Aus dem Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Entwicklung einer innovativen diagnostischen Technik unter Verwendung der xMAP®Luminex®Technologie für die simultane Detektierung von Antikörper gegen C*ooperia oncophora*, *Dictyocaulus viviparus* und *Fasciola hepatica* in bovinen Serum und Milchproben

INAUGURAL-DISSERTATION
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To my brother, Konstantinos N. Karanikolas, the one and only hero I have ever met and to my parents, Magdalini A. Argyriadou and Nikolaos K. Karanikolas, my greatest supporters in every step I have made.

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ABBREVIATIONS

Ab	Antibody
AchE	Acetylcholinesterase E
Ag	Antigen
AR	Anthelmintic Resistance
BSA	Bovine Serum Albumin
BTM	Bulk Tank Milk
BZ	Benzimidazole
CI	Confidence Interval
CV	Coefficient of Variation
COOH	Carboxylated
EDAC	(1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride)
ELISA	Enzyme-Linked Immunosorbent Assay
ES	Excretory-Secretory
FEC	Faecal Egg Count
FGS	First Grazing Season
GI	Gastrointestinal
GST	Glutathione-S-Transferase
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IHA	Indirect Haemagglutination Assay
IPTG	Isopropylthio-Galactosidde
IVM	Ivermectin
kDa	kilodalton
L1	first stage larvae
L2	second stage larvae
L3	stage three infective larvae
L4	fourth stage larvae
m	meter
MFI	Median Fluorescence Intensity
min	minutes
MLs	Macrocyclic Lactones
MSP	Major Sperm Protein
OD	Optical Density
ODR	Optical Density Ratio
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGE	Parasitic Gastroenteritis
ROC	Receiver Operating Characteristics
rpm	revolutions per minute
RT	Room Temperature
SA-PE	Streptavidin-Phycoerythrin
SD	Standard Deviation
s	seconds
S-NHS	(N-hydroxysulfosuccinimide)

1. INTRODUCTION

Infections caused by parasitic helminths belonging to the classes of Nematoda or Trematoda are of great financial and animal welfare significance to the global ruminant livestock industry. A widely distributed phenomenon comprises the growing prevalence of anthelmintic resistance (AR), showing that currently used control measures are both, costly and not sustainable in the long term. Alterations regarding epidemiology, seasonality and geographic distribution of helminthoses are most likely also influenced by climatic changes. It is additionally observed that changes concerning the environment and the livestock farming, intensification of the latter and altered management practices affect the frequency of helminth infections (for more details see http://www.gloworm.eu).

Livestock farming is crucial to the sustainability of rural European communities and therefore the social, economic and political significance on national and international basis plays an important role. Data obtained by FAOSTAT (2009) estimated that the cattle population in Europe is approaching 88 million. Current socio-economic and environmental changes will highlight the need for increased food security, obtained from sustainable intensification of agriculture (Baulcombe, 2010). Production of meat and dairy products may also increase in the future due to the increasing world population. One important key to achieve this goal is the ability of the European livestock industry to achieve sustainable optimal production levels under the pressure of continuously changing environmental and socio-economic factors.

All grazing animals are exposed to helminth infections while on pasture, any intensification of farming will lead to a decrease in the occurrence of helminth infection or diseases, however, consumer demands regarding the animal welfare issues generally include the access to pasture for grazing livestock so that this in combination with an increasing number of animals will lead to an increase of helminth infection/disease. Gastrointestinal (GI) nematodes and liver fluke have been shown to be the two most important causes of reduced productivity in ruminants by the DISCONTOOLS programme (for more information seehttp://www.discontools.eu). Frequency and intensity of these parasitic diseases increased in the European ruminant sector (van Dijk et al., 2010), mainly due to climate changes that increase the developmental success of helminths as well as the altered land use, the current farm practices and the emergence of AR (Morgan and Wall, 2009). Financial losses due to these parasitic diseases are rather difficult to estimate, however; Schweizer et al. (2005) calculated that the cost of fasciolosis in cattle approached 52 million € per year in Switzerland alone. Estimations concerning the amount of money spent within Europe for anthelmintic drugs were performed by Selzer (2009), who classified them in the order of 400 million €. Many consider such calculations to be only the tip of the iceberg of the actual cost of livestock helminthoses endemic in the different countries of the European Union (Charlier et al., 2009).

Parasitic gastroenteritis (PGE) in cattle is mainly caused by infections with *Ostertagia ostertagi* and *Cooperia oncophora.* Fasciolosis appears to be distributed worldwide and accordingly outbreak incidents are reported globally. Additionally, the lungworm *Dictyocaulus viviparus*, responsible for the considerably pathogenic bovine bronchitis, is prevalent in several regions in the EU. Control of these parasites is so far achieved by chemotherapeutic or metaphylactic use of anthelmintics. This practise has led to the development of AR. Accordingly, the development and establishment of new and sustainable strategies for monitoring and control of these parasites are urgently needed. Within such strategies, diagnostic of the causative pathogens is the most important step. Diagnostic techniques have to fulfil a range of requirements. They should be specific for the different pathogens, sensitive enough

to detect low infection levels, easy to apply for the farmers (e.g. in terms of sampling) and cost effective. Given the increasing number of farmed animals, diagnostic high throughput methods are needed. The currently available methods do lack at least one or more of the above mentioned requirements. Classical coproscopic methods are incapable to differentiate between the morphologically identical eggs of most GI nematodes and are not sensitive enough to reliably detect eggs which are excreted intermittently (e.g. *Fasciola hepatica*). Serological methods are available for the diagnosis of some parasites but often lack specificity.

The aim of this study was the development of a new diagnostic platform which enables the simultaneous detection of the three most important parasites, *C. oncophora*, *D. viviparus* and *F. hepatica*. A bead based assay was established using the xMAP® Luminex® Technology with defined serum samples obtained from parasite naïve or specifically mono-infected animals. Subsequently this assay was adapted for the use with milk samples, since milk samples are easy and cheap to obtain in practise. Additionally the newly developed technique was used to examine a large amount of samples, serum and milk, which were obtained from a harmonised spatial sampling approach within the GLOWORM project.

2. LITERATURE REVIEW

2.1. NEMATODA

Within the Cycloneuralia the group Nematoida is constituted of the two phyla, Nematoda (gr. νήμα "string") and Nematomorpha. Particularly the phylum Nematoda contains parasites of medical significance. They are found in a broad range of environmental conditions and are, in several cases, difficult to distinguish with traditional diagnostic methods. Up to now more than 26.000 of nematode species have been extensively described (Boch et al., 2006).
Morphology and basic characteristics of nematodes are extensively described in several parasitological books (Boch et al., 2006; Taylor et al., 2007). The majority of nematodes are slender, have a cylindrical form and their body is covered by a colourless layer, the cuticle, which may consist of two or three different layers. The cuticle is concealed by the underlying hypodermis, projecting into the body and forming two lateral cords, carrying the excretory canals, and a dorsal and ventral cord containing the nerves. Between hypodermis and the body cavity, a layer of longitudinal muscle cells is found.
The digestive system is tubular. The oral cavity is a simple mouth opening, surrounded by two or three lips, leading directly to the oesophagus. The latter may vary in shape (filariform, bulb-shaped, double bulb-shaped, muscular-glandular, trichuroid, rhabditiform) among different parasite species. The absence of a stomach is characteristic and the oesophagus leads directly to a muscleless intestine, where several enzymes are produced and nutrients are absorbed. In female worms it terminates in an anus, whereas in males, a cloaca is found that acts as an anus. The last part of intestine is lined by cuticle, forming the rectum, which expels waste through the anus. Movement of the food is achieved during the movements of the worm.
The nervous system consists of four peripheral nerves, the paired dorsal and ventral cords (motoneural nerves) and additionally lateral (sensory) structures.
Most nematode species have separate female and male individuals. Their reproductive system consists of thread tubes. Female organs include the ovary, oviduct and the uterus, which ends in a short vagina that opens at the vulva. In males, sperms are produced at the end of the gonad and mature through migration along its length. Each of the testes opens into a wide sperm duct, followed by a glandular and muscular ejaculatory duct connected with the cloaca. Generally males are smaller in size when compared with females, which lay either eggs or larvae. Produced eggs, embryonated or not, may differ in size and shape whereas their shell presents variable thickness, usually consisting of three distinguishable layers.
The superfamilies of trichostrongyloids and strongyloids are characterised by a free-living (pre-parasitic) phase. Different species feed on different materials such as fungi, bacteria, faecal substance, dead organisms and living tissues. During development, nematodes shed at intervals, moulting their cuticle always in four successive larval stages. In many parasitic nematodes, the cuticle of the second stage larvae (L2) is kept as a sheath around the third stage larvae (L3) stage, protecting the infective stage. External environment, temperature and humidity in particular, affect the development of these stages.
The superfamily of *Trichostrongyloidea* (gr. τρίχος „hair") contains the group of gastrointestinal (GI) nematodes such as for example *Trichostrongylus*, *Haemonchus*, *Teladorsagia*, *Ostertagia* and *Cooperia* as well as the lungworm *Dictyocaulus*, which are all of great importance to livestock. Briefly, the trichostrongyloids are small (3 to 5 mm long), often hair-like worms, which, except for *Dictyocaulus viviparus*, parasitise

the alimentary tract of animals. They are characterised by the presence of few cuticular appendages while their buccal capsule is vestigial. Male worms have a well-developed bursa and two spicules, the shape of which is important for differentiation of species. Female reproductive organs comprise ovaries, oviduct and uterus, which are usually paired, ending in a short unpaired vagina, which opens at the vulva located in the last third of the body. A short muscular organ, the ovijector, assisting egg laying, is present at the junction of uterus and vagina in some parasite species. A vulva flap may also be present (Taylor et al., 2007). Trichostrongyloids are responsible for considerably high mortality rates and widespread morbidity, especially in ruminants.
Cooperia oncophora and *Ostertagia ostertagi* are considered the most important GI nematodes in Northwest Europe (Kloosterman et al., 1984). Other parasite species responsible for parasitic gastroenteritis (PGE) in large and small ruminants across the world are *Haemonchus*, *Trichostrongylus* and *Nematodirus*.
Depending on the parasite species, hatching of eggs may occur either outside the host body, being controlled by factors such as temperature and moisture, or after ingestion in appropriate regions of the gut. Infection of the host occurs after the ingestion of the infective L3. The time needed from infection until the larvae reach maturity is defined as the prepatent period and varies among different nematode species.

2.1.1. *Cooperia oncophora*

2.1.1.1. Life cycle and epidemiology

C. oncophora parasitises in the small intestine of predominantly cattle, but also small ruminants and deer (Taylor et al. (2007). They have a direct life cycle (Figure 1), initially described by Isenstein (1963) which is divided in two distinct phases:

- Non-parasitic free-living phase: eggs are excreted by infected hosts and under optimal temperature and moisture conditions, hatch and develop via L1 and L2 into the infective L3 inside the faecal pat within 1-2 weeks post shedding.
- Parasitic phase: This phase begins with the ingestion of L3 (usually during grazing) and is characterised by the establishment of infective L3 in the host. Age and immune response of the host are the most important factors affecting the number of adults establishing in the host as well as the fecundity of the female worms. The ingested L3 exsheath and migrate into the intestinal crypts where they complete their development into adults which finally parasitise on the surface of the intestinal mucosa in the first third of the small intestine. Prepatency is approximately 2-3 weeks.

The contamination of pasture with infective L3 can either occur through the excretion of eggs by already infected hosts or through the larval population which survived in the environment. L3 are known to be able to survive on grass as well as in the soil for weeks up to months. Stromberg (1997) reviewed a wide range of environmental factors influencing development, transmission and survival of nematode larvae. Their survival is mostly influenced by humidity/moisture and temperature changes. Callinan and Westcott (1986) reported that the mean percentage of recovered L3 from herbage tends to decrease when humidity decreases and when the temperature rises from 15 to 30 °C. Agneessens et al. (1997) indicated that dry summer days lead to lower rates of *C. oncophora* in calves, while increased rainfall during the grazing season intensifies the parasite burden. This is in accordance to results obtained by O'Connor et al. (2008), where the amount of recovered L3 from pasture increased accordingly to the amount of rainfall. Desiccation appears to affect susceptible free-living larval stages, leading to their rapid killing according to Callinan and Westcott

(1986); Garcia Romero et al. (1997). Survival of desiccation on the other hand, may be achieved through the anhydrobiotic status of larvae, which leads to increased tolerance towards environmental factors (Lettini and Sukhdeo, 2006).

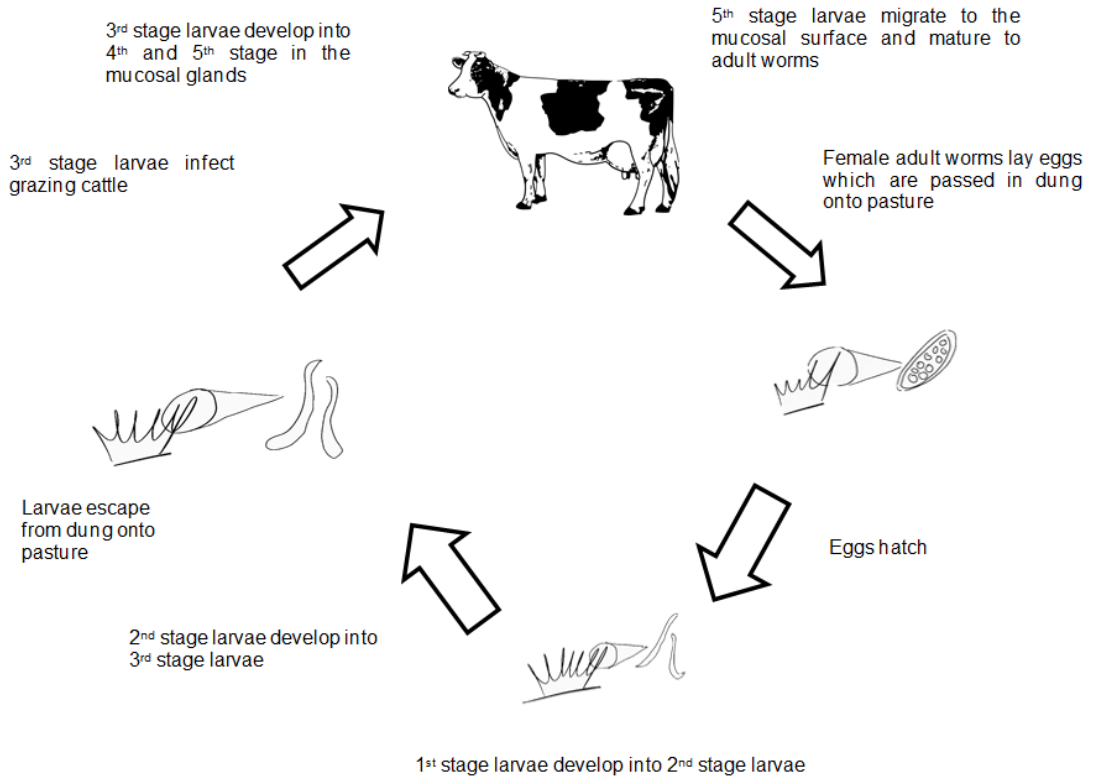

Figure 1. *Cooperia oncophora* - life cycle

C. oncophora L3 stay on pasture until ingested however it has been observed that they constantly migrate on and off herbage (Crofton, 1948). L3 need to be on pasture in order to continue their life cycle, it is assumed however that the migration into the soil extends their lifespan as it protects L3 from dehydration, high temperatures (Demeler et al., 2012; Knapp-Lawitzke et al., 2014) and UV radiation (van Dijk et al., 2009). This has also been shown in field trials where soil was identified as a potential reservoir for infective L3 (Bennema et al., 2010). Moreover, the composition of pasture in combination with the length of grass have an effect on L3 occurrence (Callinan and Westcott, 1986; Marley et al., 2006). Increased growth of both grass and legumes provides a dense “pasture cover”, protecting L3 from death by leading to a less pronounced desiccation of soil (Knapp-Lawitzke et al., 2014). Ramírez-Restrepo et al. (2005) more specifically, indicated that ingestion of *Lotus corniculatus* by grazing animals leads to a decrease of parasitic nematodes due to the condensed tannins which subsequently reduce the fertility of the adult parasites of *C. oncophora* in the intestine of sheep. Recently conducted investigations revealed that larval survival of desiccation has an influence on fitness of L3 and subsequently on fecundity of females. Furthermore it is important to notice that tolerance in regards to high temperatures and decreased humidity is different between nematode species (Chylinski et al., 2014; Leathwick, 2013). Infections with *C. oncophora* are acquired as soon as calves are turned out onto pasture (Githiori et al., 2000). Therefore every grazing animal will be infected and depending on the nematode control programme on a farm, infections can lead to clinical symptoms or mainly remain subclinical. However, upon the first contact with the parasite the immune system reacts with the

production of antibodies (Abs) against a particular nematode species. A challenge infection trial with *C. oncophora* and *O. ostertagi* in order to examine protective immunity in the subsequent housing period has been reported by Ploeger et al. (1995). They showed that in contrast to calves challenged with *O. ostertagi*, acquired and protective immunity against *C. oncophora* reinfection developed rapidly and strongly during the first grazing season (FGS). This may be due to the site of parasitism through a site-specific ability of the host to recognise pathogens and to develop certain immune responses. Additionally, it was observed that the level of exposure during infection and the timing of the infection (midsummer season) affected the development of immunity. Although the acquired immunity is in most cases not fully protective, it reduces the establishment of further parasites and also reduces the fecundity of females. Due to restrictions of anthelmintic use in lactating animals in combination with the usually only subclinical infection and the general costs of anthelmintics, most dairy cows are not regularly treated. Accordingly, infections with *C. oncophora* are widespread in Western Europe dairy cattle herds (Bisset, 1994; Kanobana et al., 2004; Karanikola et al., 2015; Kenyon and Jackson, 2012; Nogareda et al., 2006; Piekarska et al., 2013).

2.1.1.2. Pathogenesis and Clinical symptoms

Infections with *C. oncophora* are in most cases associated with *O. ostertagi* (Areskog et al., 2014; Nilsson and Sorelius, 1973). The two parasites differ in fecundity, pathogenicity, and immunological response of the host. According to Kloosterman et al. (1984) there is a reciprocal negative interaction between these two parasites. In the study of Frakena (1987) it was suggested that this interaction might be immune mediated and was caused by antigenic substances, common in both species.
C. oncophora is generally considered to be of lower pathogenicity. Other species of the same genus, for example *Cooperia pectinata* (Coop et al., 1979; Herlich, 1965) or *Cooperia punctata* (Stromberg et al., 2012) are in contrast more pathogenic. *Cooperia* spp. are all responsible for causing infections of the gastrointestinal tract which are associated with diarrhoea and subsequently reduced weight gain. Immunity to the infection is obtained 8 to 12 months following exposure to the parasite. In cases of heavy infections, catarrhal enteritis with localised villous atrophy and oedema of the intestinal mucosa is observed. Clinically cooperiosis is characterised by reduction in the appetite while, in heavily infected animals, intermittent diarrhoea is induced (Taylor et al., 2007). The economic effects of cooperiosis in adult cattle, in terms of weight gain, milk yield and reproduction are still widely unknown. In the study of Stromberg et al. (2012), which was conducted with calves experimentally infected with *C. punctata*, it was observed that the uninfected control group gained weight 7.5% more quickly in comparison to the infected animals. Additionally, *Cooperia*-infected calves consumed less dry feed per day, suggesting that cooperiosis negatively affects appetite, nutrient uptake or utilization. GI nematode infections occurring in the first 2 years of life appear to have a negative impact on later milk production particularly by reducing weight gains. Subsequently, time to reach breeding weight extends, leading to reproductive and milk-yield related costs. In the study of Barger and Gibbs (1981), uninfected cows produced 2.2 kg more milk/cow per day compared to the experimentally infected cows. Due to the small number of animals in the trials however, significance of the results was either not reported or not reached. In three other studies, no significant effects on milk yield after inoculation with infective larvae were observed (Kloosterman and Albers, 1982; Kloosterman et al., 1985; Pitt et al., 1988). Subsequently, the results from trials with experimental infections were further investigated by assessing the effect of anthelmintic treatment on milk yield. Charlier et al. (2009) reported an increase in milk production following anthelmintic treatment in 80% of the trials, with a median

increase of 0.6 kg/cow per day, leading to the assumption that anthelmintic treatment may result in improvement in milk-yield response. However, no data are currently available concerning the impact of *C. oncophora* infection on adult dairy cattle.

2.1.2. *Dictyocaulus viviparus* – The bovine lungworm

2.1.2.1. Life cycle and epidemiology

D. viviparus, the bovine lungworm, is a parasite of worldwide interest and it parasitises in the respiratory system of cattle affecting the trachea and bronchi of the hosts.
The life cycle of *D. viviparus* is direct (Figure 2). The female worms (6-8 cm long) are ovo-viviparous, producing eggs containing fully developed larvae that hatch almost immediately after their deposition. The L1 are transported up the trachea through cilia on epithelial cells, are swallowed and pass out in the faeces. The larvae are present in fresh faeces, and when observed under the microscope, their intestinal cells are filled with dark brown food granules. Consequently, the pre-parasitic stages do not require specific feeding. Under optimal conditions the L3 develop within 5 days and then seek the herbage either by their own motility or using the ubiquitous fungus *Pilobolus*, which is often growing on the surface of bovine faeces approximately 1 week after deposition. The larvae tend to migrate up the stalks of the fungi on to, and even inside, the sporangium. As soon as the sporangium is discharged, L3 are projected a distance of up to 3 m in calm air to land in the surrounding herbage. After their ingestion, L3 penetrate the intestinal mucosa and pass to the mesenteric lymph nodes where they moult. The fourth stage larvae (L4) migrate via the lymph and blood system to the lungs where it breaks out of the capillaries into the alveoli about one week after initial infection. The final moult occurs in the bronchioles a few days later and the young adults migrate up the bronchi and mature. The prepatent period lasts approximately 3 to 4 weeks (Taylor et al., 2007).
Parasitic bronchitis is relatively frequent in temperate climate areas with high rainfall rates and abundant permanent grass (Klewer et al., 2012). It occurs from June until November and leads to severe clinical pulmonary infections. In areas with tropical climate, where the disease is mainly attributed to carrier animals, its frequency increases during the flooding season. Clinical disease usually develops upon first exposure to sufficient number of infective larvae and severity as well as stimulation of an immune response is related to the number of larvae ingested (http://www.merckvetmanual.com/mvm/index.html). The disease typically affects young cattle during their first season on permanent or semi-permanent pasture since on farms, where the disease is endemic, older animals have acquired immunity. Adult cows play a significant role in the epidemiological distribution of the parasite. The presence of a low number of asymptomatic animals that excrete larvae onto pasture, is important to provide infective stages for naïve young animals (Eysker et al., 1994).

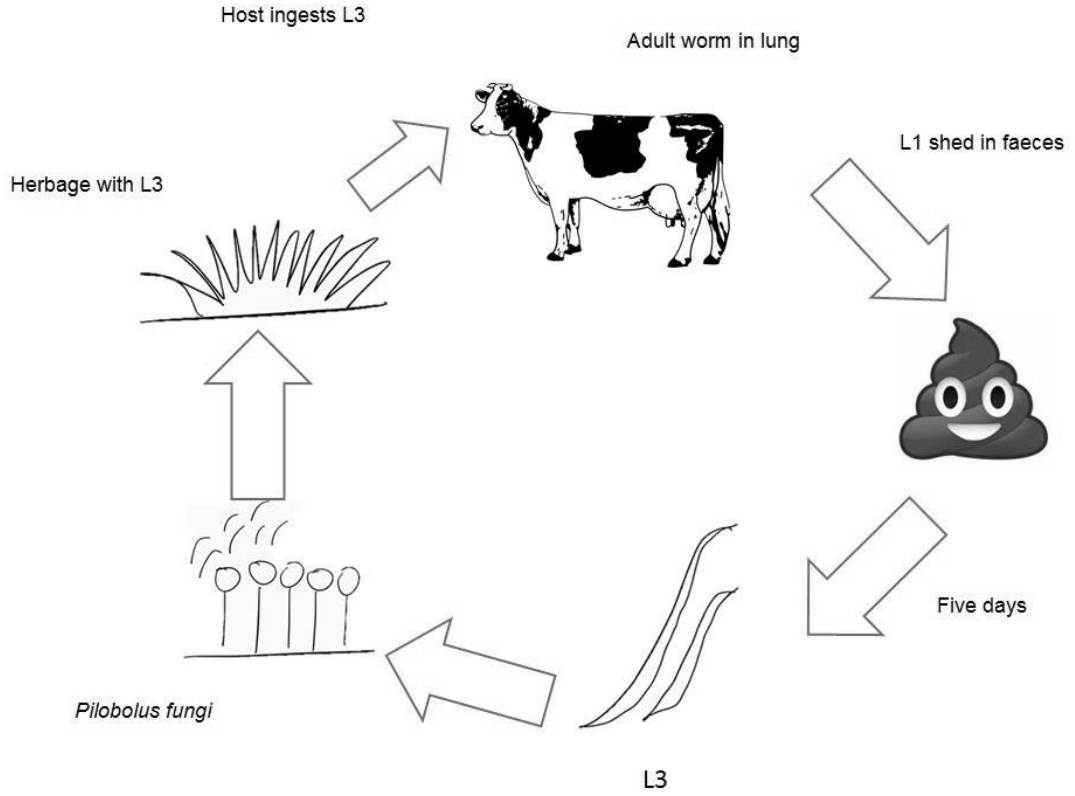

Figure 2. ***Dictyocaulus viviparus*** **– life cycle**

Lungworm infections in the northern hemisphere may persist from year to year through overwintered L3 and through animals carrying hypobiotic larvae. The phenomenon of hypobiosis occurs naturally after ingestion of larvae in late autumn and its significance in the transmission of the parasite has not yet been fully discovered. It represents an interruption or postponement of larval development inside the host with the consequence of reduced parasite metabolism (Strube et al., 2007), which enhances parasite survival during times of adverse environmental conditions (Blitz and Gibbs, 1972a, b; Gibbs, 1982). The hypobiotic larvae tend to persist in the lungs of the infected cattle, interrupt maturation into egg-laying adults until the following spring, a phenomenon frequently observed when low temperatures occur during autumn (Armour and Bruce, 1974; Blitz and Gibbs, 1972a; Eysker, 1981).

However, calves are considered to be more sensitive to lungworm infections upon their first contact since only very low numbers of overwintered larvae present on pasture early in the season have the following effect: 1) the immune system is not fully activated and therefore no sterile immunity develops, 2) the number of adult worms establishing in the host does not lead to clinical signs but 3) still lead to contamination of the pasture. Later in the season these calves can suffer from auto-infections with higher number of larvae causing moderate disease while previously unexposed calves often show severe clinical symptoms (Ploeger, 2002). Despite the fact that dairy calves are more susceptible to dictyocaulosis, autumn-born single-suckled beef calves (suckling at their mothers during the housing period) are also susceptible as soon as they are turned out onto pasture early in the summer. Contrary to that, spring-born single-suckled beef calves co-grazed with their dams until housed, only present coughing due to mild infection. In this case, the parasitic bronchitis may appear in weaned calves, grazing until the autumn season (Taylor et al., 2007). Over the last years an increase, attributed to the extensive use of

prophylactic anthelmintics in calves, which prevent exposure to the parasite at an earlier age, has been reported in adult cattle.
Increased numbers of outbreaks have been reported worldwide either due to a primary infection or to reinfection. The latter is characterised by an immune-mediated inflammation in the respiratory system as a response to the invasion of the juvenile parasite stages to the lungs (Breeze, 1985; Michel, 1957). Epidemiological studies have reported outbreaks of the disease in the Netherlands (Holzhauer et al., 2003; Holzhauer et al., 2011; Muskens and Otten, 2009), in the UK (David, 1997; van Dijk, 2004) and across the Atlantic in Canada (Wapenaar et al., 2007).
Economic losses have been estimated in several studies indicating that the financial cost per cow approaches 300 € in the UK (Woolley, 1997), whereas Holzhauer et al. (2011) reported those costs at 170 € approximately in the Netherlands, indicating a correlation between outbreaks of the disease and reduction in the milk yield and a slight increase in the number of deaths in the infected herds.
Prevalence of *D. viviparus* in Europe indicated an increasing trend in the presence of the parasite in dairy herds in the last decades. In the Netherlands lungworm infections reach 70-80% according to either the examination of individual faecal samples or the detection of specific Abs in bulk tank milk samples (BTM) (Eysker et al., 1994; Ploeger et al., 2012). In the area of Flanders, Belgium, the calculated prevalence approached 19.6% (Bennema et al., 2009), whereas in a study conducted in Sweden (Hoglund et al., 2010) higher sero-prevalence in organic herds (18%) were detected in comparison to the conventional ones (9%). An epidemiological research in Northern Germany (Klewer et al., 2012) reported *D. viviparus* exposure in 12.8% of dairy herds.

2.1.2.2. Pathogenesis and Clinical symptoms

The pathogenic effect depends mainly on the number of the ingested infective larvae, the locations of lungworms in the respiratory tract and the status of the immune system of the animal (http://www.merckvetmanual.com/mvm/index.html).

Dictyocaulosis can be divided in three distinct phases (Taylor et al., 2007):

1. prepatent phase (around 8-25 days post infection, d.p.i.): appearance of larvae within the alveoli, followed by bronchiolitis and finally bronchitis as the larvae turn into immature adults and move up the bronchi. Immature lungworms are detected in the airways and cellular infiltration of the epithelium of the bronchi by inflammatory cells, causing collapse of other groups of alveoli. Heavily infected animals may die from day 15 onwards due to respiratory failure.
2. patent phase (around 26-60 d.p.i.): associated with parasitic bronchitis, leading to a chronic eosinophilic, granulomatous pneumonia caused by the aspiration of eggs and L1 into the alveoli. Histological examination reveals hyperplasia of the bronchial epithelium, which is also heavily infiltrated by eosinophils polymorphs, macrophages and multinucleated giant cells.
3. post-patent phase (around 61-90 d.p.i.): the recovery phase in untreated calves, following dismissal of the adult lungworms. Despite an improved clinical image, the bronchi are still inflamed and residual lesions may persist. Eventually the bronchopulmonary system becomes completely normal and coughing ceases. Heavily infected animals can present flare-up of clinical signs during this phase, which frequently leads to death and is caused either by epithelisation of the lung due to the large number of type 2 pneumocytes on the alveoli (post patent parasitic bronchitis) or by bacterial infection of the poorly healed lungs, causing acute interstitial pneumonia.

Clinical symptoms of dictyocaulosis may vary from mild to severe among animals of the same herd. Mildly infected animals cough intermittently, especially during exercising. Moderate infections are characterised by the appearance of periods when the animal coughs while being at rest, tachypnoea, and in some cases hyperpnoea. Squeaks and crackles over the posterior lung lobes are a common phenomenon during auscultation. Severely infected animals are characterised by severe tachypnoea combined with dyspnoea. At this stage, animals frequently adopt a quite characteristic “air hunger” position and breathe heavily (Holzhauer et al., 2003). Side symptoms, such as deep harsh cough, squeaks and crackles over the posterior lung lobes, salivation, anorexia and mild pyrexia may also occur. In cases of massive infections, severe dyspnoea of sudden onset can occur and be followed by death within 24-48 hours (Taylor et al., 2007).
Studies regarding the effect of parasitic bronchitis on milk production of herds are limited. Holzhauer et al. (2011) estimated that milk production in 2 Dutch dairy herds declined by 15 to 20% during a severe clinical outbreak of lungworm infection, resulting in a cost of 160 € per cow present during the outbreak. The aim of a study by Dank et al. (2015) was to determine the impact of lungworm infections on dairy cattle herds on different milk production parameters such as milk yield, milk protein and fat content (%). In this study, the average milk yield was lower in infected herds (1.01 kg/cow per day), a reduction lower than the one reported by Holzhauer et al. (2011) who described a loss of 4 kg/cow per day as a result of a *D. viviparus* infection. The negative association between dictyocaulosis and milk production was stronger in late summer/autumn than in early summer, which is in agreement with the study of Bloemhoff et al. (2015). Additionally, a significantly negative relationship between *D. viviparus* infection and milk fat was reported (infected herds presented 0.14% lower average milk fat rate in comparison to negative ones). No significant association was observed between BTM status and milk protein percentage in the herds participating in the survey.

2.2. TREMATODA

The class of Trematoda, firstly described by Rudolphi in 1808, includes two different groups of internal parasitic flatworms, widely known as flukes. Trematodes include 18.000 (Littlewood and Bray, 2000) to 24.000 (Chitwood, 1999) species and are further divided in two subclasses, the Digenea (indirect life cycle) and the Monogenea (direct life cycle). The former are detected exclusively in vertebrates (final hosts) and in invertebrates (intermediate hosts) and are of great veterinary importance while the latter are reported as external parasites of fish.
The indirect life cycle includes at least one intermediate host. The definitive host is generally a vertebrate providing optimal conditions for the sexual reproduction of the flukes. The intermediate host on the other hand is the agent of dispersion and is normally a snail.
Taylor et al. (2007) provides an extensive description of the morphology and anatomy of the class of Trematoda. The majority of the flukes is dorsoventrally flattened, have a blind alimentary tract and are hermaphrodites. Their most distinctive external features are the two muscular suckers possessed by the adults for their attachment. The oral sucker is placed at the anterior end surrounding the mouth, while the ventral sucker is on the surface. The body surface consists of a tough syncytial tegument, which provides protection to the flukes against digestive enzymes produced in the gut of the host and favours the gas exchange, since trematodes lack respiratory organs.
The digestive system is simple. The oral opening, located at the anterior end of the fluke, leads to a pharynx, connected to a short oesophagus and a pair of branched,

blind-ended intestinal caeca. The excretory system comprises a large number of ciliated flame cells, which push the waste material to the exterior (protonephridia). The nervous system has a simple structure, consisting of a pair of ganglia in the head region, from which two or three nerve cords run along the ventral and the dorsal surface underneath the tegument.
Trematode parasites are simultaneous hermaphrodite, having both male and female reproductive organs. Normally two testes are found in the male reproduction parts, with sperm ducts that join together on the underside of the front half of the fluke. The female system has a single ovary leading to an oviduct that expands and forms a dilated ootype connecting to a number of vitelline glands, which produce yolk cells, surrounding the eggs.

2.2.1. *Fasciola hepatica*

The family of Fasciolidae consists of large leaf-shaped flukes, whose front ends are prolonged into the shape of a cone, at the end of which an anterior sucker is located. The ventral sucker is placed at the level of the “shoulders” of the fluke. The internal organs are branched while the cuticle is covered with spines (Taylor et al., 2007).

2.2.1.1. Life cycle and epidemiology

F. hepatica is a parasite of the liver in mainly ruminants, but fasciolosis also occurs in sheep, horses, donkeys, deer, pigs and rabbits, which are able to act as reservoirs of infection (Taylor et al., 2007). Fasciolosis also occurs in humans and is considered to be a neglected tropical disease (Hosseini et al., 2015). It is distributed worldwide and responsible for major financial losses in the animal husbandry industry due to reduced carcass weight and milk production (Behm and Sangster, 1999; Marley et al., 1999; Salimi-Bejestani et al., 2007).
The life cycle (Figure 3) of the parasite involves an intermediate host, the snail *Lymnaea* (syn. *Galba*) *truncatula*, and lasts approximately 6 to 7 weeks under optimal environment conditions (Boch et al., 2006).
Eggs are laid by adult flukes in the bile ducts, enter the intestine and are passed into the environment, where they develop and hatch, finally releasing motile ciliated miracidia. The miracidium is not able to survive in the environment for a long time without a host and as a result it must locate a suitable snail within 3 hours. As soon as the snails are infected, development into sporocyst, then into a redial stage (including asexual reproduction) and finally development into cercaria takes place. The cercaria are released from the snail and attach on firm surfaces (e.g. grass blades) were they transform into the infective metacercariae that can survive unfavourable environmental conditions. When ingested by the final host, they excyst in the small intestine, migrate through the gut wall and penetrate the liver capsule. The young flukes dig a tunnel through the liver parenchyma and after a few weeks they enter the small bile ducts, migrate to the larger ducts and finally the gallbladder, where they reach sexual maturity (Boch et al., 2006; Taylor et al., 2007).

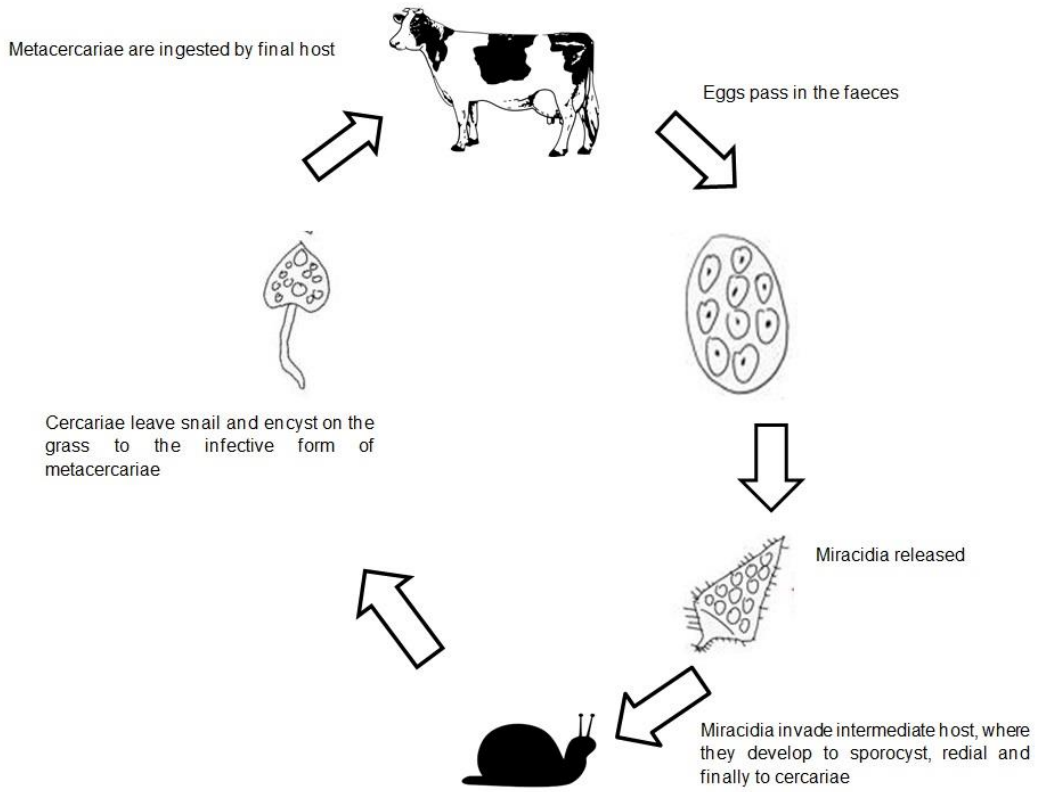

Figure 3. ***Fasciola hepatica*** **– life cycle**

Outbreaks of *F. hepatica* appear when a series of contributing factors occur together, leading to an increase in the number of infective metacercariae. These factors can be summarised in the availability of suitable snail populations and in the ideal temperature and moisture conditions (Fox et al., 2011; Mas-Coma et al., 2008; van Dijk et al., 2010). Climatic and geographical variables are known to be important in determining the risk of fluke infection, due to their effect on the survival and rate of development of the parasite on pasture and in the intermediate host, *L. truncatula*. Farm management factors may also affect the risk of cattle being infected with *F. hepatica* (Bennema et al., 2011; Morgan and Wall, 2009). Other factors including length of grazing season, proportion of fresh grass in the diet, stocking rate, type of drinking water supply, grazing on mowed pastures and herd size induce fluke infection risk through still unknown mechanisms (Bennema et al., 2011; Charlier et al., 2011; Charlier et al., 2014b).

Without the presence of *L. truncatula* the immature parasitic phases cannot develop and reach maturity. A temperature range of 10-25°C is required for both, liver fluke larval stages and the intermediate host, to enhance development and maturity of the parasite (Rapsch et al., 2008). The snails require wet mud and permanent habitats include the banks of ditches or streams and the edges of small ponds. An environmental temperature higher than 10°C is considered ideal for the development of the parasitic stages, both in the intermediate and final host. Temperatures lower than 5°C, cease the completion of the life cycle while temperatures exceeding 15°C, enhance the multiplication of snails and the development of *F. hepatica.* Climatic changes in general have an effect on the intermediate host and therefore subsequently on density, survival and dispersion of the different fluke stages in the environment (Gale et al., 2008).

As far as moisture conditions are concerned, high rainfall rates induce the development of the parasitic stages inside the intermediate host (Torgerson, 1999). The prevalence of *F. hepatica* in the UK has increased over recent years. Fasciolosis has been reported in new areas as a result of wetter summers and warmer winters

(Fox et al., 2011; Kenyon et al., 2009; Mitchell, 2002; Pritchard et al., 2005). However, in a three-year study conducted in Belgian dairy herds (Bennema et al., 2011) rainfall was negatively correlated with the presence of economically important liver fluke infections.

It has been observed that in countries with temperate climate, such as UK, fasciolosis appears with higher frequency, when the number of metacercariae on pasture increases after a period of optimal moisture conditions (May to October) (Altizer et al., 2006; Goodall and Menzeis, 1995). The explanation lays on the summer and the winter infection of the snails in which metacercariae appear on pasture from August to October and from May to June, respectively. If snails are infected in the summer, infections arise from miracidia, which have hatched either from eggs excreted in the spring/early summer by infected animals, or from eggs, which have survived the winter in an undeveloped state. Development of the parasite in the intermediate host takes place during the summer and the cercariae are shed from August until October (Goodall and Menzeis, 1995; McIlroy et al., 1990). In the so called "winter infection" metacercariae developed in snails which were infected the previous autumn. The larval development stopped during the winter and then recommenced in the season of spring (Goodall et al., 1991; Luzon-Pena et al., 1994). It is remarkable that both, the eggs and the metacercariae of *F. hepatica,* are able survive during winter, by progressing into miracidia in the late spring and then affecting the snails, and by infecting the herd in early spring respectively. The latter results in the production of eggs during mid-summer when the snails are most likely to be infected (Ollerenshaw, 1966). Metacercariae are susceptible to high temperatures and drought. In case of such conditions, the infectivity in the environment decreases. However, metacercariae have been reported to survive for several months in hay. It has been observed that in the majority of European countries, the summer infection of the snails is of high importance leading to an increase in the numbers of metacercariae annually from August to October, particularly when the summer rainfall rate in a year is rather high. On the other hand, the winter infection of snails provides the environment with high numbers of metacercariae in late spring and early summer, especially when the former months are characterised by optimal moisturising conditions (Taylor et al., 2007).

When outbreaks of fasciolosis occur in young cattle, an acquired immunity gradually develops, leading to shorter duration of the primary infection, the obstruction of the secondary infection and finally to the reduction of the number of flukes established.

In southern USA and Australia, areas that are characterised by higher temperatures compared to Europe, the epidemiology is slightly different. It has been reported that the prevalence of *F. hepatica* in countries or areas with warm climate is higher after several months of drought, mainly due to the gathering of the animals around watering places, previously inhabited by infected intermediate hosts (Taylor et al., 2007).

Several epidemiological studies have been conducted in order to provide information concerning the distribution of the parasite, especially in dairy herds. In the UK, liver fluke prevalence in adult dairy cattle has been reported to be 48% (Salimi-Bejestani et al., 2005a), 76% (McCann et al., 2010) and 80% (Howell et al., 2015) following the examination of BTM samples. In Western Europe, prevalence estimates of 37%, 50% and 61% have been reported in Belgium, Germany, and Spain, respectively (Bennema et al., 2009; Kuerpick et al., 2012b; Mezo et al., 2008).

2.2.1.2. Pathogenesis and Clinical symptoms

Pathogenesis of fasciolosis in cattle depends on the amount of ingested metacercariae and the status of the host. The parasitic phase consists of two distinguished phases. Firstly, the migration of metacercariae in the liver parenchyma,

causing liver damage and haemorrhage and secondly, the damage of the biliary mucosa of the bile ducts caused by the cuticular spines of adult flukes (Taylor et al., 2007). Reports indicate that the majority of the pathological damage caused in the liver is observed when flukes migrate through its parenchyma, before their final establishment in the bile ducts (Sanchez-Andrade et al., 2000).
In heavy infections, characterised by severe anaemia and hypoalbuminaemia, the frequency of submandibular oedema increases. Low liver fluke burden is associated with less obvious symptoms which often cannot be distinguished from other possible reasons causing reduction in the milk yield of a herd. This loss in milk production appears frequently during the winter and is considered difficult to detect (Taylor et al., 2007). In case of clinical diarrhoea, the host has often been challenged by both *F. hepatica* and *O. ostertagi* (Burden et al., 1978; Reid et al., 1967). Immunological response to the infection with *F. hepatica* provides only inadequate protection of the animals to future reinfections (Cawdery et al., 1977; Kaplan, 2001).
Chronic *F. hepatica* infections may lead to reduction in growth rates and feed conversion as observed by Oakley et al. (1979). However, most fluke infections in adult cattle are sub-clinical but economically important (Dargie, 1987; Schweizer et al., 2005). Conducted surveys have led to the conclusion that fasciolosis causes significant reduction in weight gains ranging from 4.1% to 28% in the different studies (Cawdery et al., 1977; Johnson, 1991; Marley et al., 1996; Oakley et al., 1979). Further consequence of the disease is considered the loss in the annual milk yield, which varies from 8% to 15.4%, equivalent to 0.7 and 4.2 kg per cow per day (Charlier et al., 2007; Charlier et al., 2012; Donker, 1970; Hörchner et al., 1970; Khan et al., 2011; Kuerpick et al., 2012a; Mezo et al., 2011; Randell and Bradley, 1980; Ross, 1970). Negative correlations between the Ab titre and the annual milk production have additionally been reported by Charlier et al. (2007) and Kuerpick et al. (2012a). Reduction in butterfat content has been reported (Charlier et al., 2007; Khan et al., 2011; Khan et al., 2009) or not (Charlier et al., 2012; Mezo et al., 2011). Effects on reproduction such as an increased calving interval, or delayed puberty in young animals were found (Charlier et al., 2007; Lopez-Diaz et al., 1998) or not (Mezo et al., 2011; Simsek et al., 2007). It has been observed that the size of effects depends on the breed of cow and the husbandry system used, varying between countries studied, even within Western Europe (Bennema et al., 2010; Mezo et al., 2011).

2.3. Treatment and control of trichostrongyloids

Failure of treatment against trichostrongyloids is reported even more frequently because of increasing rates of anthelmintic resistance (AR) in worm populations, which threaten the sustainability and the efficiency of the complete livestock production. In spite of the presence of various approaches to the control of these infections, effective anthelmintic drugs with a broad-spectrum activity dominate in the applied control measures. As expected these compounds were highly efficacious. However, their widespread use has led to resistant parasite populations (Demeler et al., 2009; El-Abdellati et al., 2010a; Familton et al., 2001). Control of the growing AR can be achieved by minimising the administration of anthelmintics, taking into consideration climate changes, seasonality of the parasite and so on.

2.3.1. *C. oncophora*

Control of parasites in terms of treatment as well as pro(meta)phylaxis is generally based on the use of chemotherapeutics comprising benzimidazoles (BZs), levamisole or the macrocyclic lactones (MLs) (avermectins and milbemycins), which are effective against developmental and adult stages of *C. oncophora*. In the face of increasing AR of nematode populations to the commonly used drug classes, it is important to combine treatment with the respective management measures. Due to the limitation of registered drugs available for lactating cows, in most farming systems the emphasis of nematode control applies to the young stock. Pasture-based livestock systems usually treat the young calves prior or during their FGS. Treatment at the end of the grazing period then aims at the elimination of arrested and developing larvae as well as the adult stages of the parasites. In dairy herds, topical administration of eprinomectin is suggested, since it has no withholding period in milk (Shoop et al., 1996; Taylor et al., 2007). Moxidectin is also recommended for the treatment and control of cooperiosis. Conducted surveys have indicated that treatment with the respective substance resulted in successfully preventing the established of infection (El-Abdellati et al., 2010b; Morin et al., 1996; Taylor et al., 2001).
In order to stimulate the immune system, sufficient but controlled exposure of young animals to the parasite, preventing at the same time the appearance of heavy infections, is currently suggested. This measure is known as “safe pasture” and may be achieved in the following ways:

- use of effective anthelmintics, leading to the reduction of pasture contamination with eggs during periods of the year (spring and summer in the temperate climates, autumn and winter in sub-tropical areas) when the climatic conditions favour the development of free-living larval stages and
- by resting the pasture or grazing it with other hosts, such as horses, in order to reduce the availability of infective larvae (Taylor et al., 2007).

In some cases rotational grazing of adult and young stock is suggested in order to eliminate the spreading of the parasite. This system involves rotation of paddocks in which the immune adults follow susceptible younger calves, grazing the lower more fibrous levels of the herbage, where the majority of the infectious L3 are found. Since the excretion of eggs from the adult cattle is generally extremely low, the contamination of the pasture is considerably reduced (Taylor et al., 2007).
Control and treatment in adult dairy cows is generally complicated by the limitations in terms of drug use in lactating animals. On the other hand, the majority of farmers in Europe do not consider infections with *C. oncophora* as a problem at current. One explanation could be that most infections in adult cattle remain on a subclinical level and are therefore not noticed by the majority of farmers. Recent work however has shown, that the presence of subclinical infections with GI nematodes has a considerable influence on milk production (Charlier et al., 2009).
High efficacy and broad-spectrum activity of MLs has led to their widespread and frequent use for the control of nematode infections in livestock. Even though still less often observed in cattle parasitic nematode species than in sheep nematodes, AR increased over the past decades. It has been reported in several regions such as New Zealand (Mason and McKay, 2006; West et al., 1994), Argentina (Anziani et al., 2001; Fiel et al., 2001), Brazil (Soutello et al., 2007), Europe (Coles et al., 1998; Demeler et al., 2009; El-Abdellati et al., 2010b), the United States (Gasbarre et al., 2009), Australia (Lyndal-Murphy et al., 2010; Rendell, 2010) and Japan (Kudo et al., 2014). In order to maintain the sustainability of the currently available anthelmintics, their careful use is important. The often applied metaphylactic or prophylactic strategy favours the development of AR and should, wherever possible, be replaced by targeted treatment approaches, which are considered to optimise treatment and

control of GI nematodes. They include either targeted treatments (TT), which are administrated to whole groups of animals or targeted selective treatment (TST), where anthelmintics are directed to only those animals in need, aiming to maintain productivity and further control pasture contamination (Charlier et al., 2014a; Kenyon et al., 2009; Van Wyk, 2001). The basis for TT or TST strategies however is a successful diagnosis of the respective parasite. These approaches were successfully optimised by treating groups of animals based on faecal egg count (FEC) or weight gain in small ruminants. Even though serum pepsinogen levels during housing following FGS and the levels of Abs detected in BTM samples collected from dairy cattle have been reported as possible indicators for treatment (Kenyon and Jackson, 2012), TT and TST approaches for large ruminants are still largely missing.

2.3.2. *D. viviparus*

Treatment of parasitic bronchitis is based on modern BZs, levamisole and MLs. In order to prevent clinical signs of the disease and achieve maximum efficiency, it is recommended to use these drugs at the early stages of the infection. In case of severe and well-established infection, anthelmintic treatment may even worsen its symptoms, leading ultimately to high mortality. Animals developing clinical signs while on pasture should be housed and receive anthelmintic treatment and supportive therapy (e.g. antibiotics) in case of secondary infections (Taylor et al., 2007). In order to minimise the impact of infection, anthelmintic prophylaxis has widely been used in the last decades. By application of 2 or 3 treatments during the grazing season, disruption of developing infections and stimulation of immunity to the parasite is achieved (Taylor et al., 2007). Due to the strong and protective immune response, research has focused on the development of vaccines for the control of dictyocaulosis. Over the last decades several attempts have been described, mainly based on immunisation with irradiated larvae (Cornwell, 1960; Jarrett et al., 1960) at an interval of 4 weeks. Development of high immunity level was achieved only if vaccinated calves were kept in house, totally protected from any parasitic challenge, until 2 weeks after the second dose of the vaccination. Other potential vaccine candidates showing partial protection have been described, based on secreted acetylcholinesterase (AChE), an enzyme secreted by adult worms (McKeand, 2000) or on a recombinantly expressed paramyosin (PMY), a parasite's muscle protein (Strube et al., 2015). However, up to know results have not been satisfactory, so the control or prevention of the parasite still relies on the use of anthelmintics. The same limitation of drug use in lactating animals leads to the persistence of infection in dairy cows, even though the occurrence of this parasite in Western Europe is relatively low compared to infections with GI nematodes or *F. hepatica*. Accordingly, the influence of this parasite on production parameters has not been as intensively studied. Only one recently conducted study reported a significant decline in milk production in infected herds in the Netherlands (Dank et al., 2015). The same authors also assumed that the relatively low prevalence reported in studies based on BTM Enzyme-linked Immunosorbent Assay (ELISA) data might be due to a lack of sensitivity of the ELISA and that the true prevalence is most likely underestimated. Even though no cases of AR have been reported so far for this parasite genus, the consumers demand to reduce chemical treatment in livestock in combination with a possible lack of sensitive detection systems has increased the need of developing simple but sensitive diagnostic methods.

2.3.3. *F. hepatica*

For the treatment of fasciolosis, triclabendazole, a member of the family of BZs, can be used which removes early parenchymal immature stages of the parasite as well as adults. Hillyer and Santiago de Weil (1979) proved that administration of triclabendazole is followed by a remarkable and quick reduction in the *F. hepatica* Ab titres in rabbits and rats after an experimental infection. In lactating cows, when the milk they produce is for human consumption, administration of triclabendazole is not recommended since it is characterised by an extended withdrawal period and leads consequently to the dismissal of the milk. Accordingly, this drug is not registered for dairy cows. Alternatively, other BZs such as albendazole or fenbendazole can be used, preferably during the dry period since they also have withdrawal times of 5-8 days. It needs to be noticed that those BZs are, unlike triclabendazole, only effective against adult flukes (Kuerpick et al., 2013a). Other substances, e.g. nitroxynil or clorsulon can also be used, however, restriction in registrations and the respective withdrawal period need to be taken into account.
The aim of fluke control should be based on the reduction of infection levels in snails as well as in cattle (Knubben-Schweizer et al., 2010; Parr and Gray, 2000). Several studies have described the way grazing management may be used as a control measure, either alone or in combination with flukicides. Control of snail populations with molluscicides is not permitted due to their adverse effects on the environment. Pasture drainage is another option, however in most cases this is impractical and prohibitively expensive (Roberts and Suhardono, 1996). Additionally, prophylactic administration of anthelmintics and meteorological forecasting of fasciolosis are recommended in order to control the course of liver fluke infection (Taylor et al., 2007). AR to commonly used anthelmintics has been reported in several countries and the loss of efficacy is a threat to animal health, welfare and agricultural productivity (Daniel et al., 2012; Gordon et al., 2012; Hodgkinson et al., 2013; Sargison, 2012; Sargison and Scott, 2011).

2.4. Diagnostic techniques

Nematode and trematode infections play an important role for animal welfare and are currently of great concern for the economy of the global ruminant livestock industry (Morgan et al., 2013). Constantly increasing financial costs for anthelmintic prophylaxis and treatment due to the spread of anthelmintic resistant parasite populations, as well as the often overlooked subclinical effects of the helminth infections on animal productivity (Alvarez Rojas et al., 2014; Charlier et al., 2009) have led to the need of developing new and sustainable strategies concerning the detection and subsequently the effective control of helminthoses (Mejia et al., 2011). An important step towards this end is the development of new, efficient and high-throughput diagnostic techniques. Even though more sensitive coproscopical methods have been developed (Barda et al., 2013), they usually target individual animals and are often not suitable for high-throughput diagnosis. The majority of the serological assays described are based on the detection of Abs or antigens (Ags) and can be applied to individual animals (serum samples or individual milk samples). In order to reduce costs for diagnostics, a variety of tests systems have also been used for Ab detection in BTM samples (Reber et al., 2012). Additionally, the serological methods established so far appear to lack specificity and in some cases also sensitivity, in particular when non-recombinant Ags are used (Poot et al., 1997). Most tests target only one parasite species or genus and accordingly, multiple tests have to be performed to detect mixed species infections. This leads to an increase in diagnostic costs and resulting decrease in acceptance amongst the farming communities.

2.4.1. Coproscopical diagnosis

Diagnosis of infections with GI nematodes has been traditionally based on clinical signs, grazing history on a pasture with known contamination status and FECs (Michel, 1969). Microscopic examination of faecal samples does not allow the accurate diagnosis of the parasite species inhabiting the host, since the morphology of the eggs is not characteristic for the parasite and cannot be distinguished from other GI nematodes. Differentiation of *C. oncophora* can be based on the identification of L3 following larval culture, but this requires highly skilled personnel. Diagnosis of *O. ostertagi* can be supplemented by the performance of a serum pepsinogen test and the determination of gastrin levels (Berghen et al., 1987; Berghen et al., 1993), but both are not parasite specific.

Baermann (1917) established a method in order to directly detect *D. viviparus* larvae in faeces using a sieve and funnel technique. The obtained results however, are valid only if faecal samples are collected directly from the rectum, so that contamination with soil nematode larvae is avoided and when a reasonable number of animals in the infected herd are sampled. The presence of the larvae in the faeces is restricted to the patency (21 to 70 d.p.i), lasting a few weeks post infection (Hoglund et al., 2004; Schnieder, 1992b). Despite its high specificity, the Baermann method is characterised by its overall low sensitivity. Different studies revealed that the results obtained strongly depend on a variety of factors. These include the performance of baermannisation at room temperature (RT), time used for incubation, motility of the larvae (Rode and Jørgensen, 1989) and lastly, on the ability of the researcher to differentiate *D. viviparus* larvae from other nematode larvae (Hoglund, 2006). Over the past decades, contradicting reports regarding the performance of this method have been published. Particularly the conditions (temperature and time) for the storage of samples prior to examination seem to differ considerably with recommendations between refrigeration, RT and 20-25°C (Andersen and Walters, 1973; Cremers, 1981; Eysker, 1997; Rode and Jorgensen, 1989). Additionally, the results obtained by this diagnostic technique are considered unreliable due to the fact that L1 are inactive and present variability in their motility during their development to the infective L3 (Eysker, 1997). Even lower sensitivity has been observed in adult cattle, maybe due to the higher amount of faeces being produced (Eysker, 1997; Eysker et al., 1994). Finally, the relatively short patent period in combination with small number of animals being sampled in a herd may contribute to reduced sensitivity of the method (Boon et al., 1982).

Coproscopical diagnosis of *F. hepatica* is based on the detection of the characteristic fluke eggs in the faeces of cattle (Boray, 1985) using sedimentation techniques (Happich and Boray, 1969a; Happich and Boray, 1969b; Sewell and Hammond, 1972). Microscopical discrimination of the eggs is relatively simple, since the excreted eggs display a characteristic morphology. However, similarities in structure, size and shape between the different *Fasciola* spp. may be problematic in matters of specificity, particularly in the cases where different species co-occur (Valero et al., 2009). The biggest constrain of coproscopic diagnosis is the intermittent excretion of eggs so the number of liver fluke eggs per gram of faeces does not correlate with the number of adults in the infected host. Therefore this method is characterised by low sensitivity during patency, when the number of the excreted eggs is relatively low, according to Happich and Boray (1969a). In a study conducted by Charlier et al. (2008) sedimentation and flotation, based on different amount of tested samples, were compared. It was proven that the examination of 10g of faeces provides higher sensitivity (64%) compared to that of 4g (43%). Occasional release of the eggs in the bile ducts and variability in matters of biotic potential/fecundity, according to the phase of the life cycle and the host, may affect the sensitivity of microscopical detection of the parasite (Gonzalez-Lanza et al., 1989; Sewell and Hammond, 1972). These factors lead to high occurrence of false negative results, subsequently

decreasing sensitivity (Honer, 1967). Commercially available tests systems, including FLOTAC (Cringoli et al., 2010) and Flukefinder (Foreyt, 2002), are characterised by a higher sensitivity but still limited to eggs being present in the sample taken.
Generally, and this applies for all of the parasites included in this study, the coproscopic methods are not able to detect the parasite during the pre-patent phase of infection (juvenile worms).

2.4.2. Serological diagnosis

The generally high handling costs, as well as the necessity to sample several animals, led to the development and subsequently increased use of serological methods, which can be used for individual diagnosis or herd health monitoring (Karanikola et al., 2015). Apart from ELISAs for the detection of Abs against GI nematodes, *D. viviparus* and *F. hepatica*, different tests such as the serum pepsinogen test or haemagglutination tests were established in order to facilitate diagnosis of some of the target species. Serum pepsinogen levels were used in order to assist diagnosing infections with *O. ostertagi* (Berghen et al., 1993). Detection of Abs against *D. viviparus* started with the development of different complement fixation tests, based on adult whole worm Ags, which reported the immune responses of animals to experimental and re-infection with the parasite (Cornwell and Michel, 1960; Jarrett et al., 1959; Weber, 1958). An indirect haemagglutination (IHA) test was developed in order to quantify *D. viviparus* Abs by haemagglutination (Bokhout et al., 1979). Positive correlation (80%) was calculated between serological and clinical findings on lungworm infections however; the sensitivity was relatively low whereas the number of false-positive results was high. No information is provided concerning the specific rates. Serum Abs against *F. hepatica* were detected by haemagglutination based on f2 Ag and tests were standardised by using serum from either naturally infected lactating cows or experimentally infected calves (Levieux et al., 1992). But for none of the above mentioned tests information regarding their sensitivity and specificity was provided.

2.4.2.1. Detection of C. oncophora antibodies

Keus et al. (1981b) was the first describing the establishment of a serological immunoassay for the detection of Abs against *C. oncophora* and *O. ostertagi* in calves based on saline extracts from L3, L4 and adult worms. The assay was characterised by high specificity, especially when L4 extracts when used. However, no information concerning the specificity is provided by the authors. Ploeger (1989) developed and evaluated an ELISA based on a crude worm Ag. This test showed relatively low sensitivity and specificity since it was cross-reacting with Abs produced against Ags of other helminth species or different microorganisms (de Graaf et al., 1992; van Leeuwen et al., 1992). In order to increase specificity, recombinantly expressed proteins were incorporated in the development of reliable immunoassays.
Several low molecular weight proteins, between 12-16 kDa in size, obtained from adult worms, were used in different assays described in the literature (de Graaf et al., 1993; Nieuwland et al., 1995; Parmentier et al., 1995; van Diemen et al., 1996b). In all these assays the Ags were evaluated with sera collected from animals specifically infected with *C. oncophora*. These Ags did not show any evidence of cross-reaction with serum from parasite naïve calves and *O. ostertagi* (de Graaf et al., 1993), but were not tested in regards to other GI nematodes. Poot et al. (1997) demonstrated the presence of immunodominant products of *C. oncophora* in different immunoblots of excretory-secretory (ES) products. Two cDNA sequences were recombinantly expressed in *Escherichia coli* and were tested in an ELISA, using sera from mono-infected cattle. The developed assay used a 14.2 kDa fragment und was

characterised by high sensitivity and specificity (both 100%). Cross-reactivity of the recombinantly expressed protein was tested with serum samples collected from calves specifically mono-infected with *O. ostertagi*, *D. viviparus*, *F. hepatica*, *Nematodirus helvetianus*, *Haemonchus placei* and *Trichostrongylus colubriformis*, resulting in 100% specificity. An overview of available assays, their sensitivity as well as specificity is provided in Table 1.

Table 1. Serological detection of *Cooperia oncophora* antibodies. Se%: Sensitivity%, Sp%: Specificity%, Rec.: Recombinant, CR: cross-reaction, *O. o.*: *O. ostertagi*, *D. v.*: *D. viviparus*, *F. h.*: *F. hepatica*, *N. h.*: *N. helvetianus*, *H. p.*: *H. placei*, *T. c.*: *T. colubriformis*

Ag	Standardisation	Se%	Sp%	General notes	Publication
Crude extracts of L3, L4 and adult worms	Serum from naïve & mono-infected with *C. o.* & *O. o.* calves			L3 lacks genus specificity L4 & adult Ag higher Sp	(Keus et al., 1981a)
Crude adult	Calves naturally infected			Low Se% & Sp% were determined by de Graaf et al. (1992) and van Leeuwen et al. (1992)	(Ploeger, 1989)
Partially purified adult extracts	Calves			Bands of 14.2 and 14.9 kDa were not recognised for *O. o.* and negative controls	(de Graaf et al., 1993)
Crude adult	Calves mono-infected			Fragment between 12-15 kDa	(Nieuwland et al., 1995)
Crude extracts of L3, L4 and adult worms	Calves mono-infected			L4: more specific No CR with *O. o.*, *D. v.*, *A. s.*, *F. h.*, *N. h.*	(Parmentier et al., 1995)
Crude adult	Calves mono-infected			Fragments recognised: - 14-16 kDa - 27 kDa	(van Diemen et al., 1996a)
Rec.	Calves mono-infected	100	100	Fragment of 14.2 kDa No CR with *O. o.*, *D. v.*, *F. h.*, *N. h.*, *H. p.* & *T. c.*	(Poot et al., 1997)

2.4.2.2. Detection of D. viviparus antibodies

Different ELISAs have been developed in order to detect Abs against *D. viviparus*. Seroconversion has been reported after 21 days in some studies (Gozdzik et al., 2012; Schnieder, 1993b) as well as approximately 4 to 6 weeks (de Leeuw and Cornelissen, 1993), whereas the titres may persist 4 to 7 months. Based on adult worm Ags, IgA was detected 5 w.p.i (Marius et al., 1979). ELISAs using low molecular weight (17kDa) ES products collected from L3 or adult worms were established (de Leeuw and Cornelissen, 1991, 1993) and were characterised by absence of cross-reaction when tested with *Ascaris suum*, *C. oncophora*, *F. hepatica* and *O. ostertagi*. Following the comparison of three different ELISAs, an indirect ELISA based on crude Ag, an indirect and a competitive ELISA both using partially purified Ag, de Leeuw and Cornelissen (1993) indicated that the latter indirect ELISA provided high sensitivity (97%) and specificity (97%) 4 to 6 w.p.i. in comparison to the other two methods. However, no detailed information concerning sensitivity and

specificity were provided for the other two ELISAs. The assay was further evaluated in comparative and epidemiological assays (Cornelissen et al., 1997; Hoglund et al., 2004).
In order to improve specificity, recombinantly expressed Ags were used for the development of further serological assays. Contrary to native Ags, recombinantly expressed proteins can be produced in a standardised way and a constant quality. Identification of an 18 kDa protein by Schnieder (1992a) was later shown to be encoded by a gene fragment, highly homologous to a major sperm protein (MSP) previously detected also in *A. suum*, *Caenorhabditis elegans* and *Onchocerca volvulus* (Schnieder, 1993a). Based on this Ag, Schnieder (1993b) described the development of a dipstick immunoassay that accelerated the time needed for the conduction of the examination and was characterised by high sensitivity and specificity (both over 99%).
Members of the MSP family are the main component of sperm and are found to control nematode oocyte maturation and ovulation. A serological immunoassay based on recombinantly expressed MSP has been initially developed and standardised by von Holtum et al. (2008) (sensitivity and specificity >91%) and Gozdzik et al. (2012) used a slightly modified version of this assay for the examination of individual and BTM samples (sensitivity: 97.7% and specificity: 98.1%). Large-scale epidemiological studies using recombinantly expressed MSP have been conducted (Holzhauer et al., 2011; Klewer et al., 2012) in dairy cattle herds in order to generate detailed information regarding the prevalence of the parasite (Fiedor et al., 2009; Ploeger et al., 2012). Further information regarding available immunological tests for the diagnosis of *D. viviparus* Abs are presented in Table 2.

Table 2. Serological detection of *Dictyocaulus viviparus* antibodies. Se%: Sensitivity%, Sp%: Specificity%, **(**s): serum, (m): milk, Rec.: Recombinant, CR: cross-reaction, *O. o.*: *O. ostertagi*, *C. o.*: *C. oncophora*, *D. v.*: *D. viviparus*, *F. h.*: *F. hepatica*, *N. h.*: *N. helvetianus*, *A. s.*: *A. suum*

Ag	ELISA	Standardisation	General notes	Se%	Sp%	Publication
Adult worm	(s)	Calves mono-infected with *D. v.*, *C. o.*, *O. o.*, *Trichostrongylus* & *Oesophagostomum* spp.	Abs detected 4 w.p.i.			(Marius et al., 1979)
Crude saline extract	(s)	Calves naïve & mono-infected	Some CR with *C. o.* & *O. o.* Abs detected 3 w.p.i.	100	Low	(Boon et al., 1982)
Crude & purified	(s)	Calves naïve & mono-infected with *D. v.*, *O. o.*, *C. o.*, *F. h.* & *A. s.*		97	97	(de Leeuw and Cornelissen, 1993)
Rec. MSP	(s)	Cattle naïve & mono-infected with *D. v.*, *C. o.*, *O. o.*	Fragment of 18 kDa Abs detected 30 d.p.i. No CR	>99	>99	(Schnieder, 1993b)
Partially purified	(s)	Calves naïve & mono-infected with *D. v.*, *O. o., C. o.*, *F. h., N. h.* & *A. s.*	No CR	100	99.2	(Cornelissen et al., 1997)
Rec. MSP	(s)	Calves naïve & mono-infected with *D. v.*, *O. o.*, *C. o.*	No CR	>91	>91	(von Holtum et al., 2008)
Rec. MSP	(m)	Cattle mono-infected		100	97.48	(Fiedor et al., 2009)
Rec. MSP	(s)	Calves mono-infected with *D. v.*, *C. o.* & *O. o.*	Fragment of 43 kDa Abs detected 21-28 d.p.i., No CR	97.7	98.1	(Gozdzik et al., 2012)

Ag	ELISA	Standardisation	General notes	Se%	Sp%	Publication
Rec. MSP	(m)		Adjustment of the cut-off by Fiedor et al. (2009)	100	97.32	(Schunn et al., 2012)

2.4.2.3. Detection of F. hepatica antibodies

Establishment and evaluation of several immunological assays have been extensively described in the literature, aiming at rapid and accurate diagnosis of both, early and chronic phases of *F. hepatica* infection. The majority of these assays have been fully validated using sera from calves with known infection status and are used for routine diagnosis in individual cattle. Their ability of detecting prepatent infections as early as 2 weeks, with peak concentrations reached around the 8-10 w.p.i. (Fagbemi and Guobadia, 1995; Farrell et al., 1981; Santiago and Hillyer, 1988), highlights their superiority compared to macroscopic diagnostic methods. Since the specificity of Ab responses varies during infection (Bennett et al., 1982; Hanna, 1980; Hanna and Jura, 1977; Hughes et al., 1981; Rajasekariah and Howell, 1978), a wide range of Ags have been identified and used for serological immunodiagnosis.
Several ELISAs based on ES products of adult *F. hepatica* have been described, providing satisfying sensitivity and specificity. For the DIG ELISA described by Bautista-Garfias et al. (1989), sensitivity was calculated at 97.5% while specificity reached 80%. The indirect ES ELISA described by Boulard (1985) was characterised as 96.5% sensitive and 98.8% specific. The DOT ES ELISA developed by Arriaga de Morilla et al. (1989) reached 93.1% sensitivity and 95.4% specificity. Determination of sensitivity and specificity of the three aforementioned assays was performed by Ibarra et al. (1998). No information concerning cross-reactivity of these assays with other pathogenic parasite species was provided. Salimi-Bejestani et al. (2005b) developed an ES serological immunoassay using serum from calves specifically mono-infected with *F. hepatica*, *D. viviparus*, *N. helvetianus* and *O. ostertagi* resulting in 98% sensitivity and 96% specificity. A modified in-house ES ELISA, based on the previously described method, was developed by Charlier et al. (2008). No information were provided concerning the standardisation of this assay; however, its sensitivity was reported to be 87% and its specificity 90%. These methods were all evaluated using commercially available ELISA tests and additionally either sedimentation, flotation or necropsy. Several authors have suggested the use of partially purified or well-defined Ags in order to enhance specificity. Towards this end, Cornelissen et al. (1999b) described three ELISAs for the detection of Abs in bovine serum samples using a purified epitope of Cathepsin L1. Using serum samples from calves either parasite naïve or specifically mono-infected with *F. hepatica*, he developed a peptide ELISA (sensitivity 98.9%, specificity 99.8%), an ES ELISA (sensitivity 100%, specificity 82.8%) and an indirect purified Cathepsin L like ELISA (sensitivity 100%, specificity 94.6%). Cross-reactivity was assessed by examining serum samples from calves specifically mono-infected with *D. viviparus*, *O. ostertagi*, *N. helvetianus*, *A. suum*, *Taenia saginata* and *Schistosoma matthei.* No cross-reaction was detected for any of the aforementioned parasite species for the peptide based ELISA while some cross-reactivity was found with *D. viviparus* for the other two ELISAs. In a follow-up study by Cornelissen et al. (2001), a recombinantly expressed Cathepsin L protease of approximately 28 kDa, was standardised following the examination of serum samples collected from either parasite naïve or experimentally mono-infected with aforementioned parasite species and further evaluated. It was characterised by 99.1% sensitivity and 98.5% specificity. More than a decade later, Kuerpick et al. (2013b) evaluated a recombinantly expressed Cathepsin L1, using serum samples

from either parasite naïve or experimentally mono-infected with different dosage of metacercariae, comparing its sensitivity and specificity with an ES ELISA (Charlier et al., 2008; Salimi-Bejestani et al., 2005b) and the commercially available Pourquier ELISA, based on "f2" Ag (Fasciolosis Verification Test, IDEXX, Hoofddorp, the Netherlands), a purified fraction of ES Ags (Tailliez and Korach, 1970). The latter was previously evaluated regarding sensitivity and specificity by Molloy et al. (2005), resulting in 98% and 81.3% sensitivity for serum and milk, respectively, while specificity was calculated at 100% and 95% for serum and milk, respectively. Additionally, Reichel et al. (2005), further evaluated an f2 ELISA, indicating 95% sensitivity and 98.2% specificity. The recombinantly expressed Cathepsin L1 used in this experiment (Kuerpick et al., 2013b) provided good sensitivity and specificity during different stages of infection. More precisely, sensitivity varied between 90-100% during patency, while specificity was calculated at 88.6% since some cross-reactivity was detected with serum samples collected from calves specifically mono-infected with *C. oncophora* and *D. viviparus*. The application of these serological immunoassays in the examination of individual and BTM samples has been described in literature. Salimi-Bejestani et al. (2005a) were the first to describe a milk ELISA for the detection of *F. hepatica* Abs, using ES Ag. The standardisation of the assay was performed by the examination of individual serum (*n*=1561), faecal samples (*n*=715) and BTM (*n*=61) samples. The assay was characterised by 96% sensitivity and 80% specificity. In a follow-up study, using examination of paired serum and milk samples (*n*=765), Salimi-Bejestani et al. (2007), calculated 92% sensitivity and 88% specificity using the same ES Ag, testing milk in a dilution of 1:2 contrary to the first assay where milk was tested undiluted. Lower sensitivity (60%) and specificity (90%) of an ELISA milk immunoassay was reported by Selemetas et al. (2014), using a recombinantly expressed Cathepsin L1 Ag (for more details see Table 3).

The standardisation of BTM immunoassays allowed the performance of large-scale epidemiological surveys. Typical examples of such studies are described for Belgium (Bennema et al., 2009; Charlier et al., 2007), the UK (Pritchard et al., 2005; Salimi-Bejestani et al., 2007; Salimi-Bejestani et al., 2005a), Germany (Kuerpick et al., 2013a; Kuerpick et al., 2012a; Kuerpick et al., 2012b), Sweden (Hoglund et al., 2010), Spain (Mezo et al., 2010) and Australia (Reichel et al., 2005).

Table 3. Serological detection of *Fasciola hepatica* antibodies. Se%: Sensitivity%, Sp%: Specificity%, **(**s): serum, (m): milk, Pept.: Peptide, Pur.: Purified, Rec.: Recombinant, Cath.: Cathepsin, CR: cross-reaction, *O. o.*: *O. ostertagi*, *C. o.*: *C. oncophora*, *D. v.*: *D. viviparus*, *F. h.*: *F. hepatica*, *N. h.*: *N. helvetianus*, *A. s.*: *A. suum*, *T.s.*: *Taenia saginata*, *S.m.*: *S. matheei*

Ag	ELISA	Standardisation	General notes	Se%	Sp%	Publication
ES	(s)			96.5	98.8	(Boulard, 1985)
ES	(s)			97.5	80	(Bautista-Garfias et al., 1989)
ES	(s)			93.1	95.4	(Arriaga de Morilla et al., 1989)
Pept	(s)	Calves mono-infected with *F. h.*, *D. v.*, *C. o.*, *O. o.*, *A. s.*, *N. h.*, *T. s.* & *S. m.*		98.9	99.8	(Cornelissen et al., 1999a)
ES	(s)	Calves mono-infected with *F. h.*, *D. v.*, *C. o.*, *O. o.*, *A. s.*, *N. h.*, *T. s.* & *S. m.*	CR with *D.v.*	100	82.8	(Cornelissen et al., 1999a)
Pur. Cath. L1	(s)	Calves mono-infected with *F. h.*, *D. v.*, *C. o.*, *O. o.*, *A. s.*, *N. h.*, *T. s.* & *S. m.*	CR with *D.v.*	100	94.6	(Cornelissen et al., 1999a)

Ag	ELISA	Standardisation	General notes	Se%	Sp%	Publication
Pur. Cath. L	(s)	Calves mono-infected with *F. h.*, *D. v.*, *C. o.*, *O. o.*, *A. s.*, *N. h.*, *T. s.* & *S. m.*	No CR Seroconversion at 5 w.p.i.	99.1	98.5	(Cornelissen et al., 2001)
ES	(s)	Calves mono-infected with *F. h.*, *D. v.*, *O. o.* & *N. h.*	No CR	98	96	(Salimi-Bejestani et al., 2005b)
ES	(m)	Serum samples & BTM from cattle dairy herds	Same cut-off as in (s) Milk tested undiluted	96	80	(Salimi-Bejestani et al., 2005a)
f2	(s) (m)			98 81.3	100 95	(Molloy et al., 2005)
f2	(m)	Paired samples from naturally infected cattle		95	98.2	(Reichel et al., 2005)
ES	(m)	Paired samples from naturally infected cattle	Milk tested in a dilution 1:2	92	88	(Salimi-Bejestani et al., 2007)
MM3	(m)	Serum & milk samples from different groups of animals Samples not paired	Milk tested in a dilution 1:2			(Mezo et al., 2010)
Rec.Cath. L1	(s)	Calves mono-infected with *F. h.*, *D. v.*, *C. o.* & *O. o.*	Some CR with *C. o.* & *D. v.*	95-100	88.6	(Kuerpick et al., 2013b)
Rec.Cath. L1	(m)			60	90	(Selemetas et al., 2014)

2.4. Flow cytometry-application in xMAP®Luminex®Technology

Flow cytometry is a laser-based, biophysical technology used in cell counting and sorting, biomarker and protein detection, by suspension of the cells in a stream of fluid and analysis of them by an electronic detection device. It favours the simultaneous multiparametric analysis of both the physical and biochemical characteristics of up to 100 analytes.

Multiplexed analysis is defined as the ability to perform numerous distinct assays in a single tube, using the same sample at the same time. Differently-sized beads for the concurrent analysis of different analytes was initially suggested by Horan et al. (1979). Initially, use of two distinct sizes of beads for the detection of two different Abs was described (Horan et al., 1979) and was later extended towards the use of four different-sized microspheres for the recognition of four anti-HIV Abs (McHugh et al., 1988; Scillian et al., 1989).

The FlowMetrix™ system (Luminex Corp., Austin, TX), as it was initially named – later found in literature under the name "xMAP®Luminex®Technology" (Vignali, 2000), is a platform allowing the simultaneous detection of up to 100 different analytes based on the principles of flow cytometry. The system comprises 100 bead sets and a specific software allowing the detailed analysis of the obtained results. It is used for the performance of qualitative and quantitative immunological assays for the detection of proteins as well as for the performance of DNA sequence analysis (Fulton et al., 1997).

The xMAP®Luminex®Technology is based on a bench-top flow cytometer, equipped with two lasers, providing the user with the ability to perform up to 100 simultaneous,

high-throughput examinations either of a single tube or a single well of a microtiter plate (Giavedoni, 2005; Vignali, 2000). This liquid array system consists of microsphere sets, each of which is internally dyed with a different ratio of two fluorescent dyes, red and infrared, leading to the creation of 100 distinct beads, each having a unique spectral address. Captured analytes are detected using a secondary reagent, labelled with a fluorescent reporter molecule, usually the streptavidin-phycoerythrin (SA-PE) molecule. Each labelled microsphere is cross-examined by the flow cytometer (Bio-Plex 200-Luminex Corp., Austin, TX, USA). A 635-nm, 10-mW red diode laser excites two dyes, internally placed in the individual microspheres, characterising the identity of them and subsequently classifying them into categories according to their unique spectral signal. The second laser, a 532-nm, 13-mW yttrium aluminium garnet green laser, excites the detection molecule bound onto the microsphere further quantifying the signal of interest (Dunbar and Li, 2010). Analysis of the results is achieved in almost 15 seconds and theoretically, 100 different analytes can be estimated (Perkins et al., 2006).

Application of this technology has been reported in human medicine, where multiplex assays aiming the detection of Abs, cytokines etc. have been developed and are currently commercially available (Fonseca et al., 2011; Hernandez et al., 2012; Shoma et al., 2011; Stenger et al., 2011). In the field of veterinary diagnostics, particularly regarding serological assays, only a few reports have been published (Christopher-Hennings et al., 2013). Multiplex immunoassay protocols in particular, are comparable to those described for ELISAs. Fluorescent beads are conjugated with recombinantly expressed proteins, and subsequently incubated with samples containing Abs against target species. Incubation with a secondary biotinylated Ab followed by that with a fluorescent detection molecule, the Streptavidin-Phycoerythrin (SA-PE) is preceding the analysis of the samples using specific software. Four software tools for analysis, designed to meet the multiplex testing expectations of clinical and research laboratories, have been constructed. The FLEXMAP 3D, the MAGPIX®, the Bio-Plex 100 and the Bio-Plex 200 may be applied to multiplex immunological, gene expression, genotyping, enzymatic and PCR assays (Dunbar and Li, 2010). Bio-Plex 200 was used for the conduction of this experiment (Karanikola et al., 2015).

Different microspheres are commercially available according to their properties (magnetic or not), or their applications (nucleic acid or protein assays). More precisely, the available microspheres are the following (https://www.luminexcorp.com):

- MagPlex® microspheres: versatile magnetic carboxylated microspheres, available in low concentrations of up to 500 different regions, presenting improved washing efficiency and providing the researcher with the ability to use magnetic separators instead of the expensive filter plates.
- MicroPlex® microspheres: standard non-magnetic microspheres
- MagPlex-TAG™ microspheres: pre-coupled with oligonucleotide capture sequences (Anti-TAGs), available in 150 different regions, facilitating the conduction of nucleic acid assays.
- LumAvidin® microspheres: non-magnetic microspheres, containing an avidin surface layer, capable of binding biotinylated ligands, specifically useful for the attachment of small molecules and peptides.
- SeroMAP™ microspheres: non-magnetic beads, specifically formulated aiming the reduction of non-specific binding (high background values) in serological assays.

For the needs of this study, a different version of the MagPlex® magnetic microspheres were used, available under the brand name Bio-Plex Pro™ Magnetic COOH Beads (Bio-Rad, Germany) (Karanikola et al., 2015).

2.5. Aim of the study

Currently used parasitological, immunological and molecular techniques are labour-intensive, have a high cost and are characterised by limited application either on herd basis or on large-scale surveillance. Since helminthoses involve multiple parasite species, the identification of the key species responsible for the clinical signs and the production losses is significant in order to design the appropriate treatment and control plan.
A major constraint on the effective control and management is the lack of rapid, high-throughput, routine diagnostic tests in order to assess the health status of animals and to identify the parasite species responsible for disease.
The aim of this study was to develop a new, versatile and high-throughput diagnostic assay for the simultaneous detection of specific Abs against *C. oncophora*, *D. viviparus* and *F. hepatica* in bovine serum and milk samples.

3. MATERIALS & METHODS

3.1. Materials

Material	Supplier
Kits	
DNA Clean & Concentrator™-5	ZYMO RESEARCH, Freiburg, Germany
NucleoSpin® Plasmid	MACHEREY & NAGEL, Düren, Germany
EasyPrep Pro Plasmid Miniprep kit	BIOZYM, Hessisch-Oldendorf, Germany
Protino® Ni-NTA Columns	MACHEREY & NAGEL, Düren, Germany
Micro Bio-Spin 6 Chromatography Columns	BIO-RAD, Munich, Germany
CB-X™ Assay	G-BIOSCIENCES, Darmstadt, Germany
Amine Coupling Kit®	BIO-RAD, Munich, Germany
Bio-Plex Pro™ Magnetic COOH Beads	BIO-RAD, Munich, Germany
Buffers, Solutions, Reagents	
GeneRuler™ 100bp DNA Ladder	FERMENTAS, St. Leon-Rot, Germany
Maxima® Hot Start Taq DNA Polymerase, 10x Hot Start PCR Buffer, 25mM $MgCl_2$	FERMENTAS, St. Leon-Rot, Germany
5x Phusion HF Buffer	FINNZYME, Vantaa, Finland
GR Green (GRSafe) Nucleic Acid Gel Stain	LABGENE SCIENTIFIC SA, Chatel-St. Denis, Switzerland
EDAC	SIGMA-ALDRICH, Munich, Germany
S-NHS	SIGMA-ALDRICH, Munich, Germany
Anti-V5 Ab	LIFE TECHNOLOGIES, Darmstadt, Germany
Goat Anti-Bovine IgG	DIANOVA, Hamburg, Germany
Streptavidin - Phycoerythrin	MILLIPORE, Darmstadt, Germany
IPTG	FERMENTAS, St. Leon-Rot, Germany
Gel Gode™	THERMOFISHER, Darmstadt, Germany
Hand Disinfection Solution AHD2000®	LYSOFORM, Berlin, Germany
EDTA	ROTH, Karlsruhe, Germany
Agarose NEEO Ultra Quality Roti®garose	ROTH, Karlsruhe, Germany
Consumables	
PCR-Eppendorfs (0.2ml, 1.5ml, 2ml)	BIOZYM, Hessisch-Oldendorf, Germany
PCR Single Cap 8er-Soft strips 0.2ml, PCR 96-well TW-MT Plates (white)	BIOZYM, Hessisch-Oldendorf, Germany
Filter tips (10µl, 100µl, 1000µl)	BIOZYM, Hessisch-Oldendorf, Germany
Gloves Rotiprotect-Latex (Nitryl)	ROTH, Karlsruhe, Germany
KIMTECH tissues, Rotizell®, Bacillol tissues	ROTH, Karlsruhe, Germany
Eppendorfs (0.5ml, 1.5ml, 2ml)	SARSTEDT, Nümbrecht, Germany
Sterile tubes (15ml, 50ml)	SARSTEDT, Nümbrecht, Germany
96-well round bottom plates	GREINER BIO-ONE GmbH, Frickenhausen, Germany
Aluminium foil	SARSTEDT, Nümbrecht, Germany
Devices	
Netdevice, Standard Power Pack P25, Power Pack P25 T, Electrophoresis gel chamber	BIOMETRA, Göttingen, Germany
iCycler Thermal Cycler, C1000™ Thermal Cycler, S1000™ Thermal Cycler, CFX96™ Real-Time	BIO-RAD, Munich, Germany

Syst.	
Netdevice PowerPac™ Universal Supply Electrophoresis gel chamber Sub Cell GT, Mini Sub Cell GT, Wide Mini Sub Cell GT	BIO-RAD, Munich, Germany
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	BIO-RAD, Munich, Germany
TMix	ANALYTIK JENA, Jena, Germany
Synergy 4, Take 3 Plate	BIO-TEK, Bad Friedrichschal, Germany
Pipettes 1-10µl, 100-1000µl Multipipette® x stream	EPPENDORF, Hamburg, Germany
Petri dishes	SARSTEDT, Nümbrecht, Germany
Water bath	MEMMERT, Schabwach, Germany
Minifuge RF-Heraus	SEPA-TECH, Engen, Germany
Centrifuge 54 30R	EPPENDORF, Hamburg, Germany
IKA shaker MTS 4	JANKE & KUNKEL, Staufen, Germany
IKA® VORTEX Genius 3	SIGMA-ALDRICH, Munich, Germany
nunc™ ImmunoWash 8	SIGMA-ALDRICH, Munich, Germany
EL406 washer dispenser	BIO-TEK, Bad Friedrichschal, Germany
DFC360FX Camera	LEICA MICROSYSTEMS GmbH, Wetzlar, Germany
Discovery OHAUS	OHAUS, Nänikon, Switzerland
Epoch	BIO-TEK, Bad Friedrichschal, Germany
Elma Transsonic TI-H-10	ELMA, Singen, Germany
Jenway 6051 Colorimeter	JENWAY, Staffordshire, UK
Gyro-rocker SSL3	STUART, Staffordshire, UK
MaxQ 6000	THERMOSCIENTIFIC. Darmstadt, Germany
Acculab ALC-1100.2.	SARTORIUS LABORATORY, Göttingen, Germany
pH-meter 761 Calimatic	KNICK, Berlin, Germany
Bio-Plex 200	BIO-RAD, Munich, Germany
Semi-dry blotter unit	SIGMA-ALDRICH, Munich, Germany
Syngene G: BOX	SYNGENE, Cambridge, UK
Primers & Vectors	
5'-CAC CAA TGA ATA TAC CGA TGC ACT GGC AAA ATG TAC-3' (forward primer) 5'-TTA TTC CCA ATA CAG ACA CAG AAC TTT CAG TT-3' (reverse primer)	(Poot et al., 1997)
pET151 TOPO expression vector	LIFE TECHNOLOGIES, Darmstadt, Germany
Rosetta gami® competent cells	MILLIPORE, Darmstadt, Germany
Software	
Bio-Plex 6.0	BIO-RAD, Munich, Germany
Bio-Rad CFX Manager 2.0	BIO-RAD, Munich, Germany
GraphPad Prism 5.03	GRAPHPAD PRISM, La Jolla, USA
Microsoft® Office for Mac 2011	MICROSOFT, Redmond, USA
BLAST®	National Center for Biotechnology Information, Bethesda, USA
Clonemanager professional 9	SCIENTIFIC & EDUCATION SOFTWARE, Cary, North Carolina, USA
Gene Snap 7.09.06	SYNGENE, Cambridge, UK
Sigma Plot	SYSTAT SOFTWARE, Chicago, USA
EndNote X5	THOMSON REUTERS, San Francisco, USA

3.2. Development of a serum-based multiplex assay

3.2.1. Serum samples

The standardisation of the multiplex serum assay and the determination of the cut-off values were achieved by using control sera obtained from parasite naïve animals before (negative control) and after experimental mono-infection with the target parasites *C. oncophora*, *D. viviparus* (Uppsala, Sweden) and *F. hepatica* (Ghent, Belgium). For testing specificity as well as cross reactivity, sera from animals mono-infected with other important GI nematodes, *Haemonchus contortus*, *O. ostertagi* and *T. colubriformis* were used. All animal experiments were conducted in strict accordance with the respective local legislation (Deutsches Tierschutzgesetz) and the European guideline for animal experiments (2010/63/EU). They were approved by a) the Landesamt für Gesundheit und Soziales, Berlin, Germany, under the reference number L 0088/10, b) the Ethical Commission of the Faculty of Veterinary Medicine, Ghent University, Belgium under the reference number EC2009/086 and c) the Swedish Animal Ethics Committee under the permission C4/2 (Karanikola et al., 2015).

3.2.2. Recombinant antigens

The protein used for the detection of Abs against *C. oncophora* was a 14.2 kDa ES protein (Karanikola et al., 2015; Poot et al., 1997). A codon optimised (*E. coli*) version of the open reading frame (ORF) was synthesised *in vitro* (SynthesisGene®; China). The ORF was amplified using the forward primer (5'-CAC CAA TGA ATA TAC CGA TGC ACT GGC AAA ATG TAC-3') and reverse primer (5'-TTA TTC CCA ATA CAG ACA CAG AAC TTT CAG TT-3'). PCR products were cloned into the pET151 TOPO expression vector (Life Technologies). A Rosetta gami® (Novagene) *E. coli* clone containing the pET151/CoES14.2 was cultured at 37 °C until OD_{600nm} reached 0.6. Synthesis of the ES14.2-V5-6×His protein was induced with 0.5 mM isopropylthio-galactoside (IPTG) at 37 °C for 4 h. The recombinant ES14.2-V5-6×His protein was purified from inclusion bodies using Protino® Ni-NTA columns (Macherey-Nagel, Germany) according to the manufacturer's protocol. An additional wash step using a 50 mM concentration of imidazole and 2% Tween 20 was conducted before elution with 250 mM imidazole. Purity of the eluted protein was analysed on 12% SDS–PAGE, stained with GelCode™ colloidal coomassie stain (ThermoFisher). Western blotting using an anti-V5 Ab (Life Technologies) was carried out to confirm that the target protein was obtained.
For the detection of *D. viviparus* Abs, the recombinant 43 kDa MSP, expressed as a glutathione-S-transferase (GST) fusion protein in *E. coli* BL21 (DE3) cells, as previously described by Gozdzik et al. (2012) was used. The Ag was provided by Uppsala University, Sweden.
The Ag used for the detection of *F. hepatica* was a recombinant 37 kDa Cathepsin L1 protein (Collins et al., 2004), provided by ILDANA BIOTECH, UCD, Dublin. It is an active site [$Cys^{26}Gly$] mutant expressed in the yeast *Pichia pastoris*.

3.2.3. Antigen coupling to fluorescent beads

The coupling procedure is based on a two-step carbodiimide reaction. Firstly, the activation of the carboxyl groups on the surface of the beads is required and secondly, the protein is conjugated. The activation of the beads is achieved by the incubation of the microspheres with two reagents. EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) (Sigma-Aldrich, Germany) reacts

with the carboxyl groups onto the microspheres in order to form an active O-acylisourea intermediate, which subsequently forms a more stable ester by the use of S-NHS (N-hydroxysulfosuccinimide) (Sigma-Aldrich, Germany). The formed ester reacts with the primary amine molecules (NH_2) of the proteins.
Coupling chemistry may be affected by various factors among which stand common additives to proteins and buffers (amine containing compounds such as Tris, Bovine Serum Albumine - BSA or sodium azide) interfering with the coupling reaction. Furthermore glycerol, urea, imidazole and some detergents are capable of affecting coupling chemistry. As a result, these reagents should be removed prior to the conjugation of the protein onto the beads. The molecule to be coupled should be lastly suspended in phosphate-buffered saline (PBS), pH 7.4, a buffer compatible to the solubility of ligands.
In order to remove sodium azide or imidazole, *D. viviparus* and *C. oncophora* recombinant Ags were purified by gel filtration using Micro Bio-Spin 6 chromatography columns (Bio-Rad, Germany) according to the manufacturer's protocol. Initially, all buffers were brought to RT before use. The column was inverted sharply several times in order to re-suspend the settled gel and remove any bubbles. The tip of the column was subsequently snapped off and the column was placed in a 2 ml microcentrifuge tube. It was centrifuged for 2 min at 1000 × g in order to remove the remaining packing buffer, which was finally discarded. Then 500 µl PBS, pH 7.4, were applied to the top of the column and it was centrifuged at 1000 × g for 2 min. The buffer in the collection tube was discarded. That procedure was repeated 4 additional times while on the last wash step, the tube was centrifuged for 4 min. Following that, the column was placed in a clean and labelled 1.5 or 2 ml microcentrifuge tube and 75 µl of protein sample were carefully applied directly to the centre of the column and was centrifuged for 5 min at 1000 × g. The purified protein sample was suspended in PBS, pH 7.4 and it was placed on ice until the concentrations of the Ag was determined using the CB-X™ Assay (G-Biosciences, USA). Briefly, 10µl of protein sample were transferred to a 1.5 ml centrifuge tube, 1 ml of pre-chilled (-20°C) CB-X™ was added and the tube was vortexed to mix. Then it was centrifuged at 16.000 × g for 5 min and the supernatant was carefully removed without disturbing the protein pellet. Following that, 50 µl of CB-X™ Solubilization Buffer-I and 50 µl of Solubilization Buffer II were added to the tube, which was vortexed until the protein pellet was dissolved. The CB-X™-Assay Dye was inverted 2 to 3 times to mix and then 1 ml was added to the tube, which was briefly vortexed and then left for incubation at RT for 5 min. The absorbance was read at 595nm against deionized water by transferring 200 µl assay solution to a microtiter plate well. The calculation of the protein concentration was performed by using the appropriate CB-X™ Table, provided by the manufacturer.
D. viviparus, C. oncophora and *F. hepatica* Ags were conjugated on the surface of carboxylated (COOH) magnetic beads (Bio-Plex Pro™ Magnetic COOH Beads – 1.25 × 10^7 beads/ml, Bio-Rad) using the fluorescence regions 026, 062 and 065, respectively.
Coupling reactions were performed using the Amine Coupling Kit® (Bio-Rad, Germany), following a two-step carbodiimide reaction protocol provided by the manufacturer. The stock suspension of uncoupled beads was vortexed at high speed for 30 s followed by sonication for 15 s in order to disperse bead aggregates. A 100 µl aliquot of monodisperse COOH microspheres (1.25 × 10^6 beads) was transferred to one Bio-Plex coupling reaction tube and was placed into the magnetic separator for 30 to 60 s before removal of the supernatant. The microspheres were washed once in 100 µl bead wash buffer, followed by re-suspension in 80 µl bead activation buffer. Then 10 µl of 50 mg/ml S-NHS (Sigma-Aldrich, Germany) and 10 µl of 50 mg/ml EDAC (Sigma-Aldrich), which were prepared in bead activation buffer immediately prior to their use, were added. The reaction tube was mixed gently, covered with aluminium foil and then gently agitated on a shaker for 20 min at RT.

PBS (pH 7.4, 150 µl) was added twice, always followed by vigorous vortexing. The recombinant protein was added and total volume was brought to 500 µl with PBS (final Ag concentration 5-12 µg/500 µl). Incubation was performed on a shaker at high speed (600-700 rpm) at RT for 2 h. In order to achieve higher coupling yields, some alterations of the initial protocol were made. It was observed that beads incubated at medium speed (500 rpm) as recommended had a tendency to precipitate at the bottom of the coupling reaction tube. Therefore, speed was slightly increased and the coupling reaction tube was vortexed once at high speed after 1 h to prevent precipitation.
Initially, different concentrations were used for each of the three Ags and were separately tested in order to determine the optimum Ag concentration. The amounts of the conjugated protein were the following for each bead-set: for *C. oncophora* 7 µg, 5 µg, 3.5 µg, 2.5 µg, 1.75 µg, 1.25 µg, 0.9 µg and 0.45 µg, for *D. viviparus* 5 µg, 2.5 µg, 1.25 µg, 0.66 µg and 0.45 µg, and for *F. hepatica* 5 µg, 2.5 µg and 1.25 µg. The coupled beads were placed into a magnetic separator for 1 min and after removal of the supernatant they were washed with 500 µl of PBS. The coupled beads were then re-suspended in 250 µl of blocking buffer and gently agitated at RT in the darkness for 30 min. Finally, the microspheres were washed with 500 µl of storage buffer, re-suspended in 150 µl storage buffer and stored at 4 °C in the dark. Beads were stored on the recommended conditions and always used within 4 months after coupling since decrease performance was observed thereafter (Karanikola et al., 2015).

3.2.4. Luminex multiplex serum immunoassay protocol

The assays were conducted in 96-well polystyrene, round-bottom microplates (Greiner Bio-One). The three bead-sets were initially tested in single-plex assays including negative controls as well as the respective positive control sera. These were used in a two-fold eight serial dilution series in PBS/Tween 20 (0.05%, pH 7.4) in order to identify the optimal sample dilution. Cross-reactivity was assessed by running the assay with sera from calves infected with non-target species. Then, the three bead-sets were combined in a bead-mix and a multiplex assay was performed.
Prior to each examination, beads were re-suspended by vortexing and sonication for approximately 20 s three times to avoid high numbers of aggregated beads. A 50 µl aliquot of the working microsphere mixture (concentration 100 beads/µl) was transferred into the wells, followed by the addition of 50 µl of diluted sera. The plate was incubated on a plate shaker (800 rpm) in the dark at RT for 60 min. The plate was then placed into the magnetic separator and left for separation for 60 s. The supernatant was carefully removed from each well by manual inversion. Beads were washed 5 times by adding 100 µl PBS/Tween 20 into each well to ensure absence of any undesirable or non-specifically bound Abs. The plate was then removed from the magnetic separator and 100 µl of a biotinylated secondary Ab (goat anti-bovine IgG, Dianova, Germany) diluted 1:1000 in PBS/Tween 20 were added to each well. Incubation was again conducted in darkness and at RT on a plate shaker (800 rpm) for 30 min before beads were washed as described above. Finally, 100 µl of SA-PE (Millipore) at 2 µg/ml, diluted in assay buffer, were added to each well. The plate was placed on the shaker, covered with aluminium foil and again incubated at RT on a plate shaker (800 rpm) for 30 min. The supernatant was carefully removed after magnetic separation of the beads by manual inversion and washing was performed as previously described. Assay buffer (100 µl) was added into each well and the plate was placed onto a plate shaker for approximately 15 s in order to achieve gentle agitation of the beads.
The beads were analysed using the Bio-Plex 200 (Bio-Rad, Germany) instrument following the manufacturer's instructions. A minimum of 100 events (beads) per well

was read for every bead-set and were measured in Median Fluorescence Intensity (MFI) values. All samples were analysed in duplicates in each run (Karanikola et al., 2015). To investigate reproducibility of the assays, several runs were performed using the sera from the same animals.

3.3. ELISAs used for comparison

3.3.1. Serum samples

A subset of 30 samples were tested in parallel in Sweden for the detection of *D. viviparus* Abs according to the protocol described by Gozdzik et al. (2012). In the case of *F. hepatica*, 39 serum samples were tested with DRG liver fluke ELISA while a larger subset of 363 samples were examined using the commercially available SVANOVIR®- F.hepatica-Ab ELISA.
The results obtained using the Luminex® assay were compared to existing ELISAs. *D. viviparus* Abs were detected using either an in-house ELISA for lungworm based on the recombinant MSP Ag as described in von Holtum et al. (2008) or using the modification of this ELISA as described by Gozdzik et al. (2012). Abs against *F. hepatica* were detected using either a commercially available SVANOVIR®-F.hepatica-Ab ELISA (Svanovir, Sweden) based on a crude ES Ag (Salimi-Bejestani et al., 2005b) or the commercially available DRG liver fluke ELISA (DRG Diagnostics, Germany), using recombinant Cathepsin L1 (Karanikola et al., 2015). In the case of *C. oncophora*, absence of a commercially available ELISA did not allow comparison of the Luminex® assay performance.

3.4. Development of a milk-based multiplex assay

Following the standardisation of the serum-based multiplex immunoassay, a Luminex milk assay was developed in order to provide rapid and accurate diagnosis of the target parasite species. Diagnosis by examination of BTM samples is the main tool used nowadays to detect Abs produced against GI nematodes, *D. viviparus* and *F. hepatica* in dairy cattle herds (Bloemhoff et al., 2015; Charlier et al., 2009; Kuerpick et al., 2012a).

3.4.1. Milk samples

The absence of defined milk samples from parasite-free or specifically mono-infected dairy cows is a major constraint in evaluating milk-based immunoassays. Due to the persistence of Abs despite treatment and the general more elevated Ab titres of animals after the initial infection compared to naive animals, the use of adult animals as “defined” controls is debatable. Nevertheless such samples have been used in almost all performed studies, making reported sensitivity and specificity difficult to compare. Therefore, standardisation of the milk assay was firstly based on the use of paired (serum and milk) from individual cows for the determination of cut-off values for the multiplex milk platform. These samples (n=78) were collected from herds in Lower Saxony, Germany. Serum Ab levels were obtained using the recently developed triplex serum immunoassay (Karanikola et al., 2015) and compared to milk Ab levels of the same animal. Prior to examination, milk samples were centrifuged at 1500 × g for 15 min and the whey fraction between the sediment and fat layer was recovered to avoid measurement deviations due to the impact of fat on the measured values (Witte et al., 1989). All samples were stored at -20 °C until use.

3.4.2. Performance characteristics of the multiplex milk immunoassay

In general, the same protocols and Ag concentrations were used for the conjugation of recombinant Ags on the surface of magnetic COOH beads (sections 3.2.2. and 3.2.3.). The examination of individual and BTM samples was performed according to the previously established Luminex serum immunoassay protocol (section 3.2.4.). The only difference lays on the fact that the Ag-coupled beads were incubated with 50µl of undiluted milk samples. Secondary Ab and SA-PE substrate reporter molecule were used in the same dilution and concentration, respectively, as performed during the examination of serum samples.

3.4.3. Milk samples used for comparison with ELISAs

As a second measurement comparisons between the newly developed platform and already existing ELISAs were performed. For *D. viviparus* the previously reported ELISA using the same recombinant Ag was performed by examining 30 BTM samples. For *F. hepatica*, 245 samples were tested in parallel with DRG liver fluke ELISA and 2813 were evaluated for their Ab titres using the SVANOVIR®-F.hepatica-Ab ELISA.

3.5. Field validation

The newly developed platform for the detection of Abs against *C. oncophora*, *D. viviparus* and *F. hepatica* was finally used to examine milk samples, which were collected from cattle, naturally infected with the target species, following a harmonized spatial sampling protocol (Ducheyne et al., 2015) within the GLOWORM project. Additionally serum samples collected routinely from pastured calves were used.

3.5.1. Luminex serum immunoassay

Serum samples collected in Denmark (*n*=39), Switzerland (*n*=76) and Poland (*n*=367) were used in order to validate the newly developed platform in the field. In Denmark and Switzerland the samples were taken from grazing young cattle on randomly selected farms. The sampling in Poland took place on farms previously identified for a cross-sectional survey using a two-stage sampling approach (Ducheyne et al., 2015). On a subset of those farms, 10 to 15 FGS cattle were randomly sampled.

3.5.2. Luminex milk immunoassay

BTM samples (*n*=3078) were available from a previously conducted cross-sectional study from seven areas located in four different European countries: Belgium: Flanders (*n*=1258), Germany: Lower Saxony (*n*=383) and Mecklenburg (*n*=385), Ireland: whole country (*n*=286) and Poland: Podlaskie (*n*=383) and Łódzkie (*n*=383). Extensive details are provided in Ducheyne et al. (2015). Briefly, in each country (with the exception of Ireland) the regions were selected due to the high density of dairy cattle in the respective area. A two-stage sampling approach was followed by selecting initially up to 315 municipalities per country, in which the number of

sampled farms was weighed according to three dairy cattle density categories. Within each municipality, farms were randomly selected. Sampling was performed close to or after housing of the animals (between October 2012 and January 2013).
Prior to each examination, BTM samples were centrifuged at 1500 × g for 15 to 20 min in order to recover the whey fraction between the sediment and the fat layer. The samples were stored at -20 °C until use.

3.6. Statistical analysis

GraphPad Prism® software 5.04 was used for the statistical analysis. Five parameter logistic regression curves were calculated in order to determine the optimal serum dilution for each separate bead set. Positive and negative samples as well as sera from animals infected with non-target species were compared using box plots. Serum cut-off MFI values for each parasite specific assay were obtained using receiver operating characteristics (ROC) analysis to determine at the same time sensitivity and specificity.
In the case of milk, 78 paired serum and milk samples were used for the determination of cut-off MFI values for each parasite specific assay. Serum samples were classified negative using the negative cut-off values previously reported in Karanikola et al. (2015). The values obtained for the corresponding milk samples were then used for negative cut-off determination for milk. For all target species the negative cut-off was determined using the arithmetic mean of negative milk samples plus three standard deviations (SD). This cut-off should be higher than 95% of all negative samples. To determine a positive cut-off value (only possible for *F. hepatica*), comparison of milk samples tested with DRG liver fluke ELISA (using the same recombinant Ag, (n=245) and with the Luminex® assay was performed. Since variation of MFI values for positive samples was very high and data were not normally distributed, the positive cut-off was determined by calculation of the 5% percentile of all positive samples.
For comparison with existing ELISAs (*F. hepatica* and *D. viviparus*) subsets of samples (serum and milk) were examined and calculation of Cohen's kappa coefficient was performed in order to calculate the agreement between the newly developed platforms and the commercially available immunoassays, using an online calculator (http://vassarstats.net/kappa.html). Additionally, the correlation between ELISAs and Luminex® (R^2 and Pearson r) and linear regression analysis were conducted for the newly developed milk platform.
As a proof of principle evaluation of serum and BTM samples available from a previously conducted spatial sampling approach were analysed with the new technique and were analysed using box plots. Prevalence and the 95% confidence interval (CI) of the three parasite species based on Abs were calculated for each country using OpenEpi (http://www.openepi.com) choosing the "Proportion" option and the CIs were reported as Wilson Score intervals. Pairwise comparison of the individual species prevalence between countries were conducted using the mid-p modification of the Fisher's exact test as suggested by Lydersen et al. (2009) followed by correction of p-values using the method of Bonferroni-Holm.

4. RESULTS

4.1. Luminex serum immunoassay

4.1.1. Optimal amount of protein for coupling

The optimal amount of Ag identified for the target species were 0.45 µg for both, *D. viviparus* and *C. oncophora,* and 2.5 µg for *F. hepatica*. These were determined based on the amount of conjugated protein that would provide a reliable and reproducible MFI signal, which enabled a clear differentiation between positive and negative serum control samples (Karanikola et al., 2015).

4.1.2. Optimisation of secondary antibody dilutions and SA-PE concentration

Using the optimal amount of Ag, four different secondary Ab dilutions (1:500, 1:1000, 1:2000 and 1:5000) and four SA-PE concentrations (0.5 µg/ml, 1 µg/ml, 2 µg/ml and 4 µg/ml) were tested. Optimal results were obtained for a 1:1000 dilution for the biotinylated secondary Ab and a concentration of 2 µg/ml for SA-PE (Karanikola et al., 2015).

4.1.3. Optimal serum dilution

Determination of the optimal serum dilution was based on the examination of negative and positive control sera in a two-fold dilution series ranging from 1:100 to 1:12.800 for the separate coupled bead sets. The logistic regression curves of the MFI values for all three target species enable a clear differentiation between the target species and negative control as well as non-target species (Figure 4) with relatively low background MFI values. Multiplex assays were also performed using an eight two-fold dilution series and results were comparable to those obtained in the single-plex assays (Figure 5).
For all assays R^2 values were close to 1. To achieve an optimal discrimination between positive and negative sera for all three bead sets a dilution of 1:200 was chosen (Karanikola et al., 2015).

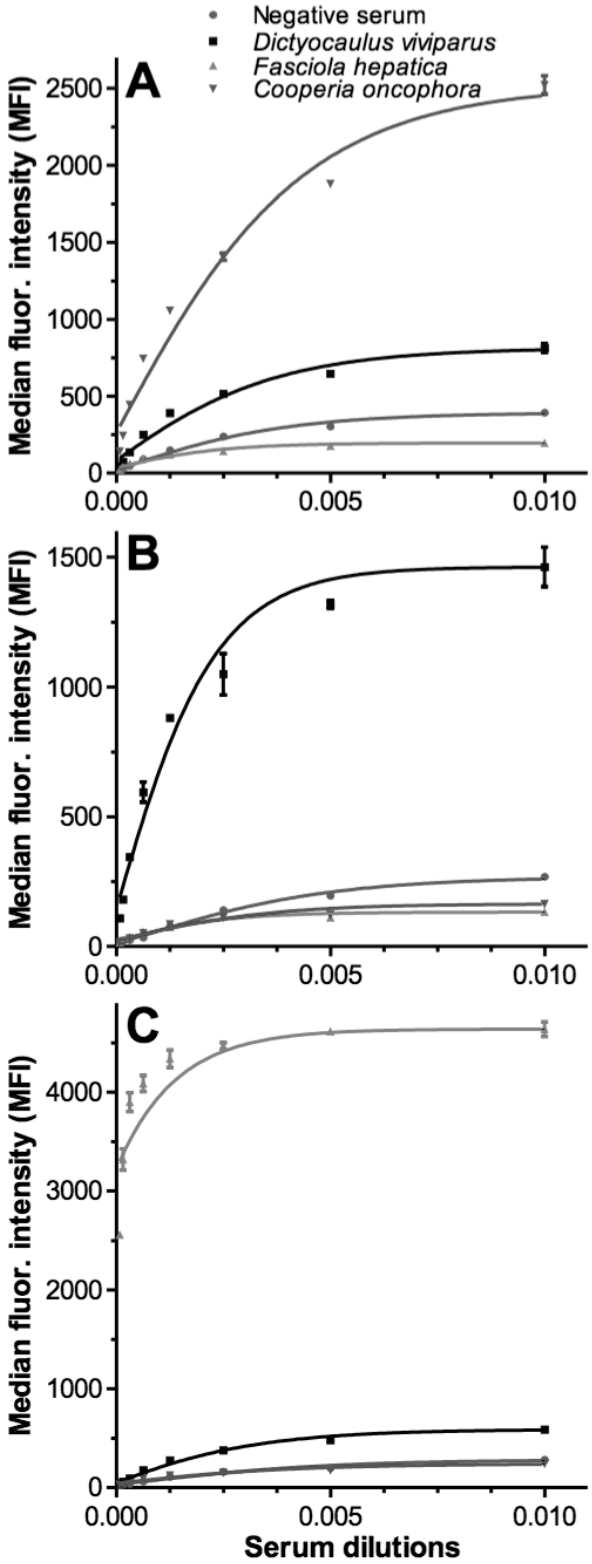

Figure 4. Results for single-plex assays using serum dilutions of animals infected with *Dictyocaulus*, *Fasciola* and *Cooperia*. Five parameter logistic regression curves were calculated based on median fluorescence intensity (MFI) values. **(A)** *Cooperia oncophora* coupled beads using sera positive for *C. oncophora* (green), *Dictyocaulus viviparus* (black), *Fasciola hepatica* (red) and negative control sera (blue). **(B)** *D. viviparus* coupled beads and **(C)** *F. hepatica* coupled beads using the same sera. Dilutions are presented as 0.005 ≙ 1:200 (Karanikola et al., 2015).

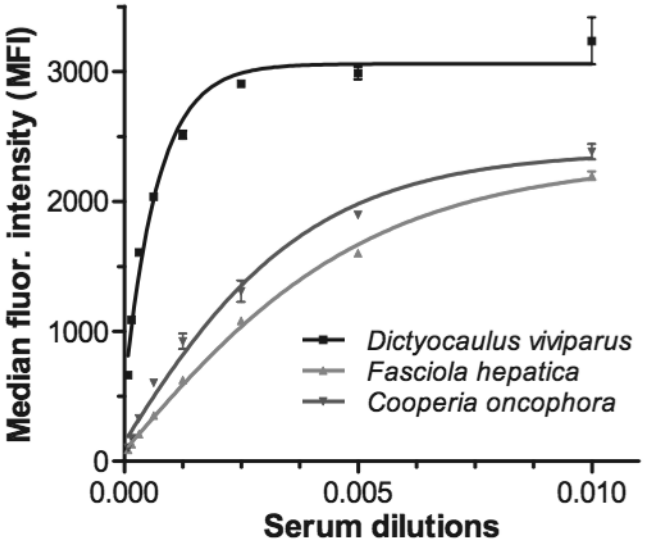

Figure 5. Results for the tri-plex assay using serum dilutions of animals infected with *Dictyocaulus*, *Fasciola* and *Cooperia*. Five parameter logistic regression curves were calculated based on median fluorescence intensity (MFI) values. Beads are coupled with recombinant antigen for the detection of *Cooperia oncophora* (green), *Dictyocaulus viviparus* (black) and *Fasciola hepatica* (red). Artificial mixtures of sera from animals infected with the target species was used. Dilutions are presented as 0.005 ≙ 1:200 (Karanikola et al., 2015).

4.1.4. Assessment of cross-reactivity and serum cut-off determination

All bead sets were examined using positive and negative control sera as well as sera from non-target species infections. Since no obvious differences were observed between single-plex and multi-plex assays, results were combined and are shown in Figure 6. Box plots indicated clear differentiation between positive and negative control serum samples for all three target species. Regarding cross-reactivity Ags used for the detection of *C. oncophora* and *F. hepatica* could clearly distinguish between infections with target and non-target species (*H. contortus*, *T. colubriformis*, *O. ostertagi*, *D. viviparus* and *F. hepatica* or *C. oncophora*, respectively). This was different for the recombinant MSP Ag, where cross-reactivity was more pronounced for sera from *C. oncophora* and *F. hepatica* infected animals; particularly for a few *C. oncophora* positive sera differences to the lowest observed MFI value for *D. viviparus* were only minimal.

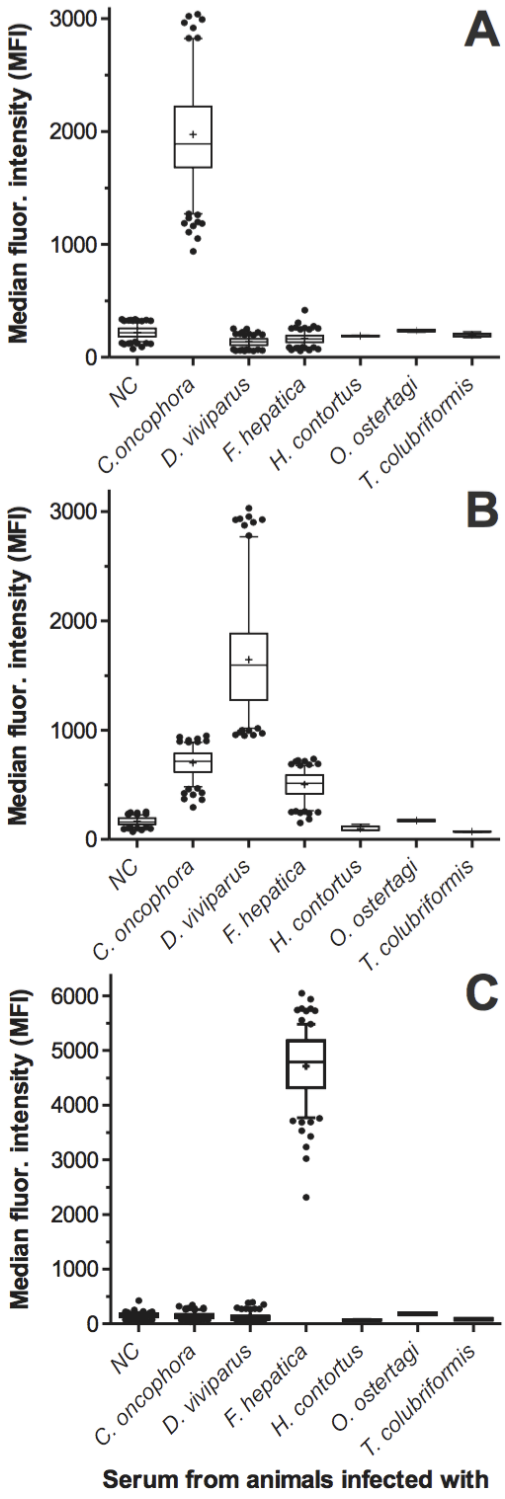

Figure 6. Cross reactivity analysis using sera from target and non-target species. Results are presented as box-plots showing the median fluorescence intensity (MFI) values obtained from multiple testing of sera from negative and mono-infected animals. Bead set were coupled with recombinant antigen for the detection of *Cooperia oncophora* **(A)**, *Dictyocaulus viviparus* **(B)** and *Fasciola hepatica* **(C)**. Whiskers represent 5% and 95% percentage quantiles and the mean is indicated by a +. Outliers are shown as individual dots (Karanikola et al., 2015).

Determination of the cut-off MFI values, sensitivity and specificity was achieved by ROC analysis separately for each bead set. Since serum samples were derived from experimentally infected animals and either clearly negative (parasite naïve prior to infection) or positive, two cut-off values were defined, one discriminating negative

and one positive, leaving grey zone in between. For *D. viviparus* the situation was slightly different with some cross-reactivity present particularly for the *C. oncophora* coupled beads, so that for this assay only one cut-off value was determined. The cut-off values with sensitivity and specificity including the 95% confidence intervals are presented in Table 4 (Karanikola et al., 2015).

Table 4. Results of the Receiver Operating Characteristics analysis for negative (neg.) and positive (pos.) cut-off values. Median fluorescence intensity (MFI), sensitivity and specificity with confidence intervals (CI) are shown (Karanikola et al., 2015).

	MFI	Specificity	95% CI	Sensitivity	95% CI
C. oncophora					
Neg. cut-off	379	99.84%	99.11-100%	100%	98.17-100%
Pos. cut-off	997	100%	99.41-100%	99.50%	97.25-99.99%
D. viviparus					
Cut-off	950.5	100%	99.32-100%	100%	97.93-100%
F. hepatica					
Neg. cut-off	340.8	99.46%	97.01-99.99%	100%	98.02-100%
Pos. cut-off	2670	100%	98.02-100%	99.46%	97.01-99.99%

4.1.5. Assay reproducibility

Serum samples were obtained from different animals on different days pre and post infection and tested multiple times independently as well as in parallel on the same plate in order to determine the reproducibility of the assay. The MFI values obtained indicated reproducible results which are shown in Table 5. The results obtained for the individual animals showed distinct immune responses, resulting in different levels of mean MFI values. Although CV were relatively high for some individuals when testing for Abs against *D. viviparus*, all individual values clearly identified the respective samples as positive (Karanikola et al., 2015).

Table 5. Results of technical reproducibility using serum from different experimentally infected animals (Karanikola et al., 2015).

	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5
C. oncophora					
N	8	8	8	8	8
Mean	2236	3778	3870	3124	2876
CV	3.29%	2.11%	3.33%	4.78%	6.12%
D. viviparus					
N	12	8	6	6	6
Mean	2332	3738	2595	1923	1472
CV	4.47%	5.32%	17.24%	24.885	9.58%
F. hepatica					
N	12	10	8		
Mean	1583	1888	4770		
CV	9.39%	9.64%	3.09%		

N number of replicates
CV coefficient of variation

4.1.6. Comparison between serum multiplex immunoassay and single ELISAs

According to Karanikola et al. (2015) the validation of this multiplex assay was performed by comparing the results obtained from the examination of individual serum samples with already existing and established assays. For the detection of *C. oncophora* Abs no commercial ELISA is currently available and therefore no comparison was conducted.
Initially, only positive and negative samples derived from experimentally infected animals pre and post infection (*F. hepatica* and *D. viviparus*) were compared, using an in-house ELISA for lungworm as described by von Holtum et al. (2008) as well as an in-house ELISA for liver fluke based on crude ES Ag. The results obtained were identical for both, negative as well as positive control sera.

To increase the number of samples tested, additionally serum samples collected during a field trial in Poland were used for comparison. Thirty-nine of these samples were analysed using the commercially available DRG liver fluke ELISAs. For the detection of lungworm no commercial ELISA is currently available. Serum samples were analysed in parallel in Sweden using an in-house ELISA (Gozdzik et al., 2012). In the latter, all samples were negative for *D. viviparus* Abs while one sample was detected positive in the Luminex assay. Due to the fact that almost all samples were negative, no kappa statistic could be calculated. The comparison with the DRG liver fluke ELISA resulted in a kappa value of 0.37 (62.7% of 0.60 maximum achievable). For this calculation the negative cut-off values for both assays were used. A larger subset of 363 samples were analysed using the commercially available SVANOVIR® F.hepatica-Ab ELISA (protocol identical to the in-house liver fluke ELISA as mentioned above) in the laboratory in Ghent. The comparison resulted in a kappa value of 0.460 (74.4% of 0.67 maximal achievable). While 67 samples were positive only in the Luminex, there were also 21 samples, which were positive in the ELISA but clearly negative in the Luminex assay.

4.2. Luminex milk immunoassay

4.2.1. Cut-off determination for individual milk samples

Negative cut-off values were determined using those of the paired serum and milk (*n*=78) samples, where corresponding serum MFI value was negative. As cut-off the arithmetic mean plus three SDs was defined (Table 6), resulting in 350.8 for *C. oncophora*, 246.3 for *D. viviparus* and 515.7 for *F. hepatica*. In the case of liver fluke, 136 samples positive in the DRG liver fluke ELISA and the newly developed Luminex® assay were available for statistical analysis. The 5% percentile of the MFI values of the ELISA positive samples was defined as the positive cut-off in the multiplex platform, resulting in a cut-off value of 1559 MFI (Table 7).

Table 6. Negative cut-off values calculated using the mean of the median fluorescence intensity (MFI) value plus three standard deviations (SD), including the 95% confidence interval (CI) for *Cooperia oncophora*, *Dictyocaulus viviparus* and *Fasciola hepatica*.

	Number of samples	Mean MFI	Mean +3SD (MFI)	95% CI of the mean
C. oncophora	68	118.1	350.8	101.8 – 149.8
D. viviparus	77	81.6	246.3	69.1 – 94.0
F. hepatica	73	157.8	515.7	130.0 – 185.7

Table 7. Positive cut-off values for *Fasciola hepatica* calculated using the 5% percentile of the median fluorescence intensity (MFI) values from samples tested positive in the DRG ELISA including minimum (Min) and the maximum (Max).

	Number of samples	Min (MFI)	Max (MFI)	Positive cut-off (MFI)
F. hepatica	136	458.5	6948	1559

Comparison of the calculated MFI values following the examination of serum and milk samples indicated complete agreement in the case of *D. viviparus*, where all samples were characterised by low Ab level.
For *C. oncophora*, 67 paired samples were detected negative, while 3 out of the 78 examined samples were found positive in both serum and milk. One sample displayed a positive MFI in serum while detected negative in milk and another sample was characterised by the opposite (serum positive/milk negative). Six out of the 78 serum samples were classified in the grey zone, while three of the

corresponding milk samples were clearly negative and the other three slightly above the determined negative cut-off for *C. oncophora*.
For *F. hepatica*, 73 paired serum and milk samples were found negative while 2 were classified in the grey zone for both serum and milk. Three serum samples were classified in the grey zone. Two of the corresponding milk samples were found positive while the other was negative.
Using the determined cut-off values, kappa values were performed for *C. oncophora* and *F. hepatica*, resulting in 0.88 (out of 0.88 possible) and 0.67 (out of 0.80 possible), respectively. The absence of positive samples (and the complete agreement regarding negative samples) kappa statistics could not be performed in the case of *D. viviparus*.

4.2.2. Comparison between multiplex milk immunoassay and single ELISAs

Validation of the developed multiplex assay was achieved by comparing the results obtained following the examination of BTM samples with the above mentioned ELISAs.
For the detection of Abs against *D. viviparus*, 30 randomly selected BTM samples were tested in parallel using an in-house developed ELISA based on the same recombinantly expressed MSP Ag (Gozdzik et al., 2012). The latter detected only negative samples contrary to the Luminex® assay where 15 samples displayed MFI values slightly above the calculated negative cut-off value. The absence of defined positive samples, did not allow the calculation of kappa values.
In the case of *F. hepatica*, 245 BTM, available from the field study, were examined using the DRG liver fluke ELISA and the newly developed platform (Figure 7).

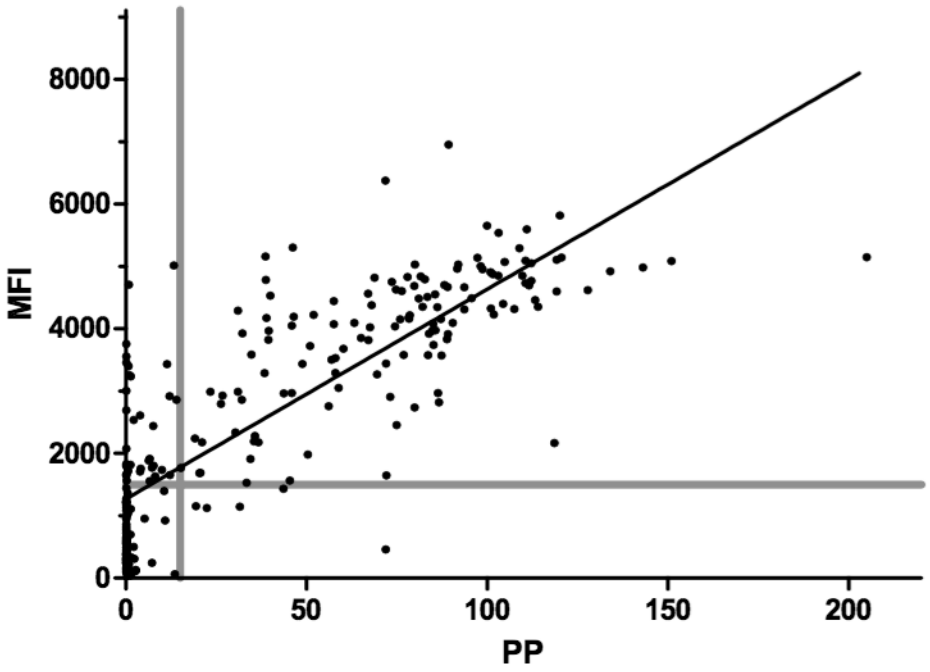

Figure 7. Correlation of results obtained by the DRG ELISA (in percent positivity; PP) and the Luminex assay (in median fluorescence intensity; MFI) for *Fasciola hepatica*. Vertical and horizontal lines represent the cut-off values for the respective assays. A Pearson correlation coefficient was calculated (r = 0.82, p<0.0001). The black line shows the linear regression line. Number of samples: 245 bulk tank milk samples.

Correlation analysis between both assays indicated a Pearson r value of 0.82, while that value was lower (Pearson r = 0.33) when Luminex® assay was correlated to the SVANOVIR® F. hepatica-Ab ELISA based on an ES Ag (Figure 8).

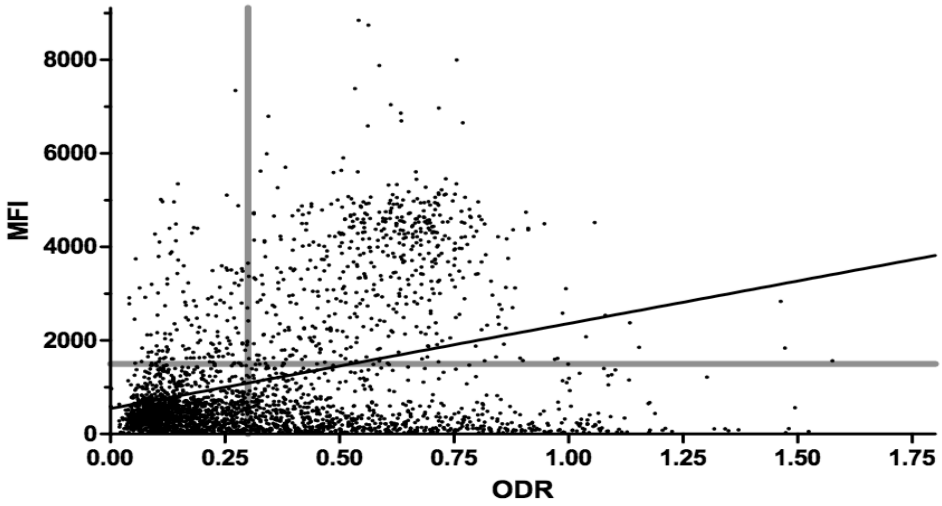

Figure 8. Correlation of results obtained by the SVANOVIR® F. hepatica-Ab ELISA (in optical density ratio; ODR) and the Luminex (in median fluorescence intensity; MFI) for *Fasciola hepatica*. Vertical and horizontal lines represent the cut-off values for the respective assays. A Pearson correlation coefficient was calculated (r = 0.33, p<0.0001). The black line shows the result of the linear regression. Number of samples: 2805 bulk tank milk samples.

Calculation of the Cohen's kappa coefficient revealed good agreement between the DRG ELISA and the Luminex® assay (0.71 of maximal achievable 0.81) while, in the case of SVANOVIR® F. hepatica-Ab ELISA kappa value resulted in 0.34 of maximal achievable 0.54, indicating fair agreement between both assays.

4.3. Field validation

4.3.1. Multiplex serum immunoassay

Analysis of field serum samples collected in Denmark, Switzerland and Poland indicated no or only low prevalence of *D. viviparus* infection in all three countries. *C. oncophora* prevalence appears to be higher in Poland in comparison to Denmark and Switzerland. In addition, Poland was characterised by increased levels of *F. hepatica* Ab titres. Significant was the number of samples classified in the grey zone, which was relatively high in all three countries participating in the study. The calculated prevalences are presented in Table 8 (Karanikola et al., 2015).

Table 8. Percentage of serum samples positive for antibodies against *Cooperia oncophora*, *Dictyocaulus viviparus* and *Fasciola hepatica*. Results for the field samples are shown per country and include 95% confidence intervals (95% CI) (Karanikola et al., 2015).

	Denmark (n=39)	Poland (n=367)	Switzerland (n=76)
C. oncophora	28.21%[a]	73.84%[b]	48.68%[c]
95% CI	16.42-43.90%	69.11-78.08%	37.78-59.71%
D. viviparus	0%[ab]	3.82%[b]	0%[a]
95% CI	0-10.68%	2.23-6.34%	0-5.77%
F. hepatica	64.10%[ac]	79.84%[b]	67.11%[c]
95% CI	48.73-77.31%	75.42-83.63%	55.91-76.65%

Percentages which do not share the same indices (a, b, c) are significantly different in a Mid-P exact test (p<0.05).

4.3.2. Multiplex milk immunoassay

The performance of the newly established triplex Luminex assay was evaluated in the field following the examination of BTM samples previously collected in Belgium (*n*=1258), Germany (*n*=768), Ireland (*n*=286) and Poland (*n*=766). In the case of the Irish milk samples, samples were taken from the same herd in successive months. For the calculation of prevalence of exposure only those samples collected in October (n=141) were used.

All detected MFI values above the negative cut-off were classified as positive in the sense of exposure to the parasite. Despite the fact that it was only possible to calculate positive cut-off values for *F. hepatica*, the distribution of MFI values for all three species (Figure 9) clearly indicates that a large number of samples display considerably higher MFI values than the negative cut-off value. This strongly suggests that many samples were obtained from either currently or recently infected herds.

The results indicated frequent occurrence of high Ab levels against *C. oncophora* in the BTM samples from all four countries (Table 9). In Belgium a significantly ($p<0.0001$) lower proportion (64.4%) of the examined herds was found positive than in all other countries. The highest prevalence of Abs against *C. oncophora* was detected in Ireland (87.9%), followed by Germany (82.4%), where especially in the region of Lower Saxony 92.2% of the herds participating in the study were positive. The number of herds tested positive was considerably lower in the case of *D. viviparus*. Only up to 38.5% of the farms were detected positive, while especially in the region of Mecklenburg, Germany only 1.3% were reached. Ab values were lowest in Germany, followed by Belgium, Poland and Ireland. All differences except the latter were significant (at least $p<0.05$).

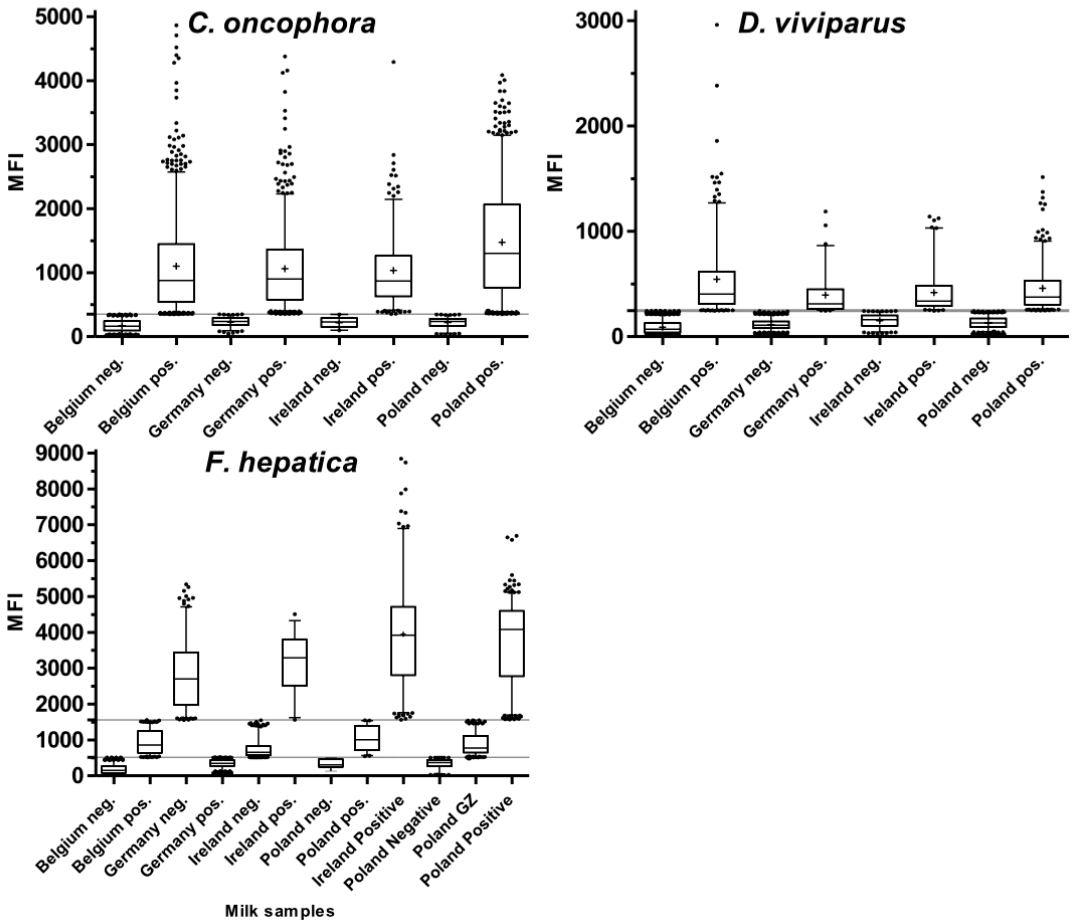

Figure 9. Box plots showing the distribution of median fluorescence intensity (MFI) values for the different countries and parasites, classified as either negative (neg.), positive (pos.) or as in the grey zone (GZ) (for *Fasciola hepatica* only). Mean MFI values are represented by a +, the whiskers show the 5 and 95% percentiles, the median is shown by a solid line. The horizontal dotted line represents the negative cut-off value, the strapped line the positive cut-off value (for *F. hepatica* only).

High numbers of BTM samples positive for Abs against *F. hepatica* were detected in Ireland (93.6%), in one region in Poland (Podlaskie), where positive samples reached 96.2% and in Lower Saxony, Germany with 85.6%. Lower prevalence of exposure was detected in Belgium, Mecklenburg (Germany) and Łódksie (Poland) where 34.3, 31.2 and 68.4% of the herds were found positive, respectively. Differences in the status between sampled regions in Germany and Poland were observed. Exposure to *C. oncophora* and *F. hepatica* was detected in higher rates in Lower Saxony in comparison to Mecklenburg. In Poland, Abs against these pathogens were remarkably higher in Podlaskie. On country level, all obtained exposure rates were significantly different from each other.

Table 9. Prevalence and 95% confidence intervals (CI) of farms tested positive (above the negative cut-off value) for antibodies against *Cooperia oncophora*, *Dictyocaulus viviparus* and *Fasciola hepatica* in bulk tank milk samples in Belgium, Germany, Ireland and Poland.

	Belgium (n=1258)	Germany (n=768)	Ireland (n=141)	Poland (n=766)
C. oncophora	64.39%[a]	82.42%[b]	87.94%[b]	83.16%[b]
95% CI	61.70-66.99%	79.57-84.95%	81.45-92.33%	80.35-85.64%
D. viviparus	23.53%[a]	9.90%[b]	32.62%[c]	38.51%[c]
95% CI	21.27-25.95%	7.98-12.21%	25.44-40.73%	35.13-42.01%
F. hepatica	34.26%[a]	41.80%[b]	93.62%[c]	80.68%[d]
95% CI	31.69-36.93%	38.36-45.32%	88.31-96.61%	77.73-83.32%

Prevalence percentages with different indicators are significantly ($p<0.05$) different between countries

5. DISCUSSION

5.1. xMAP®Luminex®Technology

Based on the principles of flow cytometry, Luminex Corp. (1997, Austin, TX, USA) initiated the commercial production of a system capable of developing innovative technologies applied to life science. xMAP®Luminex®Technology enabled the conduction of rapid, low cost and accurate large-scale studies, by simultaneously detecting multiple pathogens present in the same sample. Over the years, this technology was established in research, diagnostics, pharmaceutical and clinical laboratories. This platform combines bead-based assays with an advanced signal processing and with the identification of up to 100 analytes-pathogens at a time. The pattern of a multiplex assay comprises a suspension array system, where specific capture molecules are conjugated onto polystyrene COOH microspheres, internally dyed with different ratios of two fluorescent dyes, red and infrared. Following the completion of the incubation steps with detection reagents, beads are interrogated in the Luminex analyser by two lasers (https://www.luminexcorp.com).
This technique provides researcher with the ability to simultaneously evaluate large number of samples and analytes and is characterised by flexible multiplexing for a variety of applications since up to 100 different analytes may be detected in a single run. Different microspheres may be added or removed according to the needs of the researcher allowing the examination of multiple targets. Remarkable is the ability of the platform to eliminate test selection bias, meaning that in case of multiple cause of the disease (viral, bacterial or parasitic), no limitations are set in matters of performing different tests to recognise the cause of clinical symptoms. In matters of financial costs, xMAP®Luminex®Technology does not require expensive reagents while the total cost per analyte per sample is significantly lower than in conventional assays. The time needed for each examination is shortened thanks to faster reaction kinetics of liquid bead arrays, which do not require the conduction of enzymatic reactions (Christopher-Hennings et al., 2013).
In order to achieve highly specific multiplex assays, recombinantly expressed proteins should be used for the detection of Abs against pathogens of interest. In the literature, several assays have been described for the detection of Abs using recombinant Ags. For the detection of *Equine arteritis virus*, 8 recombinant Ags were expressed and subsequently tested using xMAP®Technology (Go et al., 2008). The results obtained were compared to an established virus neutralising test in order to detect the concordance between both diagnostic techniques. The experiment resulted in lower sensitivity for the newly developed assay based on $GP5_{55\text{-}98}$ more specifically, however, it was recommended by the authors as a supplementary diagnostic test that would provide accurate, rapid and convenient screening of infected horses. Based on these results and the generally better performance of recombinant Ags in ELISA in matters of specificity and detection of prepatent infections, three recombinant Ags were used in the present study (Karanikola et al., 2015) for the detection of Abs against *C. oncophora* (Poot et al., 1997), *D. viviparus* (Gozdzik et al., 2012) and *F. hepatica* (Collins et al., 2004).
xMAP®Luminex®Technology is widely used in human medical diagnosis (Tighe et al., 2015) but only few reports have been published in the field of veterinary medicine, particularly regarding serological assays. In the literature, several authors have proceeded to the conduction of experiments based on Luminex®Technology, describing either single- or multiplex immunoassays, aiming to increase sensitivity and specificity of the assays. More specifically, a commercially available multiple serologic test for the detection of Abs against 4 different avian viruses (*Infectious*

bursal disease virus, *Newcastle disease virus*, Infectious bronchitis virus and avian reoviruses), under the brand name xMAP Flock Monitor (Biovet USA Inc., Bloomington, MN) indicated good correlation to the existing ELISAs when samples were tested under the same conditions (Christopher-Hennings et al., 2013). Ab responses to avian *Influenza A virus* have also been evaluated using this technique by Watson et al. (2009). Great progress has been made in the porcine disease diagnostics using bead-based multiplex immunoassays. The detection of Abs against *Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV) (Langenhorst et al., 2012) in pen-based oral fluid samples, appeared as an efficient and cost-effective approach to monitor the syndrome. Serologic responses concerning the infection with *Trichinella spiralis* and *Toxoplasma gondii* (Bokken et al., 2012; van der Wal et al., 2013) have additionally been studied and led to the development of multiplex assays. Gimenez-Lirola et al. (2012) reported the development of a multiplex immunofluorescence assay for the early detection of Abs against Gram-positive assay to detect *Erysepelothrix* spp. bacteria using a recombinant polypeptide from the major surface protective Ag which was characterised by higher sensitivity when compared to existing ELISAs. It is worth mentioning that xMAP®Luminex®Technology has been also been used for the detection of porcine inflammation markers, such as cytokines (Bjerre et al., 2009; Bongoni et al., 2013; Johannisson et al., 2006; Wyns et al., 2013). In equine disease diagnostic assays, Luminex® Technology was used to develop a Luminex assay for the detection of Abs specific to *Equine arteritis virus* (Go et al., 2008). Lyme disease bead-based immunoassays have been established for horses and dogs, indicating the wide range this technology applies to (Wagner et al., 2011a; Wagner et al., 2011b). Development of assays for the multiple detection of cytokines in horses has also been described (Wagner and Freer, 2009).

5.2. Multiplexing in bovine diagnostics

Monitoring bovine herds regarding particular pathogens has been achieved through the use of xMAP® Technology. A number of multiplex assays has been developed for the detection and differentiation of pestivirus species from different host, i.e. *Bovine viral diarrhea virus 1* and *2* (BVDV-1 and BVDV-2, respectively), *Classical swine fever virus* and *Border disease virus* (Deregt et al., 2006). The detection of Abs produced against 4 bovine viruses, such as BVDV, *Bovine respiratory syncytial virus*, *Bovine parainfluenza virus 3* and *Bovine herpesvirus 1* was achieved by coupling crude viral lysates on the surface of beads. The assay was characterised by good sensitivity however, when a recombinant protein was used for the detection of Abs against BVDV as a singleplex assay, sensitivity was relatively higher (Anderson et al., 2011).

Several steps have been taken towards the development of multiplex assay for the confirmation of the presence of cytokines in milk and plasma samples. Dernfalk et al. (2007) indicated that IL-1β, IL-6 and TNF-α could be simultaneously detected in bovine samples collected from animals with confirmed clinical mastitis. They furthermore proved absence of cross-reactivity between these analytes and provided future research with a basis for optimising this kind of multiplex assays.

The development of innovative diagnostic techniques based on Luminex® Technology was the aim of the present study. Finally, two high-throughput multiplex immunoassays were established and evaluated under field conditions for the detection of Abs against *C. oncophora*, *D. viviparus* and *F. hepatica* in bovine serum and milk samples. For both assays, the future inclusion of other parameters such as levels of pro-inflammatory cytokines or acute phase-proteins, additional parasite species would allow to obtain a herd health status not only in terms of one particular pathogen.

5.3. Multiplex immunoassay for the simultaneous detection of antibodies against *C. oncophora*, *D. viviparus* and *F. hepatica* in bovine serum samples

Current immunoassays, available for the detection of the three target species, are only capable of detecting Abs against one parasite per examination run. Another drawback of the existing diagnostic techniques is the extensive use of crude or ES Ags, which limit the specificity of the assays. In the literature, several serological immunoassays have been established using recombinant Ags for the diagnosis of *C. oncophora*, *D. viviparus* and *F. hepatica* infection. However, only for the latter a commercial ELISA is available.

xMAP®Luminex®Technology has been widely described in the literature since it improves sensitivity and specificity of serological immunoassays. The establishment of fluorescent immunoassays is based on the serum samples collected from animals, experimentally infected with the parasites of interest. The use of recombinantly expressed proteins reassures high specificity of the assay and eliminates the need to perform animal experiments for the production of ES or crude Ags. In the present study, a high-throughput immunoassay was developed for the screening and quantification of Abs by conjugating recombinant Ags in different concentrations onto COOH magnetic microspheres in order to determine the optimal ones (Karanikola et al., 2015).

The Amine Coupling Kit® (Bio-Rad, Germany) was used for the coupling of the three recombinant Ags on the surface of the COOH beads, following the instructions of the manufacturer. Minor modifications were reported in order to achieve higher conjugation yield. The initial step of the protocol describes vortexing of the stock of uncoupled beads at speed 7 for 30 s, followed by a 15 s sonication. This step was repeated three times in order to avoid high rates of aggregated beads, which would reduce the surface available for the conjugation of the protein and subsequently lead to unreliable MFI values measured by the Bio-Plex 200 software. Secondly, it was observed that magnetic microspheres incubated at medium speed (500 rpm) as recommended tend to precipitate at the bottom of the coupling reaction tube. For this reason, the incubation speed was slightly increased and the reaction tube was vortexed once at high speed after 1h of incubation in order to avoid precipitation. Another modification made in the initial protocol is the amount of the protein coupled. According to the manufacturer, the optimal amount of protein should be between 5 and 12 μg. In the present study different Ag concentrations were tested in order to find the optimal one. Coupled beads were stored on the recommended conditions (4 °C in the dark) and always used within 4 months after coupling since decreased performance was observed thereafter. It was observed that Ag concentrations of 0.45 μg for *C. oncophora* and *D. viviparus* and 2.5 μg for *F. hepatica* were the optimal for the conduction of the present experiments, concentrations by far lower than those initially recommended. Using low amount of protein provided the advantage of having a higher stock of Ag for future use while at the same time it was justified by the use of low Ag concentrations in ELISA immunoassays previously described for the target species. Another explanation of this disagreement with the manufacturer may lay on the fact that this coupling kit was standardised using human molecules for conjugation, having different molecular weight. In the literature concerning the application of Luminex® Technology in veterinary medicine, only a few publications referred to the optimal concentration of the conjugated protein while others followed the instructions provided by the manufacturer. To our knowledge, this was the first study, where such low Ags concentrations were reported in a multiplex fluorescence assay.

The assays were conducted in 96-well polystyrene, round-bottom microplates (Greiner Bio-One). The three bead-sets were initially tested in singleplex assays including negative controls as well as the respective positive control sera. These were used in a two-fold eight serial dilution series (1:100-1:12.800) in PBS/Tween20

(0.05 %, pH 7.4) in order to identify the optimal sample dilution. Cross-reactivity was assessed by running the assay with sera from calves infected with non-target species. Then, the three bead-sets were combined in a bead-mix and a multiplex assay was performed.

Standardisation of Ab detection in the serological assays was performed using the defined negative and positive control sera, collected from parasite naïve animals before (negative control) and after experimental mono-infection with the target parasites *D. viviparus*, (100 larvae over 5 consecutive days) *C. oncophora* (30,000–40,000 larvae) and *F. hepatica* (500 metacercariae). For testing specificity as well as cross reactivity, sera from animals mono-infected with other important GI nematodes, *H. contortus*, *T. colubriformis* and *O. ostertagi* were used. Information concerning cross-reactivity is frequently not available in the protocols described by the manufacturer of commercially available kits. It is found in the literature that for several of the developed and in the field evaluated in-house ELISAs that assessment of cross-reactivity has not been tested or at least not been reported. Evaluation of each coupled bead-set with sera from calves infected with other important for the livestock parasite species (*H. contortus*, *O. ostertagi* and *Tr. colubriformis*) indicated absence of cross-reactivity for all non-target species for all three bead-sets.

For the *C. oncophora* recombinant 14.2kDa protein absence of cross-reaction was reported by Poot et al. (1997) following the examination of serum samples obtained from animals specifically infected with *O. ostertagi* and *D. viviparus* with an in-house established ELISA. This observation is in complete agreement with the results obtained in the present study, where no cross-reactions were observed for any of the parasite species tested, reporting sensitivity and specificity of 100% in both assays.

The recombinant 43kDa MSP Ag, coupled onto *D. viviparus* bead-sets, previously described by Goździk et al. (2012) was characterised by high sensitivity (97.7%) and specificity (98.1%) when used in an ELISA. In the case of the Luminex® based assay a few MFI values were very close to the lowest values of lungworm-infected calves; however, no overlapping of the results occurred. When the obtained MFI values following the examination of sera from negative and infected with *H. contortus*, *O. ostertagi* or *T. colubriformis* calves with the *D. viviparus* beads were compared with sera positive for *C. oncophora* and *F. hepatica*, the latter were substantially elevated. This background prevented the definition of two cut-off values separating clearly negative and clearly positive from the intermediate grey zone, as it was in the case of beads for detection of *C. oncophora* and *F. hepatica* Ab.

For the detection of liver fluke the recombinant Cathepsin L1 Ag was used. The absence of cross-reactivity with positive sera collected from calves infected with the other two target and other non-target species, resulted in 100% sensitivity and specificity. Such high rates of these two parameters have not been previously reported. Kuerpick et al. (2013b) reported the presence of two false positive results out of the 13 lungworm-infected animals and one out of the four *C. oncophora* infected animals, resulting in sensitivity between 90 to 100% and specificity of 88.6%. Cornelissen et al. (1997) obtained similar findings with five out of 191 animals infected with *D. viviparus*, one out of 31 animals infected with *C. oncophora* and one out of 55 animals infected with *O. ostertagi* (sensitivity 99.1%, specificity 98.5%). The currently observed absence of cross reactivity might not be confirmed in the Luminex® assay upon the use of significantly higher number of animals infected with non-target species. However, a similar level of cross reactivity as described above in the ELISAs would still be an improvement in comparison to systems using complex Ags such as ES Ag with reported specificities between 83-96% (Charlier et al., 2014b).

Following the establishment of the multiplex immunofluorescence assay, field serum samples collected in Denmark, Switzerland and Poland were used in order to validate the multiplex platform in the field. The samples collected from Poland in particular, were previously tested with two established *F. hepatica* ELISAs. One

immunoassay was using an ES (*n*=363), commercially available under the brand name SVANOVIR® F.hepatica-Ab ELISA (Svanovir, Sweden), and the second was the DRG liver fluke ELISA (DRG Diagnostic, Germany) based on the recombinant Cathepsin L1 Ag. In the case of *D. viviparus* an in-house developed recombinant MSP ELISA for the diagnosis of lungworm (Gozdzik et al., 2012) infections was used in order to compare the newly developed assay with existing immunoassays. The results obtained were compared with the MFI values calculated with the Luminex® Technology, since ELISA is frequently used as a "gold standard" for the evaluation of multiplex developed assays. However, correlation between ELISA assays varies among publications (Baker et al., 2012; Dernfalk et al., 2007; Elshal and McCoy, 2006; Klein et al., 2012; Pickering et al., 2002; Willman et al., 2001). Comparison of the results obtained with the Luminex and the other ELISAs in the present study indicated good agreement between these assays and the newly developed multiplex platform, especially when the same recombinant Ags were used. The examination with recombinant MSP ELISA in Sweden revealed only negative ODR values for the tested samples while one sample was detected positive in the Luminex assay, explainable by the potentially higher sensitivity the Luminex method. Due to the fact that almost all samples were negative, no kappa statistic could be calculated. The comparison with the DRG liver fluke ELISA resulted in a kappa value of 0.37 (62.7% of 0.60 maximum achievable). For this calculation the negative cut-off values for both assays were used. A larger subset of 363 samples were analysed using the commercially available SVANOVIR® F.hepatica-Ab ELISA in the laboratory in Ghent, Belgium. The comparison resulted in a kappa value of 0.460 (74.4% of 0.67 maximal achievable). While 67 samples were positive only in the Luminex, there were also 21 samples which appeared positive in the ELISA but clearly negative in the Luminex assay. Samples were tested with the commercially available DRG ELISA, using the recombinant Cathepsin L1 Ag for the diagnosis of *F. hepatica* infections. The comparison between the DRG ELISA and Luminex was complicated since the latter used five different categories for the classification of the examined samples. It was therefore unclear how to handle positive and negative in multiplexing samples, which were detected as "questionable" or "low to moderate infestation" in the ELISA. The majority of the deviations were due to the higher number of positive samples detected in Luminex assay, while there was one sample classified as negative in the Luminex and at the same time positive in the ELISA. The second immunoassay used for the validation of the new fluorescent method was an ES liver fluke ELISA, previously described by Salimi-Bejestani et al. (2005b), adopted and modified by many laboratories, which developed their in-house ELISAs. Since more data were available for the ES ELISA better comparison between these two assays could be performed. This resulted in a better kappa value however there was a high number of samples positive in one of the two techniques while being negative in ELISA or in the grey zone in the Luminex® immunoassay. Taking into consideration the use of recombinant Ag in the newly developed tri-plex assay, higher specificity was expected than using crude or ES Ags, leading at the same time to lower sensitivity since not all animals might develop Abs against Cathepsin L1 protease. Additionally, the proved high rate of false positive results detected by the ES ELISA might be an explanation for the lack of complete accordance between the two assays, mainly due to the co-infection of animals with pathogens showing cross reactivity with *F. hepatica*.

The newly developed platform was used for the screening of the infections in samples, calculating prevalence rates in Denmark, Poland and Switzerland. The results obtained show no or low rates of lungworm infection in all three countries. *C. oncophora* appears in higher rates in Poland (73.8%) in comparison to Denmark (28.2%) and Switzerland (48.7%). Additionally in Poland, increased levels of liver fluke were detected. The number of samples classified in the grey zone is considerably high in all three sampled countries.

5.4. Multiplex immunoassay for the simultaneous detection of antibodies against *C. oncophora*, *D. viviparus* and *F. hepatica* in bovine milk samples

Standardisation of the above described multiplex serum immunoassay was achieved following the testing of dilution series (1:100-1:12.800) of control sera obtained from parasite naïve animals, before and after their infection with the target species. Specificity and sensitivity, determined by examining sera from both, parasite free calves and those specifically mono-infected with other GI nematodes (*H. contortus*, *O. ostertagi* and *T. colubriformis*) were both high (99,46-100%). In the serum assay, no cross-reactivity was observed between the target species as well as with the other GI nematodes of interest (Karanikola et al., 2015), leading to the assumption that the use of the same Ags will also successfully enable the specific detection of Abs against the target parasites in milk.

The newly developed immunoassay, apart from reducing the costs, time and sample volume needed for testing, provides the ability of including or excluding the parasites of interest during each run. The use of this technique may be considered a valuable tool in the hands of farmers and veterinarians since it allows the collection of one sample, which is sent to only one laboratory for the simultaneous detection of three target species.

The multiplex platform was based on the conjugation of recombinant Ags for the three target species onto COOH magnetic microspheres following the modified coupling protocol described by Karanikola et al. (2015). The optimal conjugated amounts were 0.45 μg for *C. oncophora* and *D. viviparus* and 2.5 μg for *F. hepatica*. The optimal dilution of biotinylated secondary Ab and the concentration of SA-PE conjugate were set at 1:1000 and 2 μg/ml respectively.

Immunological diagnostic techniques provide higher sensitivity and specificity in comparison to coproscopical methods (Anderson et al., 1999; Charlier et al., 2008). The quantification of parasite specific Abs in BTM samples is considered an advantage since milk based assays are less invasive, cheaper and allow the conduction of large-scale surveys as well as monitoring of Ab levels on herd basis or diagnosis of individual animals. In comparison, serum based techniques are labour-intensive in terms of the time needed for collection of each sample and do not provide results on herd level. In recent years, advances have been achieved towards the development and evaluation of different milk ELISAs, replacing serological methods (Charlier et al., 2007). However, classical ELISAs are only able to detect Abs against one parasite and have additionally often limitations in specificity due to the use of ES or crude extract Ags. In the case of *F. hepatica* the rate of false positive results detected by the ELISA in milk samples using ES Ag increased when co-infections existed (e.g. mastitis). ELISAs based on recombinant Ags aiming to increase the specificity have also been described, but only one using Cathepsin L1 for detection of *F. hepatica* is commercially available yet (DRG ELISA; DRG Diagnostics, Germany). Using a recombinant instead of ES Ag, the results of the analysis of paired samples in the current study revealed a high degree of correlation, suggesting that no particular problems with the specific detection of Abs against *F. hepatica* occur in the Luminex milk assay.

In contrast to single target ELISAs, xMAP® Technology is a platform enabling simultaneous quantitative detection of multiple analytes in a single assay, based on the principles of fluorescence (Giavedoni, 2005). High sensitivity and specificity achieved by the use of recombinant Ags, reduction in the time needed for assay performance and data acquisition as well as lower costs stand among the advantages offered by this technique (Vignali, 2000). Luminex® is widely used in human diagnostic, where a considerable variety of kits are commercially available, but has only recently been introduced to the field of veterinary medicine (Christopher-Hennings et al., 2013). In cattle diagnostics a Luminex® bead-based immunoassay has been described by Dernfalk et al. (2007) for the detection of cytokines in milk

and plasma samples. IL-1β, IL-6 and TNF-α could be simultaneously detected in bovine samples collected from animals with confirmed clinical mastitis proving absence of cross-reactivity between these reagents and providing future research with a basis for optimisation this kind of multiplex assays.

Evaluation of milk immunoassays is usually performed by calculating the correlation of the results with those obtained after the examination of respective paired serum and milk samples and determination of sensitivity and specificity when serum is considered the gold standard. Since defined milk samples from parasite-free or specifically mono-infected dairy cows are not available, paired samples (serum and milk) for individual cows were used for the determination of cut-off values for milk. These samples (*n*=78) were collected from herds in Lower Saxony, Germany. Serum Ab levels were obtained using the recently described triplex assay (Karanikola et al., 2015) and compared to milk Ab levels of the same animal.

In contrast, in the serum assay, two cut-off values were established for both *C. oncophora* and *F. hepatica*. Animals that were never exposed to the parasite had MFI values below the negative cut-off. The positive cut-off was developed to identify all animals with patent infections – although recently infected animals might still show MFI values above this positive cut-off. The grey zone between these cut-off values typically includes animals in the prepatency or currently non-infected animals with decreasing Ab levels. In contrast, only one cut-off could be determined for *D. viviparus* due to the very close MFI values obtained between animals specifically infected with *D. viviparus* and those infected with *C. oncophora* and *F. hepatica* infected animals (Karanikola et al., 2015).

In a survey performed by Fiedor et al. (2009) a *D. viviparus* BTM ELISA was correlated with faecal and serum samples collected from individual heifers and cows. The results indicated good correlation between BTM ODR and the calculated mean herd serum ODR. Additionally, the obtained BTM values reflected the proportion of animals showing clinical signs. Good correlation between serum and milk immunoassays has also been reported (Ploeger et al., 2012) for *F. hepatica*. In the present study, examination of paired milk and serum samples was performed, resulting in substantial agreement for all three assays. However, this analysis partially suffers from the fact that for *C. oncophora* only few or in case of *D. viviparus* no positive samples were available for this comparison. For *F. hepatica* the number of positive samples was sufficiently high and two ELISAs were commercially available, so Pearson r was calculated. Correlation was substantially better between the Luminex assay and DRG ELISA (Pearson r=0.82) using the same recombinant Cathepsin L1 Ag than for the comparison with the SVANOVIR® F. hepatica-Ab ELISA (Pearson r=0.33) which uses an ES Ag. Calculation of kappa values confirmed substantial agreement with the ELISA based on the same recombinant Ag (0.81) while fair agreement was obtained for the ELISA using an ES Ag (0.54).

The ELISAs described up to now for the detection of Abs against *D. viviparus* (Fiedor et al., 2009) and *F. hepatica* (Salimi-Bejestani et al., 2005a) in BTM samples are characterised by high sensitivity and specificity. Calculation of these two parameters was based on paired samples collected from animals kept either under controlled conditions or in areas endemic for the parasite, respectively. However, in none of these studies defined milk samples from parasite naïve cows were used, since this kind of samples are generally unavailable, and sensitivity as well as specificity calculations were based on large number of paired samples. In the present study, sensitivity and specificity could not be calculated since the required defined positive (from mono-infected animals) and negative individual milk samples were again not available and the number of paired samples used were not high enough to display sufficient numbers of positive and negative samples. However, since the same Ags were used in the previously reported serum multiplex immunoassay (Karanikola et al., 2015), similar high sensitivity and specificity can also be expected. Comparisons between different immunoassays for the detection of Abs against *D. viviparus* have

recently been reported by Ploeger et al. (2014). These authors compared the results obtained with a previously commercially available Dutch BTM ELISA (based on the former Ceditest®, Lelystad, The Netherlands) as that was reported by Cornelissen et al. (1997) with the ELISA modified by Schunn et al. (2012). Despite the fact that the two methods are based on different Ags, a significant correlation was obtained but sensitivity and specificity were quite different and also differed from those previously reported for the respective technique. The MSP-based ELISA showed high specificity but much lower sensitivity than the Dutch BTM ELISA. However, the latter was considerably inferior regarding specificity. This highlights the fact that sensitivity and specificity obtained using samples derived from experimental animals are often not confirmed when field samples are used.

In the samples obtained from the field survey, Abs against *C. oncophora* were detected in high rates in all four European countries in the investigated regions. To the knowledge of the authors, this is the first epidemiological overview of the infection status of dairy herds in Europe with this specific parasite conducted with milk samples. Additionally, Githiori et al. (2000) reported the use of the recombinant *C. oncophora* ES 14.2 Ag ELISA for a retrospective study performed on serum samples. These authors also compared the crude Ag ELISA with the recombinant ES 14.2 ELISA and found a strong correlation between both. Regarding the detection of Abs against the other important gastro-intestinal nematode *O. ostertagi* it would have been beneficial to include it in the current study, but unfortunately no recombinant Ags suitable for diagnostic were identified. Further work is urgently needed and once a suitable Ag will be available, the Luminex assay offers the advantage of an easy inclusion of this target species into the existing assay.

Contrary to *C. oncophora*, *D. viviparus* was detected at considerably lower levels in all examined regions of the four European countries participating in the current study. The results obtained are slightly different from those previously reported in studies conducted in Belgium (Bennema et al., 2009) and Germany (Klewer et al., 2012), where herd prevalence of 19.6% (vs 23.4% in the multiplex assay) and 6.6 to 22.3% (vs 18.7% in the multiplex assay) were reported, respectively. Remarkably, the lower prevalence observed in the aforementioned studies were detected in samples collected in autumn, which corresponds to the time the samples in the present study were obtained. The situation was slightly different for Ireland, where 32.6% of the examined BTM samples were found positive. A recently reported survey of *D. viviparus* in Irish dairy farms (using and ELISA with MSP Ag) found 53.4% prevalence (*n*=277) in BTM samples collected in November (Bloemhoff et al., 2015). These authors observed an extremely rapid rise from 2.2% (obtained in August) to 53.4% (in November). Since the samples in the current study were collected in October, the difference in prevalence might be due to the earlier sampling time.

For *F. hepatica* a prevalence of 37.3% was previously reported for Flanders, Belgium, by Bennema et al. (2009), which is in agreement with that obtained by the Luminex milk immunoassay (34.3%). For Germany two studies, performed in 2008 and 2010 have been reported (Kuerpick et al., 2013a; Kuerpick et al., 2012b). Both used an ELISA based on ES Ag with a cut-off value of an ODR >0.8, which is believed to indicate production losses due to fasciolosis. The prevalence for Mecklenburg-Vorpommern of 9.6% and Lower Saxony of 29.4% reported by Kuerpick et al. (2013a) differs considerably from those reported by Kuerpick et al. (2012b), where prevalence in Lower Saxony between 53-59% and 18.4-49.5% were found in 2008 and 2010, respectively. In the present study, moderate (between 31.2 and 52.5%) rates of increased Ab levels against *F. hepatica* were found, varying between the different sampling regions. One possible explanation for the higher rates obtained in the aforementioned survey might be the use of the high cut-off by Kuerpick et al. (2013a), while in the present study a cut-off only for clearly negative samples was used. Similar observations were made in Poland, where one of the two regions (Podlaskie, 92.5%) had higher rates than the other (Lodskie, 68.4%). Ireland

on the other hand showed high rates (93.6%) of samples above the negative cut-off value, a result higher than that reported by Selemetas et al. (2014), who stated that 67% of Irish dairy were exposed to the parasite. The higher rates in the present study might be again due to the use of a negative cut-off, while Selemetas et al. (2014) excluded the dubious samples from the positive ones. In another study (Selemetas and de Waal, 2015), 83% of the Irish dairy farms have been exposed to *F. hepatica*, a rate closer to that detected by the newly developed Luminex milk immunoassay.

6. SUMMARY

For the diagnosis and the identification of *C. oncophora*, *D. viviparus* and *F. hepatica*, coproscopical, molecular and serological techniques are available. For standard diagnosis, mainly coproscopical and serological methods are currently used. The sensitivity and specificity of the different techniques are extremely variable. Serological assays have the advantage of their implementation as herd health monitoring tools. All currently available assays are only capable of diagnosing Abs against one parasite and particularly serological assays are often limited in their specificity due to the use of complex crude or ES Ags.

A high-throughput multiplex fluorescence immunoassay was successfully developed for the simultaneous detection of Abs produced against *C. oncophora*, *D. viviparus* and *F. hepatica* in bovine serum and milk (individual and BTM) samples. It is characterised by low costs and time, high reproducibility and more importantly, by high sensitivity and specificity, due to the use of recombinant Ags. The platform was furthermore characterised by absence of cross-reactivity with other important GI nematodes of livestock (e.g. *H. contortus*, *O. ostertagi* and *T. colubriformis*) when examined with positive sera from experimentally infected calves. Future examination of positive control serum samples from animals specifically mono-infected with other parasite species, such as *Taenia saginata*, *Paramphistomum cervi* etc. is envisaged.

The conduction of one assay (in a 96-well format) can be performed in three hours, which is less or similar than those times usually needed for the performance of a classical ELISA. This multi-plex, multi-well format allows performance of large-scale epidemiological studies. Validation of the newly developed platform for both kinds of samples was performed using established, commercially available ELISAs that have been widely applied in the field. Correlation between the assays was good, especially when the same recombinant Ags were used.

Since this platform is modular, it on one hand enables inclusion of other parasite species e.g. *O. ostertagi*, *H. contortus*, important biomarkers and herd health parameters in the future or on the other hand exclusion of individual target species for farms where these species are irrelevant in order to limit the costs. In particular, inclusion of *O. ostertagi* should be a major aim for the future, though recombinant Ag suitable for diagnosis are currently not available.

Finally, the newly developed serum triplex Luminex assays was used to analyse field serum samples collected in Denmark, Switzerland and Poland. The results obtained show no or low rates of lungworm infection in all three countries. *C. oncophora* appears in higher rates in Poland in comparison to Denmark and Switzerland. Additionally in Poland, increased levels of liver fluke were detected.

The first use of this technique in a field survey following the collection of BTM samples from four European countries (Belgium, Germany, Ireland and Poland) revealed generally high exposure to *C. oncophora* and considerably less exposure to *D. viviparus*. For *F. hepatica* the situation was slightly different with two countries (Ireland and Poland) showing high exposure rates and the other two more moderate ones. Further optimisation of the assay (e.g. determination of positive cut-off values), the inclusion of more paired samples from endemic and non-endemic areas is envisaged in the future.

7. ENTWICKLUNG EINER INNOVATIVEN DIAGNOSTISCHEN TECHNIK UNTER VERWENDUNG DER XMAP®LUMINEX®TECHNOLOGIE FÜR DIE SIMULTANE DETEKTIERUNG VON ANTIKÖRPER GEGEN *COOPERIA ONCOPHORA*, *DICTYOCAULUS VIVIPARUS* UND *FASCIOLA HEPATICA* IN BOVINEN SERUM UND MILCHPROBEN

Für die Diagnose und auch Identifikation von *C. oncophora*, *D. viviparus* und *F. hepatica* sind sowohl koproskopische, molekulare als auch serologische Methoden verfügbar. In der Routinediagnostik kommen hauptsächlich koproskopische und serologische Methoden zum Einsatz. Die Sensitivität und Spezifität der verschiedenen Methoden ist jedoch extrem unterschiedlich. Serologische Verfahren haben den Vorteil, daß man sie relative einfach in der Überwachung des Gesundheitszustandes von Herden einsetzen kann. Allen momentan verfügbaren Verfahren is jedoch gemeinsam, daß sie jeweils nur Antikörper gegen einen bestimmten Parasiten detektieren und außerdem, aufgrund der Verwendung von komplexen Ganzwurm-Antigenen oder exkretorisch-sekretorischen Antigenen, hinsichtlich ihrer Sensitivität und Spezifität limitiert sind.
Im Rahmen der vorliegenden Arbeit konnte erfolgreich ein fluoreszens-basiertes Multiplex-Verfahren mit hoher Proben-Durchsatzzahl für die Bestimmung von Antikörpern gegen *C. oncophora*, *D. viviparus* und *F. hepatica* in bovinen Serum- und Milchproben (Einzelproben und Tankmilchproben) entwickelt werden. Dieses neue Verfahren zeichnet sich einerseits durch den geringen Kosten- und Zeitaufwand pro Probe und andererseits durch seine gute Reproduzierbarkeit, Sensitivität und Spezifität (durch die Verwendung rekombinanter Antigene) aus. Desweiteren war das Ausbleiben von Kreuzreaktionen mit anderen wichtigen Nematoden von Weidetieren (z.B. *H. contortus*, *O. ostertagi* und *T. colubriformis*) charakteristisch, was sich durch Untersuchung von Seren definiert-infizierter Kälber zeigte. Die Untersuchung von möglichen Kreuzreaktionen mit anderen Parasitenarten wie *Taenia saginata*, *Paramphistomum cervi* etc., ist bezüglich der weiteren Validierung anzustreben.
Die Durchführung einer Platte (im 96-well Format) kann in drei Stunden durchgeführt werden, was kürzer oder genauso lang ist wie die Zeit, welche für die Durchführung eines klassischen ELISAs benötigt wird. Durch die gleichzeitige Untersuchung auf mehrere Parasiten ermöglicht dieses multi-plex/multi-well Format somit die Durchführung größerer epidemiologischer Studien. Die Validierung des neuen Verfahrens für beiden Probenarten, Serum und Milch, wurde durch die parallele Untersuchung der Proben in kommerziell erhältlichen, zugelassenen ELISAs erreicht. Die Korrelationen waren im Ganzen gut, insbesondere dann, wenn das gleiche rekombinante Antigen verwendet wurde. Da diese Multiplex-Plattform modular aufgebaut ist, ermöglicht es sowohl den zukünftigen Einschluß anderer Parasitenarten (z.B. *O. ostertagi*, *H. contortus*) als auch anderer wichtigen Biomarker oder Gesundheitsparameter. Insbesondere der Einschluß von *O. ostertagi* sollte ein wichtiges Ziel in der Zukunft sein, auch wenn es zur Zeit keine geeigneten Antigene für die Diagnose dieses Parasiten gibt. Genauso können auch bestimmte Parameter weggelassen werden wenn diese auf bestimmten Betrieben keine Rolle spielen um somit Kosten senken zu können.
Das neue Triplex-Serum Verfahren wurde abschließend für die Untersuchung von in Dänemark, der Schweiz und Polen gesammelten Serumproben angewandt. Die erzielten Ergebnisse zeigen nur gering oder keine Antikörpertiter gegen *D. viviparus* in allen drei untersuchten Ländern. *C. oncophora* scheint in Polen häufiger vorzukommen als in Dänemark und der Schweiz. Außerdem wurden in Polen mehr erhöhte Antikörpertiter gegen *F. hepatica* detektiert.

Zusammenfassung

Der erste Einsatz des neuen Verfahrens erfolgte in einer größeren Feldstudie mit der Untersuchung von Tankmilchproben aus vier europäischen Ländern (Belgien, Deutschland, Irland und Polen). Hierbei wurden generell hohe Antiköpertiter gegen *C. oncophora* und deutlich geringere gegen *D. viviparus* festgestellt. Hinsichtlich *F. hepatica* war die Situation etwas anders, wobei in zwei Ländern (Irland und Polen) hohe und in dem dritten nur ein mittlerer Antikörpertiter ermittelt wurden. Die weitere Optimierung des neuen Verfahrens, wie z.B. die Etablierung von Positiv-Grenzwerten mit einer höheren Anzahl gepaarter Proben ist zukünftig vorgesehen.

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9. PUBLICATIONS

9.1. Peer-reviewed publications

Development of a multiplex fluorescence immunological assay for the simultaneous detection of antibodies against *Cooperia oncophora*, *Dictyocaulus viviparus* and *Fasciola hepatica* in cattle

First author: Sofia N. Karanikola
Co-authors: Jürgen Krücken, Sabrina Ramünke, Theo de Waal, Johan Höglund, Johannes Charlier, Corinna Weber, Elisabeth Müller, Slawomir J. Kowalczyk, Jaroslaw Kaba, Georg von Samson-Himmelstjerna, Janina Demeler

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9.2. Presentations from 2012 until 2015

"Development of a new diagnostic method for the detection of multiple parasitism in cattle." 28.09.2012: "Parasitologisches Kolloquium", Institut für Parasitologie und Tropenveterinärmedizin, FU Berlin

"Development of a new diagnostic method for the detection of multiple parasite infections in cattle." 08.10.2012: Presentation during EU-Project GLOWORM (KBBE 2011.1.3-04, No 288975) Bristol, UK, Work package 1

"Luminex technology in the diagnostics of *Dictyocaulus viviparus* and *Fasciola hepatica* in cattle." 16.09.2013: Presentation during EU-Project GLOWORM (KBBE 2011.1.3-04, No 288975) Dublin, Ireland, Work package 1

"Luminex technology in the diagnostics of *Dictyocaulus viviparus*, *Fasciola hepatica* and *Cooperia oncophora* in cattle." 12.04.2014: "Parasitologisches Kolloquium", Institut für Parasitologie und Tropenveterinärmedizin, FU Berlin

"Luminex technology in the diagnostics of *Dictyocaulus viviparus*, *Fasciola hepatica* and *Cooperia oncophora* in cattle." 17.07.2014: Presentation during ParaTrop conference, Zurich, Switzerland

"Luminex technology in the diagnostics of *Dictyocaulus viviparus*, *Fasciola hepatica* and *Cooperia oncophora* in cattle." 15.12.2014: Presentation during EU-Project GLOWORM (KBBE 2011.1.3-04, No 288975) Ghent, Belgium, Work package 1

"Luminex technology in the diagnostics of *Dictyocaulus viviparus*, *Fasciola hepatica* and *Cooperia oncophora* in cattle-Epidemiological examination of 3000 samples collected from 4 different European countries." 11.02.2015: "Parasitologisches Kolloquium", Institut für Parasitologie und Tropenveterinärmedizin, FU Berlin

"Luminex technology in the diagnostics of *Cooperia oncophora*, *Dictyocaulus viviparus* and *Fasciola hepatica* in bovine serum and milk samples." 30.06.2015: Presentation during the Deutsche Veterinärmedizinische Gesellschaft (DVG) conference, Stralsund, Germany

References

"Luminex technology in the diagnostics of *Cooperia oncophora*, *Dictyocaulus viviparus* and *Fasciola hepatica* in bovine serum and milk samples." 20.08.2015: Presentation during the 25th International conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), Liverpool, UK

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Declaration

11. DECLARATION

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 19.07.2016 Sofia N. Karanikola