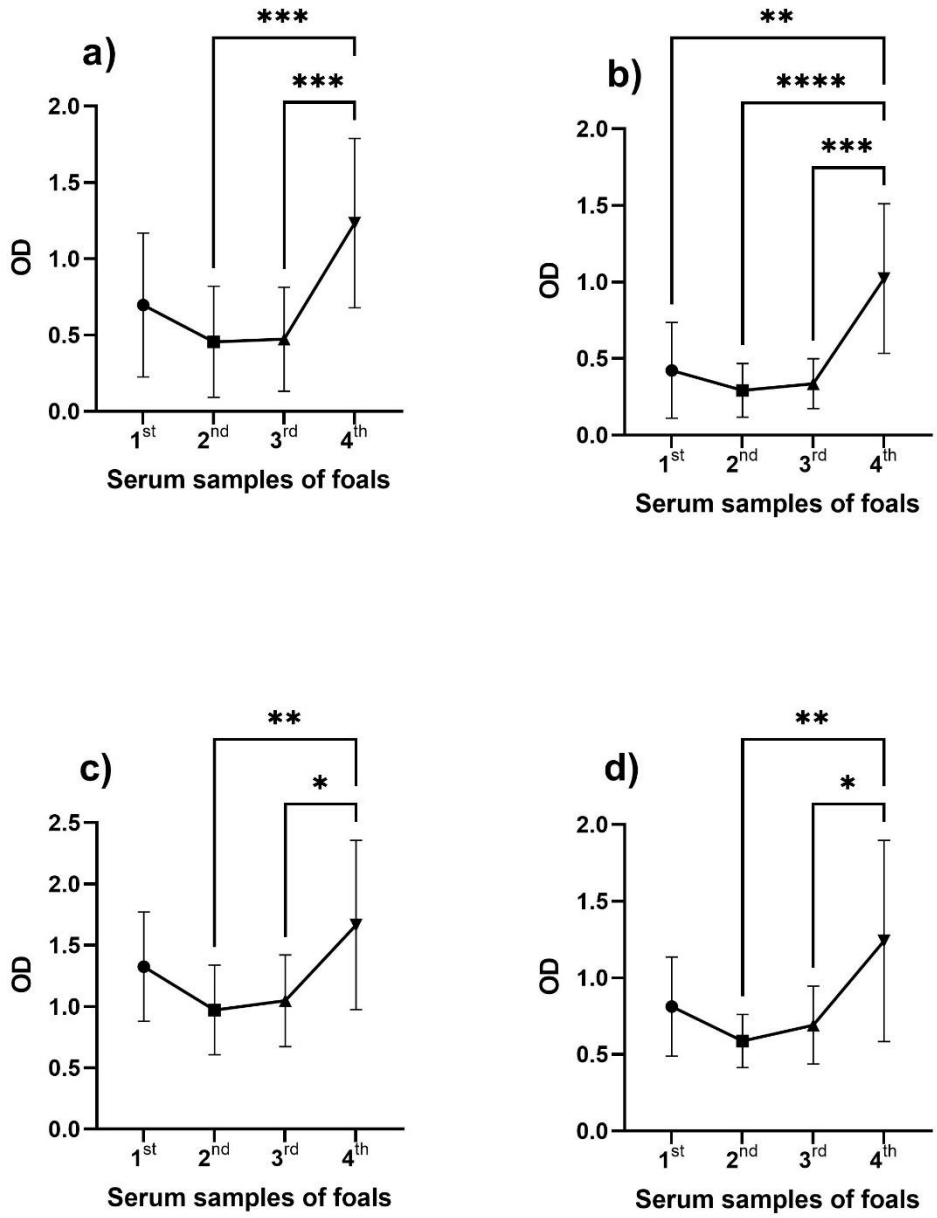

*Figure 6. Mean antibody levels for all included foals shown as OD on the y-axis and serum sample collection occasion (1^{st} – 4^{th}) on the x-axis. Analysis of the: a) AsHb antigen using conjugate one, Anti-Horse IgG (Fc)-HRP. b) AsHb antigen using conjugate two, Anti-Horse (whole molecule)-Peroxidase Antibody. c) L3-Lung antigen using conjugate one Anti-Horse IgG (Fc)-HRP. d) L3-Lung antigen using conjugate two, Anti-Horse (whole molecule)-Peroxidase Antibody. Significant differences between the sampling's occasions were analysed using nonparametric one-way ANOVA and Dunn´s multiple comparisons test and indicated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.*

4.2.2 Correlation between mare and foal antibody levels

There was a significant correlation between antibody levels in mare and foal at the first sampling occasion for the AsHb ELISA with conjugate one ($r = 0.8924$, $p <$

0.0001) and conjugate two (r = 8626, p < 0.0001) (Figure 7). No significant correlation (conjugate one: r = 0.4911, p = 0.0630, conjugate two: r = 0.3662, p = 0.1794) was however identified with the L3-Lung ELISA (Figure 7).

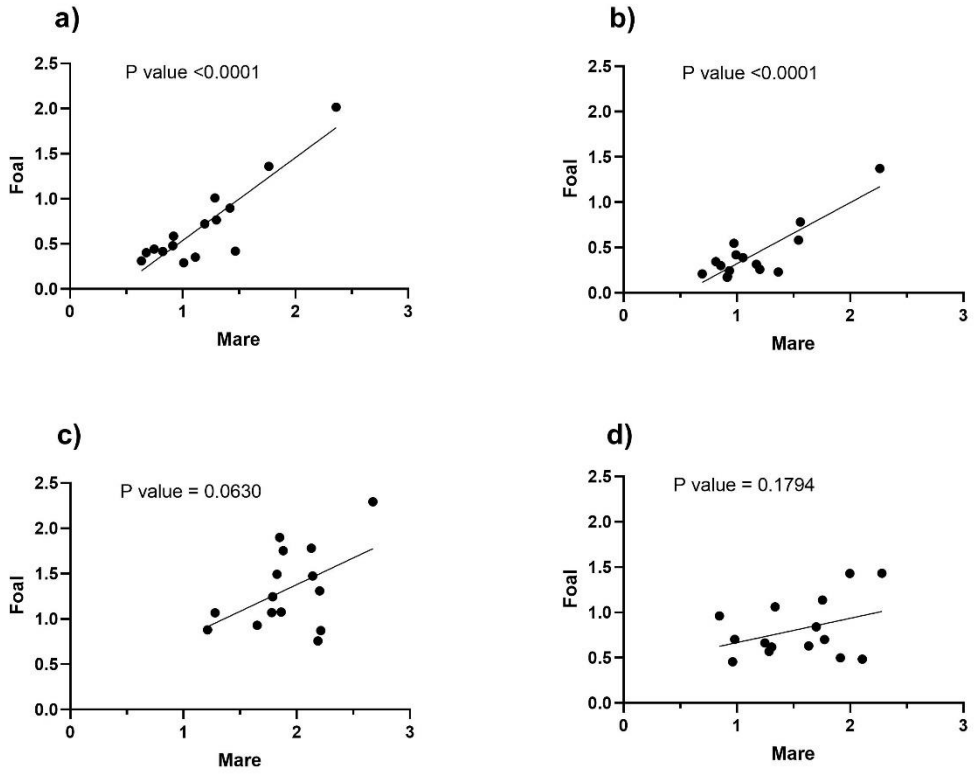

Figure 7. Simple linear regression of correlation of antibody levels in all included mares and foals at the first sampling occasion. Analysis of the: a) AsHb antigen with conjugate one, Anti-Horse IgG (Fc)-HRP. b) AsHb antigen with conjugate two, Anti-Horse (whole molecule)-Peroxidase Antibody. c) L3-Lung antigen with conjugate one Anti-Horse IgG (Fc)-HRP, and d) L3-Lung antigen with conjugate two, Anti-Horse (whole molecule)-Peroxidase Antibody. OD levels of the mares are shown on the x-axis and the corresponding foal on the y-axis. Regression was calculated by the Pearson r correlation test.

4.3 Faecal examination

All foals included in the study acquired a patent infection. As shown in Table 1, *Parascaris* spp. eggs were detected in the foals´ faeces when they were 13-19 weeks old. Some foals showed patency before the collection of the fourth serum sample, some after, and a few the same week as the fourth serum sample was collected.

None of the included mares shed any *Parascaris* spp. eggs in the faeces when they were examined in the beginning of the sample collection period, indicating the absence of a patent infection.

5. Discussion

Antibodies against *Parascaris* spp. could be detected from the foals using the AsHb ELISA and L3-Lung ELISA validated for *A. suum* by Vlaminck *et al.* (2012) and Vandekerckhove *et al.* (2017). The AsHb ELISAs show stronger significance between the second to fourth and the third to fourth samples than the L3-Lung ELISAs, however both were found to be significant. Both conjugates (Anti-Horse IgG (Fc)-HRP and Anti-Horse (whole molecule)-Peroxidase Antibody) worked well and seemed to be relative equivalent to each other in both the AsHb and L3-Lung ELISAs. Our study therefore indicates the possibility of developing a serodiagnostic test with further research including a larger sample size and possibly increasing the number of farm locations.

For clinical application of this serological method, a cut-off value needs to be determined to differentiate between infected and not infected foals. In the studies by Vlaminck *et al.* (2012) and Vandekerckhove *et al.* (2017) a cut off value separating positive and negative infections were determined at 0.5 OD mm for AsHb ELISA and 0.25 OD mm for L3-Lung ELISA. However, these values cannot be extrapolated to our study directly. For a cut-off value to be concluded for *Parascaris* spp. there is a need for negative controls, as used in the studies of Vlaminck *et al.* (2012) and Vandekerckhove *et al.* (2017). However, optimal negative controls would be foals reared in environments completely free of *Parascaris* spp. contamination, which would be complicated to achieve using privately owned foals from stud farms as the parasite has a prevalence around 50% (Lind & Christensson 2009). In addition, the parasite can remain viable for many years in the environment (Nielsen & Reinemeyer 2018). Therefore, the negative control foals would probably have to be reared experimental, which is ethically questionable. Another option would be to sample foals for blood before they receive their first colostrum.

It would also be interesting to investigate the antibody response in relation to the infection dose of positive controls as in this study we had no method of determining the infective dose, only that the foals became patently infected at the end of the study. In the study by Vandekerckhove *et al.* (2017), piglets were infected with specific doses of *A. suum* to compare the dose given with the antibody response. This would however be a challenge to apply in a study of foals, as they are mostly privately owned and naturally infected. However, including more stud farms with

various degrees of *Parascaris* spp. contamination is an option to investigate if the infection pressure in the environment affects the antibody level of the foals.

To reach a cut-off value for *Parascaris* spp. with serology, the time of collection also needs to be investigated further. The optimal time for when to sample the foals needs to be explored to limit false positives due to maternal antibodies or false negative due to not reached sufficient levels of antibodies in response to infection. Since some of the foals had generally higher antibody levels throughout the study in comparison to other foals (Figure 5), there is a possibility of generating false negative and false positive results depending on what level the cut-off value would be set to. In the study by Vandekerckhove *et al.* (2017), *A. suum* could be detected with the L3-Lung ELISA in six-week-old, infected piglets. In our study the significant variations in antibody levels did not occur until the fourth samplings, which is equivalent to changes in antibody levels are not notable until examined at around 13 to 16 weeks when the fourth blood sample was taken, which is notably later than for the piglets. To determine the optimal timepoint for testing, samples need to be collected more frequently around the time of the third and fourth sampling occasions, to see when the antibody levels begin to raise.

There was an indication of passive transfer of antibodies from the mares to the foals in our study, as the foals´ antibody levels showed an initial decrease between the first and second sampling occasion. Antibody levels then reach a plateau between the second and third sampling occasion, and then increase between the third and fourth sampling occasion (Figure 5). This pattern is similar to the antibody changes from passive transfer from the mother as the offspring first receive antibodies via the colostrum, which will decrease over time, to be replaced with the foal's own antibody production initiated by an infection. In addition, the correlation of mare and foal antibodies at the first sampling at two to three weeks of age was significant for the AsHb ELISA (Figure 7). However, it should be taken into consideration that the blood samples from the mares were collected when the foals were two to three weeks old. As the foals receive the colostrum the first day post-partum and the colostrum is formed in the udder during the week prior to the foaling (Sjastaad *et al.* 2016), antibody levels in the mares might have changed slightly with time.

The passive transfer of maternal antibodies is in correspondence with the conclusion drawn by Burk *et al.* (2016) when examining antibodies against *Parascaris* spp. excretory-secretory products on western blot. Burk *et al.* (2016) speculated that the maternal antibodies would interfere with a diagnostic test, however our study showed that the maternal antibodies decrease quickly and are replace by the foal's own antibodies somewhere between our third and fourth sampling occasion. This corresponds to an antibody level increase somewhere between week nine and ten to about week 14. As patent infection was detected by faecal examination between week 13-19, the serology has a possibility earlier diagnosis.

The mares had relatively high antibody titres for being deemed uninfected by the standard faecal examination (Figure 7). It is possible that they have a high antibody level to *Parascaris* spp. due to being exposed to the eggs continuously at the stud farm, but are averting the infection in an early stage. There could however be a risk of cross-reaction with antigens from other parasites, which has been a challenge in serodiagnostic for *A. suum*. Fan & Su (2004) found cross-rection between *A. suum* and *Toxacara canis*, as well as *Angiostrongylus cantonensis* for some of the antigens used when evaluating cross-reactions of different antigens with ELISA in experimentally infected mice. Similarly, Iglesias *et al.* (1996) observed high antigenic cross-reaction between *Anisakis simplex* and *A. suum*, when examining cross-reaction if different antigens for ELISA of *A. simplex* with ascaridoids and some other non-ascaridoid nematodes. It needs to be taken into consideration and further investigation that antigenic cross-reaction might be possible with equine parasites from other clades for this serological method.

Eventually, a serological technique for *Parascaris* spp. based on saliva would also be beneficial, as collection of saliva is less invasive than a blood sample. In addition, it would be easier in practise since the owners themselves can order a swab, collect saliva, and send it to a laboratory without having to involve a veterinarian. An easier accessible diagnostic tool for owners could also increase the surveillance of *Parascaris* spp. on farms. Saliva-based ELISA has been an alternative method for diagnostics for the horse tapeworm, *A. Perfoliate*. The saliva-based ELISA has been reported to have sensitivity of 83% (Lightbody *et al.* 2016), which is comparably high to the coprological method used for the parasite (Jürgenschellert *et al.* 2020). Therefore, whilst collecting blood samples for serology in this study, saliva samples were also collected with EquiSal TM saliva swabs for potential further studies concerning comparison of antibodies presented in serum to in saliva.

6. Conclusion

Results from the study showed that antibodies could be detected with the AsHb and L3-Lung ELISAs. Although antibodies were detected there is a need for further studies with a larger sample group, including negative controls to exclude the risk of misinterpreting results. It would be advantageous to develop this serological method for clinical diagnostic application and perhaps in the future use it as a tool for targeted deworming of infected foals instead of the routine use of anthelmintics. A targeted treatment plan for *Parascaris* spp. would be beneficial to limit the resistance development and reduce the amount of anthelmintic substances passed into the environment. This diagnostic method could also be applicable in prevalence studies and other research.

The potential of developing a serological diagnostic tool is evident in this study and there might also be an interest of expanding the investigation further and examine the possibility of serology by saliva samples for easier and non-invasive sampling.

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Popular science summary

Parascaris spp. are large intestinal roundworm parasites which commonly infects foals in their first year before they acquire an immunity to the parasite. Foals get infected by swallowing eggs from contaminated pastures and stables, where the eggs can survive and contaminate for many years. Once the ingested eggs reach the intestine they hatch into larvae. The larvae will thereafter travel from the intestine through the liver to the lungs. The damage done in the lungs will cause the foals to cough which helps the larvae to move up the airways and then be swallowed back into the intestines. Back in the intestine they mature into reproductive adult roundworms which can be up to 50 cm in length. The size and number of parasites can in severe cases be so immense that they block the intestine and cause obstruction that can lead to the foal's death without medical or surgical intervention. The prognosis even if treated surgically is guarded. However, the infection can pass without symptoms or can be milder than obstruction, but still affect the foal's wellbeing. Example of these associated symptoms can be tiredness, weight loss, impaired growth, enlarged abdomen, and coughing and nasal discharge when the larvae are passing through the lungs.

The way of diagnosing *Parascaris* spp. infection in a foal is currently by examining their stool for parasite eggs. However, the eggs are not present in the faeces until 10-14 weeks after the initial infection of the foal as the larvae needs to migrate through the foal and then have the time to mature into reproductive adult roundworms. This means that the foals can already be at risk for intestinal obstruction before it is possible to determine if they are infected. Therefore, most foals are routinely dewormed 2-3 times in their first year to avert this serious risk.

Three deworming drug substances are registered for the treatment of the parasite, however over the years there have been many reports of the parasites developing resistance to these drug substances. Therefore, the need for an alternative diagnostic method is necessary for the possibility of limiting the overuse of anthelmintics substances which is the cause of the emerging resistance.

A diagnostic method for detecting antibodies in serum from blood has been developed for a related roundworm from pigs, *Ascaris suum*. The method is called ELISA (Indirect Enzyme-linked Immunosorbent Assay). Two different antigens have been validated for the pig parasite, one called AsHb that has been derived from the adult roundworm and one called L3-Lung derived from the larval stage of the

parasite. As the pig and horse parasites are closely related, this study examined if antibodies for *Parascaris* spp. could be detected with the same method used for the pig roundworm.

A total of 15 foals and their mothers were followed throughout this study. Blood samples were collected when the foals were about two weeks old and then at three more occasions with about 4-week intervals. The mares were also sampled for blood in the beginning of the study to see if antibodies were passed on from the mothers to their foals via colostrum. Faeces were collected to confirm infection of the foals and see when the foals started to shed *Parascaris* spp. eggs. Serum was analysed at the University of Ghent and the faecal examinations were mostly analysed directly upon returning to the lab on the day of collection.

Results showed that antibodies could be detected with both ELISA methods. There was significant correlation of antibodies passed over from the mare to its foal in the colostrum for the AsHb ELISA. When examining the antibody levels of the foals over time, the foals had initially high antibody levels due to transfer of antibodies from mare to foal in the colostrum. Thereafter the antibody level decreased as the maternal bodies were diminishing in the foal. When the last serum sample was collected at about 14 weeks, the antibody levels had increased significantly compared to the second and third sample occasions at about six and 10 weeks, which is due to the foals´ own antibody production against *Parascaris* spp. infection.

On the other hand, no negative controls were sampled in this study as most foals are infected with this parasite, and therefore the antibody levels can only be compared between the different sampling occasions. Negative controls are however necessary to be able to derive a cut-off value to differentiate between infected and non-infected foals for clinical application.

For further studies it would be beneficial with an antibody test using saliva instead of serum as saliva sampling is less invasive, and owners can collect it themselves. A method of antibody test of saliva has already been developed for the horse tapeworm and could perhaps be applicable for *Parascaris* spp. Therefor saliva samples were collected alongside the serum samples for further studies.

This study showed the potentiality of an antibody test for *Parascaris* spp., but a larger sample size and negative controls are needed for further development. If an antibody test is developed, it could be used to find infection of *Parascaris* spp. easier and could be incorporated into a strategy of only treating foals with a positive result and thereby lead to limit the use of anthelmintic substances, which is beneficial for both constraining the emerging resistance and less residual leakage of anthelmintic substances to the environment.

Acknowledgements

Special thanks to my supervisor, Frida Martin, and assistant supervisor, Eva Tydén, for all the support and help with collecting and analysing the material and guiding me through the subject, participating stud farms and their personnel for their cooperation and contribution to the project, and of course, all foals and mares included in the project for calmly accepting our handling when gathering samples.

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