Invited review

Heartworm disease – Overview, intervention, and industry perspective

Sandra Noack a, John Harrington b, Douglas S. Carithers c, Ronald Kaminsky d, Paul M. Selzer a, *  

a Boehringer Ingelheim Animal Health, Binger Str. 173, 55216, Ingelheim am Rhein, Germany  
b Boehringer Ingelheim Animal Health, 1730 Olympic Drive, 30096, Athens, GA, USA  
c Boehringer Ingelheim Animal Health, 3239 Satellite Blvd, 30096, Duluth, GA, USA  
d paraC Consulting, Altenstein 13, 79685, Hag.Ehrsberg, Germany

ARTICLE INFO

Keywords:  
Heartworm disease  
Dirofilaria immitis  
Macrocyclic lactones  
Mechanism of action  
Anthelmintic resistance  
Animal health

ABSTRACT

Dirofilaria immitis, also known as heartworm, is a major parasitic threat for dogs and cats around the world. Because of its impact on the health and welfare of companion animals, heartworm disease is of huge veterinary and economic importance especially in North America, Europe, Asia and Australia. Within the animal health market many different heartworm preventive products are available, all of which contain active components of the same drug class, the macrocyclic lactones. In addition to compliance issues, such as under-dosing or irregular treatment intervals, the occurrence of drug-resistant heartworms within the populations in the Mississippi River areas adds to the failure of preventive treatments. The objective of this review is to provide an overview of the disease, summarize the current disease control measures and highlight potential new avenues and best practices for treatment and prevention.

1. Introduction

Companion animals, specifically dogs and cats, are hosts to a variety of external and internal parasites (Selzer and Epe, 2021). One of the most important endoparasites in companion animal health, from both pathologic and an economic perspective, is Dirofilaria immitis, the filarial nematode parasite that causes heartworm disease. In dogs, the disease is caused by young adult and adult parasites provoking pathology in the pulmonary arteries. Canines act as the definitive host, so sexual reproduction occurs in the pulmonary arteries and microfilariae are released into the circulatory system (McCall et al., 2008b; Bowman and Atkins, 2009). D. immitis infections also occur in cats, and the disease is usually more severe in this atypical host. In cats, severe disease, or even death, can be caused by just a few developing immature or adult filariae. The adult worms are often shorter than those found in dogs and rarely produce microfilariae (Venco et al., 2015). The life cycle of D. immitis is similar to the pathogenic filarial parasites of humans, Onchocerca volvulus, Brugia malayi, and Wuchereria bancrofti, in that they are all transmitted by arthropod vectors (Tahir et al., 2019). D. immitis is distributed across the globe, being endemic in countries on six continents (Simón et al., 2012). From an animal health perspective, dogs and to a lesser extent cats are the most important animals amongst the numerous mammalian hosts that can be infected by D. immitis (Simón et al., 2009; Moroni et al., 2020; Selzer and Epe, 2021). Thus, this review will focus primarily on heartworm disease in dogs and include relevant background on cats where appropriate. Other filarial parasite species have also been reported in dogs including Acanthocheilonema reconditum, Cercopithifilaria bainae, and Onchocerca lupi. Among these, O. lupi is of clinical significance, and has been reported not only in dogs, but also in cats and humans in North America (Dantas-Torres and Otranto, 2020).

Treatment of an established D. immitis infection in dogs requires a prolonged regimen of drug treatment, exercise restriction and sometimes even surgery (Bowman and Atkins, 2009; Carret et al., 2019). Therefore, the current practice is to control heartworm disease through prevention, which currently utilizes a single class of drugs, the macrocyclic lactones (MLs), including e.g. ivermectin, milbemycin oxime and moxidectin (Wolstenholme et al., 2016). Ivermectin is considered an essential medicine by the World Health Organization (World Health Organization, 2019) based on its efficacy against human filarial parasites. The drug has been successfully applied in the Americas (Lakwo et al., 2020) and has led to the elimination of onchocerciasis in four out of six endemic countries (Sauerbrey et al., 2018). Ivermectin has and is still contributing substantially in mass drug administration campaigns in Africa to control human pathogenic filarial parasites (Kim et al., 2015; Lakwo et al., 2020). However, successful elimination of onchocerciasis might be endangered by...
ivermectin-resistant *O. volvulus* populations (Osei-Atweneboana et al., 2011; Nana-Djeunga et al., 2014). Similarly, successful prevention of heartworm disease in dogs may be compromised by ML-resistant *D. immitis* (Hampshire, 2005; Bourguinat et al., 2015).

In this review, we give an overview on heartworm diseases, outline the industry’s perspective on its control, the currently available treatments, their potential mode of action and the issues involved that lead to selection of resistance. In addition to the general distribution of *D. immitis*, reasons for both the increasing and decreasing prevalence in certain geographic areas will be discussed. Advances in the diagnostic capabilities, as well as the potential for the discovery of novel treatments based on new technology platforms will also be addressed.

As a note for the reader and in accordance with the World Association for the Advancement of Veterinary Parasitology (WAAVP, https://www.waavp.org) we used the term “dirofilariasis” (Kassai et al., 1988). However, in many publications the disease is also referred to as “dirofilariosis” or occasionally also as “dirofilariasis”, which is important to consider when reviewing the literature (Ashford, 2001; Kassai, 2006).

2. Heartworm biology

2.1. Taxonomy and lifecycle

Within the phylum of Nematoda, *Dirofilaria immitis* (Figs. 1 and 2) belongs to the superfamily Filarioidea, commonly known as filarial parasites or filariae. The definite host of the filarial parasites are always vertebrates and the intermediate hosts are arthropods, often mosquitoes. The *Filarioidea* are grouped into three families, the *Filaridae*, *Setariidae*, and *Onchocercidae*. The *Onchocercidae* include the human pathogenic species *O. volvulus*, causing onchocerciasis also known as river blindness, as well as *W. bancrofti* and *B. malayi*, both causative agents of lymphatic filariasis (Deplazes et al., 2016; Mehlhorn, 2016). The *Onchocercidae* also contain *D. immitis* and *Dirofilaria repens*, both causing dirofilariosis in carnivores, a major global threat to companion animal health and welfare (Bowman and Atkins, 2009; Capelli et al., 2018). Other animal pathogenic species of the *Filarioidea* are *Parafilaria multipapillosa* (*Filaridae*) causing hemorrhagic subcutaneous nodules – equine summer bleeding – and *Setaria digitata* (*Setariidae*), a pathogenic parasite of cattle and water buffalo in Asia (Deplazes et al., 2016; Mehlhorn, 2016). The larval stage of *S. digitata* can cause fatal cerebrospinal nematodosis in goats, sheep and horses (Perumal et al., 2016) and infections in humans can cause abscesses, allergic reactions, enlarged lymph nodes, eye lesions, and lung inflammation (Rodrigo et al., 2014).

Like the majority of filariae, *D. immitis* has no free-living stages and exhibits a complex lifecycle involving multiple developmental stages in both, the definitive mammalian host and the mosquito vector (Fig. 2). The adult worms (macrofilariae) live as obligate endoparasites mainly in the lobar arteries and main pulmonary artery of the canine hosts. In dogs with high worm burden, adult *D. immitis* can also be found in the right ventricle. Females measure up to 30 cm and males around 18 cm. Females are ovoviviparous; they release sheathless microfilariae in the blood which are 250–300 μm in size. Various mosquito species can acquire the *D. immitis* microfilarial stage in a bloodmeal from an infected host. Once ingested by a mosquito, the microfilariae migrate within hours from the midgut to the Malpighian tubules where they will morphologically change into various “sausage” forms representing first stage larvae (Sawyer and Weinstein, 1963; Shang Kuan and Prichard, 2020). However, reports for *D. immitis* described these changes using *in vitro* cultivated microfilariae which were unable to develop in those systems beyond the L1 stage (Kuan and Prichard, 2020). Nevertheless, the principle development stages of *D. immitis* within the mosquitoes are confirmed by observations on the related *B. malayi* (Erickson et al., 2009). Filariae harvested from infected mosquitoes at various time intervals could be distinctly differentiated into microfilariae, which migrate and develop within hours into intracellular L1 stages. These

Fig. 1. Adult *D. immitis* parasite. Photograph of a mass of adult *D. immitis* worms removed from a dog at necropsy. Most are female worms, but the coiled posterior end of a male worm can be seen in the middle of the mass.
shorter and non-feeding L1 stages undergo a first molt resulting in intracellular remaining L2 stages. They molt for a second time to become finally infective \textit{B. malayi} L3 stages (Erickson et al., 2009), which can be transmitted to another vertebrate host. Development of \textit{D. immitis} and migration in the canine host, as well as the host immune response, are largely uncharacterized until adults appear in the pulmonary arteries approximately 6 months after infection (Bowman and Atkins, 2009; Simón et al., 2012; Deplazes et al., 2016; Mehlhorn, 2016).

Cats are less suitable hosts for \textit{D. immitis} than dogs (McCall et al., 2008b) and most worms in cats do not develop to the adult stage. Those cats with adult \textit{D. immitis} usually harbor 1–3 worms only. These worms are also smaller than those found in dogs and they rarely produce microfilariae.
2.2. The disease

Young adult and adult *D. immitis* heartworms cause disease of dogs, initially a vascular disease that can progress to impaired blood flow, eventually affecting the vascular and pulmonary system, and in severe cases the right heart chambers. First symptoms include a mild persistent cough and reluctance to exercise. Dogs may manifest decreased appetite and become exercise intolerant. Eventually, the damage to the pulmonary endothelium and vascular occlusion from worm death will reduce cardiac output. The resulting pulmonary hypertension may lead to compensatory right-side heart enlargement and progress to right heart failure. A less common sequelae occurs when a sudden obstruction of blood flow through the lungs caused by large numbers of adults in the pulmonary arteries, reduces the flow to the point where worms migrate and become aberrantly located in the right atrium, ventricle and often in the vena cava (Fig. 3). This form of blockage is called caudal syndrome and will cause a life-threatening form of heart failure (Bowman and Atkins, 2009; Simón et al., 2012; Ames and Atkins, 2020).

Cats are much less immunologically tolerant of heartworm infections, and as a result manifest clinical signs different from dogs. In cats, even the death of immature *D. immitis* in the pulmonary arteries can cause severe, pulmonary symptoms (Atkins and Litster, 2006). This condition has been named heartworm associated respiratory disease (HARD). Clinical signs manifest as chronic coughing, labored breathing, vomiting and even sudden death, with no other apparent clinical signs. It has been experimentally verified that as early as 70-90-day-old *D. immitis* infections resulted in induced severe pulmonary airway, interstitial, and arterial lung lesions (Dillon et al., 2017). In contrast to dogs, cats have pulmonary intravascular macrophages, which can be modulated by parasite products and may contribute to the different outcome of the disease in cats (Dillon et al., 2008). Many of these symptoms mimic those of asthma in cats, requiring differential diagnosis because treatment and prognosis of these two diseases is different (Garrity et al., 2019).

*Dirofilaria immitis* has zoonotic potential and thus could be considered a Public Health issue. Humans can be accidently infected with *D. immitis*, and in many cases, these infections will progress, developing through the tissue stages, and reaching the pulmonary vasculature. In the pulmonary vasculature of humans the young adult worms will die, resulting in development of pulmonary nodules, but will remain asymptomatic. Thirty-three cases of such pulmonary dirofilarial infections were reported in Europe (Simón et al., 2012). However, the exposure of humans to *D. immitis* is likely to be higher, as analysis of 250 serum samples from northern Spain for specific IgG antibodies against *D. immitis* revealed a seroprevalence of 11.6% (Morchón et al., 2010). Similarly, 6.1% of 668 investigated people in Portugal were found to be seropositive for *D. immitis* (Fontes-Sousa et al., 2019). Although *D. immitis* infections in humans are typically asymptomatic, they can cause great concern as radiographically, the resulting coin lesions are indistinguishable from lung cancer (Simón et al., 2012).

2.3. Vectors

Heartworm abundance and distribution are closely linked with mosquito vector biology and ecology. Any mosquito species that allows for the successful development of microfilariae into the infective L3 stage and subsequent migration to the proboscis can be a competent vector of *D. immitis* (Ledesma and Harrington, 2011). Over 60 species of mosquitoes are capable of supporting the development of L3 *D. immitis* (Ludlam et al., 1970). More than 20 different species were detected with infective L3 in various field studies (Scoles, 1998; Ward, 2005; Bowman and Atkins, 2009; Ledesma and Harrington, 2011). Nine species have been identified as major potential vectors, all with well-known developmental and biological parameters allowing for the development of national forecast models for canine heartworm risk (Brown et al., 2012). These species are *Aedes aegypti*, *Ae. albopictus*, *Ae. canadensis*, *Ae. sierrensis*, *Ae. tritivittatus*, *Ae. vexans*, *Anopheles punctipennis*, *A. quadrivinculatus*, and *Culex quinquefasciatus*. Mosquito interspecies competition, which depends on various factors such as humidity, vegetation, and urbanization, can lead to a distribution of transmission competent species. Vector and environmental maps have been investigated for the occurrence of these species, suggesting that presence of *C. quinquefasciatus*, *Ae. sierrensis*, *A. punctipennis*, or *Ae. quadrivinculatus*, or *Ae. canadensis* is associated with higher heartworm prevalence while other mosquito species are estimated to decrease prevalence rates (Wang et al., 2014). Like many filariae, *D. immitis* microfilariae exhibit a circadian rhythm, which will have an impact on the vector capacity and thus, on the epidemiology of heartworm, because chance for transmission of parasites increases with higher overlap of the mosquito activity pattern and microfilarialia peak in the host (Iongia et al., 2017; Evans et al., 2017).

In Europe, several species of mosquitoes have been found infected with *D. immitis* including *C. ppienis* in Italy (Cancrini et al., 2006), Spain (Morchón et al., 2007), and Turkey (Yıldirim et al., 2011); *C. theileri* on the island of Madeira, Portugal (Santa-Ana et al., 2006), and on the Canary Islands, Spain (Morchón et al., 2012); *Ae. vexans* in Turkey (Yıldirim et al., 2011) and *Ae. albopictus*, *A. caspius*, *A. maculipennis*, and *Coquillettidia richardi* in Italy (Cancrini et al., 2003, 2006). More recently, Montarsi et al. (2015) identified *Ae. (Finlaya) koreicus* as a new vector for *D. immitis* in Europe. In most areas of Europe, the activity of these species is limited to the period of time between spring and summer. An uptick of *D. immitis* infections in dogs has been projected into currently heartworm-free areas, as has already been observed for *D. repens*. This is possibly due to climatic changes favourable for survival and spread of *D. immitis* in mosquito vectors (Genchi et al., 2011).

While many mosquito species can transmit *D. immitis*, in Australia the primary vector is *Ae. notoscriptus* (Russell and Geary, 1996). In Japan, *C. ppienis pallens* and *C. tritaeniorychus* are the major vectors for *Dirofilaria* transmission. In all, at least 16 mosquito species are known to play a role in heartworm transmission in Japan (Aka, 2011). The primary vector species in Brazil are *Ae. taeniorynchus* (73.9%), *Ae. scapularis* (20%) and *C. quinquefasciatus* (2.5%). However, it was suggested that in contrast to the analysis performed for the USA (Wang et al., 2014), the composition of the mosquito population in the investigated area is not such a critical factor in the distribution of heartworm infections in South America (Labarthe and Guerrero, 2005).

2.4. Wolbachia

Most filarial species harbor and depend on bacterial endosymbionts. This is also the case for *D. immitis* (Simon et al., 1995; Bandi et al., 1999) and *D. repens* (Grandi et al., 2008) as both rely on the rickettsia-like endosymbiont *Wolbachia* for embryogenesis, development and survival (Ferri et al., 2011; Taylor et al., 2013). Genome analysis from *Brugia malayi* and their *Wolbachia* endosymbiont population showed that the *Wolbachia* genome encodes genes sufficient for the biosynthetic pathway of purines and pyrimidines, heme and riboflavin, none of which are completely encoded in the *Brugia* genome (Foster et al., 2005; Ghedin et al., 2007). Those findings were confirmed by genome analysis of the *Wolbachia* population of *D. immitis*. These *Wolbachia* also encode enzymes that are missing in the *D. immitis* genome like biosynthesis of heme, purine and pyrimidines. Additionally, the *Wolbachia* population from *B. malayi* contains genes necessary for folate synthesis (Godel et al., 2012). The essential metabolic contributions of *Wolbachia* renders them a valid drug target to control filariae (Slatko et al., 2010; McCall et al., 2014b; Landmann, 2019; Turner et al., 2020).
3. Prevalence and diagnosis

3.1. Prevalence

_Dirofilaria immitis_ infections in dogs and cats have been identified throughout the world in tropical and temperate regions (Simón et al., 2012; Genchi and Kramer, 2019) while the occurrence of _D. repens_ is restricted to the Old World (Fig. 4). A few anecdotal reports suggest the presence of _D. repens_ in Mexico and Chile as well (Lopez et al., 2012; Ramos-Lopez et al., 2016). Two opposing phenomena currently influence the prevalence and spreading of dirofilariosis. The prevalence of _Dirofilaria_ infections appears to be increasing worldwide mainly due to climate changes and the accompanying spread of competent mosquito species such as _Ae. albopictus_ and _Ae. koreicus_ (Montarsi et al., 2015). In contrast, heartworm prevalence is decreasing in some regions including Japan (Oi et al., 2014) and Northern Italy, likely due to a higher awareness and intensified control of the disease (Genchi and Kramer, 2019; Mendoza-Roldan et al., 2020). The latter observation may indicate that the distribution and thus the risk of heartworm infection could be reduced by a higher awareness of veterinary practitioners and a concomitant increase in preventive treatment of dogs.

However, a decreasing heartworm prevalence, particularly in the Mediterranean, may also be due to the overall reduction of the mosquito population. The general correlation of mosquito abundance and risk of disease transmission is well established for malaria (Kitron and Spielman, 1989) and it was demonstrated that depending on local conditions for mosquito populations, there is less or more risk of malaria transmission (Parham and Michael, 2010). Efforts to eliminate malaria in Europe and the Mediterranean date back as far as the late 19th century with a widespread achievement of elimination in the 20th century (Peachem et al., 2010). Today, an additional important effect is the ongoing reduction of insect populations due to industrialization, urbanization and application of insecticides (Goulson et al., 2015). Thus, heartworm prevalence may continue to decline in some endemic areas because of reduction of vector populations in these regions. In contrast, climate changes will lead to a northward spread of mosquito vectors, leading to higher disease risk in presently non-endemic areas (Medlock et al., 2012; Cella et al., 2019; Hertig, 2019). This forecast spread of _Anopheles_ spp. and as a result malaria could also be applicable to the prevalence of dirofilariosis, assuming the mosquito migration includes species which are competent intermediate hosts for _Dirofilaria_ spp.

The prevalence of _D. immitis_ in cats is estimated to be 5–20 fold lower than in dogs (Montoya-Alonso et al., 2017; Garrity et al., 2019). In a heartworm endemic area in northern Italy, a prevalence in dogs of 29% and in cats of 4.7% was shown (Venco et al., 2011). A similar ratio of prevalence rates was detected in central Italy with 5.6% in dogs and 1.6% in cats (Traversa et al., 2010).

In dogs, _D. immitis_ is prevalent in all the Americas with a few exceptions such as Chile where no heartworm cases were found in surveys (Laborde and Guerrero, 2005; Simón et al., 2012; Maggi and Kramer, 2019; Dantas-Torres and Otranto, 2020). In the USA, mean prevalence rates are generally between 1% and 12% (Lee et al., 2010; Little et al., 2014, 2021), but can be locally quite high. Florida, the most southeastern state in the USA, exhibits a 28% prevalence rate (Hays et al., 2020), and rates as high as 48% were observed in the Gulf Coast regions (McCall et al., 2008b; Bowman et al., 2009). These reports have been confirmed by more recent surveys of the American Heartworm Society (https://www.heartwormsociety.org/in-the-news/558-ahs-announces-findings-of-2019-heartworm-incidence-survey, accessed November 25th, 2020), which revealed that the top five states in heartworm incidence in 2019 were in the southeast (Mississippi, Louisiana, South Carolina, Arkansas and Alabama) (Fig. 5), similar to the report of Little et al. (2021), which showed highest incidence in Mississippi, Louisiana, Arkansas, Alabama, and Texas. The general distribution has not changed compared to 2016. No state is free of _D. immitis_ infections (Little et al., 2021). Prevalence rates are much lower in regions with colder climates. In Canada, for example, a study conducted in 1993 determined the prevalence as 0.24% (Slocombe and Villeneuve, 1993). A more recent study revealed a prevalence of 3.9% in shelter dogs in Ontario, Canada (Jacobson et al., 2020). Climate change may also drive an increase in heartworm prevalence in previously preclusive cold regions (Bowman et al., 2016).

No detailed European surveys on the distribution of _D. immitis_ have been reported, but surveys conducted at national or regional level are available. A few surveys have also focused on _D. repens_ in Europe due to the zoonotic potential in humans, even though the infection in dogs is

![Fig. 4. Presence of _D. immitis_ and _D. repens_ infections throughout the world. Analyzing the number of dogs at risk for _Dirofilaria_ infection, in Asia approx. 148 million dogs are at risk, in Latin America and Europe approx. 98 million dogs each, in North America approx. 80 million, in Africa approx. 50 million, and in Oceania approx. 6 million (Lopez et al., 2012; Simón et al., 2012; Ramos-Lopez et al., 2016; Genchi and Kramer, 2015; Boehringer Ingelheim internal analysis).](image-url)
often asymptomatic (Salamatin et al., 2013; Moskvina and Ermolenko, 2018). Furthermore, a substantial decrease of *D. immitis* infections has been observed in some endemic areas such as Northern Italy and the Canary Islands (Spain) (Genchi and Kramer, 2019). In contrast to the reduction of prevalence in those areas, increased transmission in Central and Northern Europe has been observed and may be attributed to climate changes (Simon et al., 2012; Capelli et al., 2018; Szell et al., 2020). Even in areas as far north as Finland, Estonia and Siberia, autochthonous cases have been reported (Jokelainen et al., 2016; Pietikainen et al., 2017; Genchi and Kramer, 2019). An additional factor contributing to the spread of dirofilariosis is the movement of positive dogs from endemic countries to formerly heartworm-free countries like Germany (Genchi et al., 2014). In addition, increasing occurrence and climate-change driven spread of reservoir hosts in wildlife, e.g. the golden jackal (*Canis aureus*), seem to play a significant role, too (Szell et al., 2020). A special case appears to be Austria, where only recently the introduction of *D. repens* was confirmed in mammals and in the mosquito vectors *A. algeriensis* and *A. maculipennis* (Fuehrer et al., 2016). Most cases of dirofilariosis were imported cases, but climate analysis indicates that *D. immitis* also has the capacity to establish itself in the lowland regions of Austria, given that the host and a number of competent culicid vectors are present (Fuehrer et al., 2016).

In Australia, *D. immitis* has been reported in all states with historical prevalence up to 100% in the Northern Territory (Welch et al., 1979). Today, prevalence is considered to be low throughout Australia (Nguyen et al., 2016). Dirofilariosis is also present in the near and far East and in Asia. In China prevalence ranges from 2% to 15% (Li et al., 2013). Interestingly, a novel *Dirofilaria* species, *Candidatus D. hongkongensis*, was identified in Hong Kong (Yilmaz et al., 2016, 2019) which also occurs in India (Pradeep et al., 2019). In Japan, heartworm prevalence decreased within a decade by about half in shelter dogs, from 46% in 1999–2001 to 23% in 2009–2011 (Oi et al., 2014). Notably, no *D. immitis* was detected in Israel while it was observed in other Middle East countries (Genchi and Kramer, 2019). Information on the prevalence of dirofilariosis in Africa is limited (McCall et al., 2008b). However, reports on the presence of *D. immitis* and *D. repens* are increasing in recent years. Dirofilariosis has been observed in Tunisia, Algeria and Mozambique, although the more dominant filarial species in dogs appears to be *Acanthocheilonema dracunculoides* (Genchi and Kramer, 2019).

There are more than 470 million dogs and 370 million cats worldwide (https://www.statista.com/statistics/1044386/dog-and-cat-pet-population-worldwide/accessed October 10th, 2020), and the populations continue growing. More than 200 million dogs live in North America, Europe, Australia and Japan where prophylaxis and treatment probability are expected to be high (Boehringer Ingelheim internal analysis), making heartworm prevention a highly attractive market segment.

### 3.2. Diagnosis

The Companion Animal Parasite Council (CAPC, https://capcvet.org/) and the American Heartworm Society (https://heartwormsociety.org) recommend that all dogs, including those on heartworm prevention, should be tested annually using both antigen and microfilarial tests. Different parasitological, serological or molecular tests are available to detect different life stages of heartworm taking into account that not all life stages may be present in an infected dog at any given time point (Little et al., 2018). Microscopic diagnosis for microfilariae can be performed utilizing direct blood smears or from concentrated blood (modified Knott’s test) and enables differential diagnosis to other filariae, particularly *Acanthocheilonema spp* and *D. repens* (Magnis et al., 2013). However, this technique may not be suitable for mixed filarial infections, and is, compared to other diagnostic tests, rather insensitive, and a test should not be defined as negative unless at least 1 ml of blood has been investigated (American Heartworm Society, 2020; Panarese et al., 2020).

The knowledge of any circadian rhythm can have an impact on diagnostic reliability when diagnosis is based on identification of microfilariae. However, *D. immitis* microfilaremia rhythm does not show a clear pattern. While in Romania microfilaremia peaks during night (Ionica et al., 2017), another study could not detect any consistent pattern even under standardized environmental conditions (Lovis et al.,

![Average number of cases per reporting clinic](image-url)
In a smaller study with experimentally infected dogs, a 24-h periodicity that decreased in magnitude as the dog and infection aged was apparent, adding the age of the infection(s) as another factor contributing to the complexity of interpreting potential circadian rhythms (Evans et al., 2017a).

Several serological tests based on enzyme-linked immunosorbent assays (ELISA) or immunochromatographic (ICT) tests have been developed for the detection of *D. immitis* adult antigens for in-clinic use (Barr et al., 2011; Lee et al., 2011; Henry et al., 2018; Little et al., 2018). The available highly specific antigen tests (98%–100%) detect only mature infections about 7 months post infection (McCall et al., 2001; Carmichael et al., 2017; Henry et al., 2018). At least one female *D. immitis* is needed and infections with very low worm burden may not be detected (Courtney and Zeng, 2001; Atkins, 2003). However, current commercial antigen tests were found to reliably detect infections in which more than one female adult *D. immitis* was present (Genchi et al., 2018). Furthermore, antigens may not be detected in some dogs, unless the samples are heat pretreated to release antigens from immune complexes (Little et al., 2018). Heat pretreatment is generally not recommended, with the notable exception of patients manifesting clinical signs and living in endemic areas which have not been under consistent preventive use (Little et al., 2018). Further development of assays resulted in tests suitable for diagnosis of multiple infections at the same time. Two of such rapid in-clinic tests, the FLEX4 assay (Abaxis, Union City, CA) and the SNAP assay (IDEXX Laboratories, Inc., Westbrook, ME) were recently evaluated for the detection of various *Anaplasma*, *Borrelia* and *Ehrlichia* species, and for *D. immitis* (Liu et al., 2018). While specificities for both rapid assays ranged from 98% to 100%, their sensitivities differed substantially for various pathogens and were 88.2% (FLEX4) versus 94.1% (SNAP) for *D. immitis* (Liu et al., 2018).

Molecular diagnosis is particularly appropriate for dogs suspected of being infected, but with microfilariae-negative results. It was demonstrated that in samples with only four microfilariae per ml, a multiplex PCR assay was able to give reliable positive results and to distinguish between *D. immitis* and *D. repens* (Gioia et al., 2016; Ferreira et al., 2017). Radiography and echocardiography are additional test methods for confirming the diagnosis and moreover, to assess the severity of heartworm disease (American Heartworm Society, 2020), particularly in cats (Berdoulay et al., 2004; Garrity et al., 2019).

---

**Fig. 6.** Structure of MLs marketed against heartworm infections. The macrocyclic core ring structure (top) indicates the regions where the MLs differ from each other. C13 is marked and highlighted in bold, where the main difference between the two major classes of MLs resides. Residues specific for each individual ML are visualized in blue. Modified following Prichard and Geary (2019).
4. Disease control

4.1. Macro cyclic lactones

Starting with the introduction of ivermectin in the canine market in 1987 (Heartgard®), until today, heartworm prevention is achieved almost solely through regular administration of active pharmaceutical ingredients (APIs) from the same chemical class, the macrocyclic lactones (MLs). The first MLs active against parasites – the avermectins, fermentation products of *Streptomyces avermitilis* – were discovered in 1975 from a soil sample collected in Japan (Burg et al., 1979; Campbell, 2012). Subsequently, in 2015, half of the Nobel Prize in Physiology or Medicine was awarded jointly to Campbell and Omura for their outstanding contribution to the discovery of the avermectins (Voorhis et al., 2015).

The MLs can be divided into two groups, the avermectins (abamectin, ivermectin, eprinomectin, and selamectin) and the milbemycins (milbemycin oxime and moxidectin) (Shoop et al., 1995; Vercruysse and Rew, 2002). All contain a common 16-member ML ring. The main structural difference between both classes resides at C13 of the macrocyclic ring: avermectins contain sugar residues, whereas milbemycins are protonated (Fig. 6). For further details on classification of the different MLs, for example the differences between avermectin 1- and 2-subsets or A and B series, please refer to Shoop et al. (1995) and Prichard and Geary (2019).

4.1.1. Ivermectin

The soil-dwelling bacterium *Streptomyces avermitilis* was first discovered by Satoshi Omura in a soil sample from Kawana on the southeast coast of Honshu, Japan in 1973. As anecdotes are told, Omura always carried plastic bags with him to collect specimen and found his most promising sample in the woods next to a golf course (Voorhis et al., 2015). Extracts from cultures from this strain were sent to Merck laboratories to be tested in anthelmintic screening in 1974, where it showed promising activity against nematodes and many ectoparasites (Burg et al., 1979; Chabala et al., 1980; Omura, 2008; Campbell, 2012). William C. Campbell had the active components purified and identified the avermectins in 1975. The more effective chemical derivative ivermectin was subsequently commercialized, entering the Animal Health market in 1981 initially for livestock (Omura and Crump, 2014; Voorhis et al., 2015; Laing et al., 2017). The drug’s potential in human health to fight onchocerciasis was confirmed a few years later and it was registered in 1987 and immediately provided free of charge for control of river blindness (branded as Mectizan®) (Molyneux and Ward, 2015; Ashour, 2019; http://www.mectizan.org/resources/2014-annual-highlights accessed November 27th, 2020). The World Health Organization lists ivermectin amongst the essential medicines (World Health Organization, 2019), and mass drug administration campaigns in Africa rely on its efficacy to control human filarial parasites (Kim et al., 2015).

Ivermectin is a chemically modified, dihydro derivative of naturally produced avermectin B1, composed of >80% 22,23-dihydro-avermectin B1a and <20% 22,23-dihydro-avermectin B1b at initial launch (Fig. 6) (Campbell, 1981; Campbell et al., 1983), whereas today’s ratio is 90 to 10% (https://online.uspnf.com DocID: GUID-2506EB22-023C-4689-BE0C-392C296F1803_4-en-US). It shows activity against a broad spectrum of parasitic nematodes after both oral and parenteral administration, but not against cestodes or trematodes. In addition, it has activity against many arthropods like fleas, lice, mites and some tick species (McKellar and Benchoua, 1996; Martin et al., 2020). While it is effective against microfilariae, L3 and L4 stages, it is not efficacious against adult but does reduce fertility (Martin et al., 2020). Ivermectin is marketed as oral, topical and injectable formulations, including long-acting injectables and boluses against endo- and ectoparasites of animals (Soll et al., 1988; Prichard et al., 2012).

While the antiparasitic activity of ivermectin is dependent on glutamate-gated chloride channels of endo- as well as ectoparasites (Arena et al., 1995; Wolstenholme and Rogers, 2005; Wolstenholme, 2012; Weber and Selzer, 2016), this API may target many more processes within different organisms. This includes modulation of other types of ligand-gated channels (Wolstenholme and Rogers, 2005), diverse modes of action in cancer treatment (e.g. Sharmeen et al., 2010; Yin et al., 2015; Nambara et al., 2017; Wang et al., 2018; Intuyod et al., 2019; Jiang et al., 2019; Zhang et al., 2019; Tang et al., 2020), inhibition of viral replication in several flaviviruses (Mastrangelo et al., 2012), regulation of metabolism through the farnesoid receptor in diabetic mice (Jin et al., 2013), and improvement of allergic skin inflammation by reducing activation of allergen-specific T cells (Ventre et al., 2017). Very recently, activity against SARS-CoV-2, the causative virus for COVID-19, has also been hypothesized for ivermectin based on observations in *in vitro* assays (Caly et al., 2020) and in hospitalized patients (Rajter et al., 2020). Although ivermectin is very safe, it is not without toxicity in mammals. While filariae and other nematodes are exquisitely sensitive to the drug, micromolar doses are often associated with activity in mammalian cell culture experiments. Therefore, it seems unlikely to reach effective and safe doses for antiviral therapy in humans (Schmith et al., 2020).

4.1.2. Eprinomectin

Eprinomectin or 4′-epi-acetylamino-4′-deoxyavermectin B1 was developed exclusively for veterinary medicine use as the first topical endectocide for all cattle, including lactating animals (Shoop et al., 1996a, 1996b). It is a semi-synthetic derivative of avermectin B1 or abamectin, consisting of two homologs, B1a (not less than 90%) and B1b (not more than 10%), which differ by a methylene group. Eprinomectin first entered the market in a topical formulation against internal and external parasites of cattle including lactating cows (Shoop et al., 1996b; Holste et al., 1997, 1998; Pitt et al., 1997; Williams et al., 1997; Rehbein et al., 2005). As it showed good bioavailability and systemic activity also in cats following topical application (Kvaternick et al., 2014), it was included in a topical endectoparasitic combination product together with fipronil, (S)-methoprene, and praziquantel for cats (Broadline®) (Baker et al., 2014; Rehbein et al., 2014).

4.1.3. Abamectin

Abamectin remains the only ML that is used in both animal health and crop protection (Bai and Ogbourne, 2016). It consists of avermectin B1a (>90%) and avermectin B1b (<10%). Abamectin is approved in Australia for preventive use against heartworm in dogs, however only in endo- and ectoparasiticide combination products that include oxibendazole and praziquantel (https://portal.apvma.gov.au/pbcris accessed November 27th, 2020).

4.1.4. Selamectin

Selamectin is a semisynthetic monosaccharide oxime derivative of doramectin (25-cyclohexyl-25-de(1-methylpropyl)-5-deoxy-22,23-dihydro-5-(hydroxymino)-avermectin B1 monosaccharide). Doramectin has been identified as the most potent nematicide in a series of new avermectins prepared by mutational biosynthesis, having a cyclohexyl group in the C25 position of the avermectin ring (Dutton et al., 1991; Goudie et al., 1993). Selamectin was selected for its efficacy against *D. immitis*, gastrointestinal nematodes, fleas and ticks in 1999 (Banks et al., 2000; Bishop et al., 2000; Prichard and Geary, 2019). It is available as topical formulation for dogs and cats, while doramectin is marketed for ruminants and swine only.

4.1.5. Milbemycin oxime

The milbemycins were initially isolated in 1967 as fermentation products from *Streptomyces hygroscopicus*, and subsequently from *Streptomyces cyaneogriseus* – epiaclindamino-4′-deoxyavermectin B1 (not more than 10%), which differ by a methylene group. Eprinomectin first entered the market in a topical formulation against internal and external parasites of cattle including lactating cows (Shoop et al., 1996b; Holste et al., 1997, 1998; Pitt et al., 1997; Williams et al., 1997; Rehbein et al., 2005). As it showed good bioavailability and systemic activity also in cats following topical application (Kvaternick et al., 2014), it was included in a topical endectoparasitic combination product together with fipronil, (S)-methoprene, and praziquantel for cats (Broadline®) (Baker et al., 2014; Rehbein et al., 2014).

Selamectin was selected for its efficacy against *D. immitis*, gastrointestinal nematodes, fleas and ticks in 1999 (Banks et al., 2000; Bishop et al., 2000; Prichard and Geary, 2019). It is available as topical formulation for dogs and cats, while doramectin is marketed for ruminants and swine only.
28-epoxy-5-hydroxyimino-25-ethyl/methyl milbemycin) was derived (Takiguchi et al., 1980, 1983; Prichard et al., 2012). Milbemycin oxime is available in an oral formulation, consisting of a mixture of 70–80% milbemycin A4 oxime and 30–20% milbemycin A3 oxime, for heartworm prevention in dogs and cats. In addition, milbemycin oxime shows efficacy against immature and adult stages of other parasitic roundworms, hookworms, whipworms, and lungworms, and mites (Garfield and Reedy, 1992; Holm, 2003; Prichard et al., 2012).

4.1.6. Moxidectin

Exploration of fermentation products from S. cyaneogriseus in 1983 revealed not only a new source of milbemycin, but also the new ML nemadectin (F-29249e) (Carter et al., 1987, 1988). Addition of a methoxime moiety at C-23 and a substituted olefinic side chain at the 25-position to nemadectin yields moxidectin (Ranjan et al., 1992). Heartworm prophylaxis products administer moxidectin orally, topically, or as injectable. Moxidectin has been approved for human use against river blindness (https://www.fda.gov/drugs/drug-approvals-and-databases/drug-trials-snapshots-moxidectin accessed November 25th, 2020).

Compared to the avermectins, moxidectin inhibits Pgp-mediated rhodamine123 transport with 10 times lower potency (Lespine et al., 2007). Moxidectin is very lipophilic and has a long half-life, which makes it particularly suitable for long-acting injectable formulations, e.g., ProHeart-6® and ProHeart-12® preventives in canines, and Cydectin® LA for prevention and treatment of gastrointestinal nematodes in cattle (Prichard et al., 2012; Prichard and Geary, 2019). Topical moxidectin products obtained FDA approval for elimination of microfilariae in heartworm-positive dogs, diminishing adverse reactions, which occur normally due to high microfilarial counts in infected dogs (McCall et al., 2014a). A combination product containing moxidectin, sarolaner and pyrantel to obtain protection against endo- and ectoparasites has been marketed recently (Simparica® Trio) (Kryda et al., 2019; Becskei et al., 2020).

4.2. Non-macrocyclic lactone treatments

4.2.1. Diethylcarbamazine citrate

Diethylcarbamazine citrate (DEC) (Fig. 7) is the oldest heartworm preventive, discovered in 1947 as a derivative of piperazine. It shows both microfilaricidal and adulticidal activity, presumably by increasing fibrillary susceptibility to innate immune attack (Sutton et al., 1985; El-Shahawi et al., 2010). It was first used to control human filariasis around the world (Hawking, 1962), and made it to the animal health market in products for heartworm prophylaxis in the 1960s (Paillet et al., 1968; Prescott et al., 1978). In contrast to other preventives, it has to be given daily (https://apvma.gov.au accessed December 2nd, 2020).

As diethylcarbamazine citrate is on the World Health Organization’s List of Essential Medicines (World Health Organization, 2019) for treatment of filariasis including lymphatic filariasis, tropical pulmonary eosinophilia, and loiasis (Chitkara and Sarinas, 1997), its use in animal health has been limited, with only a few products still marketed.

4.2.2. Adulticide treatment using arsenamide sodium and melarsomine dihydrochloride

The adulticide arsenamide (thiacetarsamide) sodium (Caparsolate®) (Fig. 7) was used for treatment of adult D. immitis since the 1940s. Treatment needed to be administered intravenously, and dogs had to be hospitalized during initial treatment to handle possible hepatotoxic and nephrotoxic side effects (Raynaud, 1992). In the 1990s, melarsomine dihydrochloride (Immiticide®) (Fig. 7) supplanted thiacetarsamide as an adulticide, as it provided easier administration as well as increased safety and efficacy (Raynaud, 1992; Rawlings et al., 1993a; McTier et al., 1994; Maksimovich et al., 1997). Still today, melarsomine dihydrochloride is the first line adulticide treatment for heartworm infections. Even though this therapy reduces the need for hospitalization of dogs, strict exercise restriction is required to limit thromboembolic effects (Raynaud, 1992; Rawlings et al., 1993b; Bowman and Drake, 2017; American Heartworm Society, 2020). Treated dogs should be carefully monitored for adverse effects such as increase in pulmonary pressure due to dying or dead worms, as well as intense inflammatory reactions against the parasite or its Wolbachia endosymbionts (Kramer et al., 2008; Ames et al., 2020).

To achieve complete elimination of adult heartworm infections, both the American Heartworm Society and the European Society of Dirofilariosis and Angiostongyllosis propose protocols with 2–3 month pre-treatment with an ML combined with an antibiotic against Wolbachia (such as doxycycline, see below) prior to the administration of doses of Immiticide® (European Society of Dirofilariosis and Angiostongyllosis, 2017; American Heartworm Society, 2020). The reason for this pre-treatment is to help close the susceptibility gap in the efficacy of MLs and Immiticide® against some heartworm stages (Bowman and Drake, 2017). The combined delayed treatment provides efficacy by initially using MLs to eliminate susceptible larvae and prevent new infections, while allowing older worms to develop further and become susceptible to melarsomine dihydrochloride. In addition, the risk of severe pulmonary thromboembolism is reduced due to the staged killing of the adult parasites (Carretón et al., 2014; American Heartworm Society, 2020). As some studies provided evidence that melarsomine dihydrochloride may be effective already against worms from two to four months of age, the adulticidal treatment protocol might be improved further (McCall, 2005; McCall et al., 2010; Bowman and Drake, 2017; American Heartworm Society, 2020; Carretón et al., 2019).

Adulticide therapy using melarsomine is not considered safe for cats, as worm death in cats is associated with a high risk of pulmonary thromboembolism and anaphylactic reactions (Pennisi et al., 2020). Surgery known as worm embolectomy is an alternative to relying on melarsomine dihydrochloride (Immiticide®), as worm death in cats is associated with a high risk of pulmonary thromboembolism and anaphylactic reactions (Pennisi et al., 2020).

4.2.3. Doxycycline for supportive treatment

Doxycycline-mediated clearance of Wolbachia in Onchocerca and Dirofilaria infections demonstrated that Wolbachia are required for...
filarial larval development, embryogenesis and long-term viability (Turner et al., 2020). Treatment with doxycycline (Fig. 7) successfully killed third- and fourth-stage heartworm larvae in experimentally infected dogs (McCall et al., 2011). However, a major draw-back of doxycycline is the long treatment duration needed to eliminate the required 90% of Wolbachia for a sustainable effect (Turner et al., 2020). In addition, long-term application of doxycycline in dogs is often associated with low tolerability and severe gastro-intestinal side effects (Savadelis et al., 2018). Nevertheless, for adulticidal treatment, a combination of doxycycline with monthly doses of ivermectin showed a superior microfilaricidal and adulticidal efficacy compared to the drugs given alone (Bazzocchi et al., 2008; McCall et al., 2008a). Moreover, doxycycline seems to enable a shorter treatment regimen by eliminating Wolbachia prior to the first adulticidal dose of melarsomine dihydrochloride (Carreton et al., 2019). Reducing the burden of Wolbachia in D. immitis prior to adulticide treatment proved to be more efficacious with fewer inflammatory reactions and lower risk of fatal pulmonary thromboembolisms (Bazzocchi et al., 2008; Kramer et al., 2008; Nelson et al., 2017). The combination of doxycycline, ivermectin and melarsomine significantly reduced the severity of arterial lesions and thrombi (Kramer et al., 2008). The American Heartworm Society (2020) recommends a therapy including ivermectin or moxidectin, doxycycline and melarsomine.

4.3. The mode of action of macrocyclic lactones

At a biochemical level it is well established that MLs are potent allosteric agonists of some nematode glutamate gated chloride channels (GluCl, Fig. 8) (Duce and Scott, 1985; Scott and Duce, 1986; Arena et al., 1992; Cully et al., 1994; Yates and Wolstenholme, 2004), with GluCl sequence diversity within nematode genomes providing both sensitive and insensitive subunits (Wolstenholme 2012). These channels belong to a large superfamily of cys-loop ligand gated ion channels characterized by a conserved extracellular region of amino acids that form a loop, closed by disulfide bonded cysteine residues (Connolly and Wafford, 2004). Genetic analysis has revealed that GluCl occur in many nematodes and arthropods including filariae but the size and composition vary between species (Williamson et al., 2007). While GluCl are limited to invertebrates (Wolstenholme, 2012; Buckingham et al., 2020), members of this superfamily span vertebrate and invertebrate lineages and include diverse receptors with distinct ligand specificity, such as the vertebrate glycine- and 5-HT-receptors (Lynch 2004; Barnes et al., 2009). MLs are promiscuous allosteric modulators of these related channels, for example ML can act as agonist on human glycine receptors (Shan et al., 2001) and both as agonist and inhibitor on various insect and human gamma-amino-butyric acid gated channels (Lees et al., 2014; Estrada-Mondragon and Lynch, 2015). However, because of GluCl’s exquisite ML sensitivity, therapeutic relevance of other channels is most likely irrelevant for heartworm disease therapy. Nicotinic acetylcholine receptors as well may be potentiated or attenuated by MLs (Raymond et al., 2000). Despite the channel promiscuity of MLs, a strong line of evidence from multiple experimental systems and species implicate GluCl as the relevant nematode targets, perhaps the most compelling data coming from resistance mutagenesis studies in Caenorhabditis elegans (Dent et al., 2000). Indeed, the pentameric ligand gated ion channels are well-established molecular targets for nematodes and arthropods that have provided for a spectrum of veterinary parasiticides that act at distinct binding sites (Weber and Selzer, 2016).

Observations in a homopentameric GluCl from C. elegans indicate the allosteric signal to adopt an open channel conformation is induced when MLs bind to a site created by the interface of the first (M1) and third (M3) transmembrane helices of adjacent subunits (Hibbs and Gouaux, Fig. 8). Illustration of the binding site and opening of LGCC in response to IVM. (A) Five GluCl subunits, each consisting of four alpha helical structures (M1-M4) and an extracellular domain not shown here, adopt a pentameric tertiary structure. The transmembrane region is arranged with each subunit perpendicular to the plane of the membrane and the M2 helices (orange cylinders) of each subunit lining the interior channel. Chloride ions (green circles) flow down the concentration gradient (indicated by the green arrow) when IVM (yellow/orange wedge) binds at the interface of two GluCl subunits, inducing a shift in the helices and tilting of the M2 helices away from the center of the channel, effectively widening the extracellular portion of the channel. (B) An illustration of the electrophysiological response of GluCl to IVM. The closed state is represented by a static electrical potential (flat line) and circle with narrow pore, precluding chloride ion flow. Addition of IVM (yellow/orange arrow) induces an irreversible open channel conformation, illustrated by the right part of the trace and the circle with a large pore.
Subunit heterogeneity can create structural diversity in the interfacial ML-binding site that can, in turn, result in differential GluCl sensitivity, as well as differential agonized or inhibited response of the ligand-gated chloride channel (LGCC) to ML (Estrada-Mondragon and Lynch, 2015; Atif et al., 2019). Indeed, within parasitic nematodes, GluCl sequences can be divided into alpha- and beta-subunits, with alpha-subunits generally displaying sensitivity to IVM and beta-subunits presenting glutamate but not IVM-gated channels (Wolstenholme and Rogers 2005). With respect to ML structure, the benzofuran group fused to the 16-membered macrocyclic lactone ring participates directly in binding at the subunit interfaces (Hibbs and Gouaux, 2011). The structurally variable spiroketal group may mediate ML-affinity by applying spatial constraints on site occupation, providing a plausible explanation for the different potencies displayed by structurally variable MLs (Martin et al., 2020). The binding site is buried in the hydrophobic region of the phospholipid bilayer and is in fact normally occupied by phospholipids (Althoff et al., 2014). Accordingly, it has been demonstrated that ivermectin first partitions into the phospholipid bilayer and diffuses laterally in the membrane before displacing lipids from and binding to GluCl (Atif et al., 2019). Closed channels adopt a structure in which the second transmembrane helix (M2) of each subunit lines the pore and aligns parallel to other subunit M2 helices and perpendicular to the plane of the membrane (Althoff et al., 2014). In the ML-bound state the M2 helices tilt away from the center of the pore resulting in a larger diameter opening at the extracellular membrane surface (Hibbs and Gouaux, 2011; Althoff et al., 2014). Prolonged opening of GluCl results in neuronal hyperpolarization, which precludes action potential propagation essentially silencing the associated neuron.

MLs evoke an array of phenotypes from various nematode species. Inhibition of pharyngeal pumping, reduction of oviposition, and decreased motility are all well characterized outcomes of treating nematodes in vitro (Wolstenholme and Rogers, 2005). In alignment with these observations, ML-sensitive GluCl localize to tissues and cells that mediate the respective phenotypes. For example, GluCl-subunits localize to the pharynx in C. elegans, Haemonchus contortus and Ascaris suum, others are expressed in motor neurons and motor neuron commissures in C. elegans and H. contortus, and the amphids in H. contortus (Wolstenholme and Rogers, 2005). However, a direct link between these phenotypes and the in vivo mode of action in ML-based prevention of heartworm disease remains hypothetical. In some cases, evidence suggests that such phenotypes as motility are not relevant to the in vivo MoA for heartworm disease prevention (Wolstenholme et al., 2016; Evans et al., 2017b). Indeed, the concentration of ML required to elicit paralysis in vitro (4.6 μM) (Evans et al., 2013) is several orders of magnitude higher than an extrapolated tissue concentration sufficient to provide 100% protection against heartworm disease (3.4 nM) (Daurio et al., 2011).
The MP3, Td2008 and Jd2009 isolate, were resistant to all tested MLs, particularly ivermectin and milbemycin oxime. The resistance pattern was demonstrated in dogs under various treatment protocols starting from one dose only (Snyder et al., 2011) up to monthly treatments (Bourguinat et al., 2015) and was independent of the application route (Pulaski et al., 2014). The Td2008 and Jd2009 D. immitis isolates were able to develop to adult heartworms in dogs despite of 5 or 9 monthly repeated ivermectin treatments following experimental infection (Bourguinat et al., 2015). Importantly, moxidectin still showed efficacy against strains that showed resistance to other MLs. The sensitivity of one of these drug-resistant D. immitis isolates, the MP3 isolate, was evaluated against various MLs in dogs with the result that only moxidectin was 100% effective (Blagburn et al., 2011). The reasons for this observation have not been elucidated yet but may be a higher intrinsic potency of moxidectin particularly against filariae, a significantly different pharmacokinetic profile, or a different mechanism of resistance (Pritchard and Geary, 2019). However, moxidectin resistance was also reported to occur in four ML-resistant D. immitis isolates (JYD-34, ZoeMO, ZoeLA and AMAL) (McTier et al., 2017) which could be partially overcome by increasing dose and treatment frequency (McTier et al., 2019). With the documented evidence, there is no doubt that there is genuine ML resistance present in the field. Several important questions remain: How prevalent are such parasite populations, and what is the risk of further distribution of drug resistance in other areas? What are the mechanisms behind resistance, and could resistance be diagnosed on an epidemiological level? However, for the majority of heartworm prophylaxis, todays ML-based drugs are still of good value, because resistance appears to be present mainly in the Mississippi River areas.

4.5. mdr1 mutations in collies and related breeds

While in general MLs are considered to be safe for most mammals, some dog breeds including collies and shepherds are prone to moderate to severe neurological effects. The genetic reason underlying this susceptibility is a 4 base pair deletion mutation leading to a frameshift in the multi-drug resistant (mdr1) transporter gene (nt230 (del4) mdr1 mutation; Fig 10) (Mealey et al., 2001; Geyer et al., 2005). The mdr1 (del4) mutation in these dogs can be traced back to a common ancestor in Great Britain around 1873, before formal breeds were registered and genetically isolated (Neff et al., 2004; Gramer et al., 2011).

The P-glycoprotein MDR1 belongs to a family of membrane-bound ATP-binding cassette transporters (ABC transporters) (Dean et al., 2001) and acts as a drug efflux pump across the blood-brain barrier. MDR1 plays an important role in elimination of many drugs from the mammalian central nervous system, including humans (Fig 11) (Mealey et al., 2008). The channel was first isolated and characterized from Chinese hamster ovary cells that had developed resistance to chemotherapy drugs by overexpression of MDR1 (Juliano and Ling, 1976). While mice with a deficient mdr1 gene showed no obvious phenotype in general, all mice of a colony infected with mites showed enhanced drug sensitivity and subsequently died after treatment with ivermectin (Schinkel et al., 1994). Not only MLs, but also many structurally unrelated drugs, toxins, and xenobiotics can also be substrates for MDR1, with distinct affinities and binding modes for different classes of substrates (Schinkel et al., 1996; Srikanth and Gaudet, 2019).

Sensitivity to ivermectin is increased already for heterozygous mdr1 (+/-) but especially for homozygous mdr1 (–/-) dogs, lacking expression of functional MDR1 (Mealey et al., 2008; Merola and Eubig, 2012). Although all marketed products consider this fact and therefore provide ML doses for heartworm prevention that are well tolerated by mdr1 (del4) deficient dogs, it is advised to genetically pre-test collies, shepherds and related breeds for mdr1 (del4) mutations before treatment with MLs (Geyer and Janko, 2012; Stiedl and Weber, 2017). The prevalence of at least one mdr1 (del4) allele, either as mdr1 (+/-) or mdr1 (–/-), can be as high as 75% for Collies (Table 1), but is almost nonexistent for other breeds, including breeds which share some history with the affected dog breeds (e.g., Bearded Collie, Anatolian Shepherd...
It is estimated that about 1–2% of all dogs in the northern hemisphere carry such a mutation (Neff et al., 2004; Geyer et al., 2005; Mealey et al., 2005; Gramer et al., 2011; Tappin et al., 2012; Monobe et al., 2015; Mizukami et al., 2016; Dekel et al., 2017; Marelli et al., 2020). Across geographies, the prevalence of MDR1 deficiency in collies is comparable (Geyer and Janko, 2012).

Today, with further technological advancement and the rise of an integrated health management, it is rather straightforward to test dogs for this mutation using genetic tests on cells obtained by cheek swabbing or a blood sample (e.g. Stiedl and Weber, 2017; Lee et al., 2019; Silvestro et al., 2019). It is not only reasonable to know the risk of one’s own dog before treatment with MLs, but this information is also used by many dog breeders, selecting for mdr wt dogs to increase the value of the pups and thus, hopefully outbreeding the mdr1(del4) mutation in the future.

In addition to the nt230(del4) mdr1 mutation, more than 30 single nucleotide polymorphisms have been identified in the canine mdr1 gene which might affect the transport function or expression level (Geyer and Janko, 2012). One can speculate that these polymorphisms might be the reason for increased drug sensitivity of some dogs that lack the deletion.

### 4.6. Market products

Most heartworm preventives today are available as oral formulations, while only few topicals and two injectable formulations are marketed. To illustrate distinctions in differently regulated markets, we focus on marketed products in the USA, Europe, Japan and Australia. In these geographies, heartworm active APIs take different market shares – either based on local registrations, differences in marketing, or customer preferences (Fig. 12).

#### 4.6.1. USA

International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines for the registration of anthelmintic products have been largely adopted by the U.S. Food & Drug Administration (FDA) in its Guidance for Industry (GFI) system, e.g., GFI #90 (VICH GL7) Efficacy of Anthelmintics: General Requirements, GFI #111 (VICH GL19) Efficacy of Anthelmintics: Specific Recommendations for Canine, and GFI #113 (VICH GL20) Efficacy of Anthelmintics: Specific Recommendations for Feline (https://www.fda.gov/animal-veterinary/guidance-regulations/guidance-industry accessed November 30th, 2020). At present, two laboratory dose confirmation studies and one multisite field safety and effectiveness study must be
conducted to demonstrate heartworm preventive efficacy, following the principles of Good Clinical Practice (GCP) as described in GFI #85 (VICH GL9) “Good Clinical Practice.” The FDA has historically required 100% efficacy in these studies for registration. The Center for Veterinary Medicine (CVM) is currently evaluating alternative approaches for the design of studies conducted to show effectiveness (FDA, 2018).

While there are several approved heartworm preventives for dogs and cats, only one has been approved for ferrets to date (Advantage Multi for Cats). Table 2 summarizes the products approved for use in the USA.

4.6.2. European Union

EU Regulation 2019/6 currently governs the centralized marketing authorization procedure for both human and veterinary medicines (amending EU Regulation 726/2004 relating to authorization and supervision of veterinary medicines) (https://eur-lex.europa.eu/eli/reg/2019/6/oj accessed November 30th, 2020), while national registrations can be requested from the respective national authorities. In addition, the respective VICH guidelines have to be followed: VICH GL7 Efficacy of Anthelmintics: General Requirements, VICH GL19 Efficacy of Anthelmintics: Specific Recommendations for Canine, and VICH GL20 Efficacy of Anthelmintics: Specific Recommendations for Feline (https://vichsec.org/en/guidelines/pharmaceuticals/pharma-efficacy/anthelmintics accessed November 30th, 2020). In general, most new, innovative medicines are submitted to EMA for centralized authorization, while most generics and over-the-counter medicines use national marketing authorization. Expansion of marketing authorization to other EU member states can be obtained by either a mutual recognition procedure or a centralized procedure. Nevertheless, data requirements and standards for authorization of medicines in the EU are the same, irrespective of the authorization route.

Combination products dominate the European market for heartworm prevention (Table 3).

4.6.3. Japan

In Japan, the Ministry of Agriculture, Forestry and Fisheries (MAFF) holds jurisdiction over affairs concerning veterinary medicinal products. Regulations include the Law and the Enforcement Ordinance of the Law for Ensuring the Quality, Efficacy, and Safety of Drugs and Medical Devices, (Enforcement Ordinance No. 11, 1961), and the Control Regulations of Veterinary Medical Products (Control Regulations, Ministerial Ordinance No. 107, 2004). Besides local guidelines established for registration studies by MAFF, further globally harmonized VICH guidelines on quality, safety, and efficacy have to be considered for registration (http://www.maff.go.jp/nval/ accessed November 30th, 2020). Preventive treatment using oral products containing only one API dominate the market, including many generics (Table 4). Adulticidal products based on melarsomine dihydrochloride (Immiticide and generics thereof) have been authorized, but are no longer available in Japan, as their production and sales have been discontinued.

4.6.4. Australia

All agricultural and veterinary chemical products sold in Australia have to be registered by the Australian Pesticides and Veterinary Medicines Authority (APVMA). The “Efficacy and target animal safety general guideline (Part 8)” in conjunction with the adopted VICH guidelines as well as the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines have to be followed. More than 95% efficacy against D. immitis is required for registration.

While preventive products containing abamectin and diethylcarbamazine citrate as well as melarsomine treatment are available for dogs...
only, all other products are also approved for use in cats. Some moxidectin-based products are also approved for use in ferrets, while some selamectin-based products can be used in rabbits, too. For adulticidal treatment, only melarsomine dihydrochloride as Immiticide is registered (Table 5).

### 5. The way forward

#### 5.1. Drug discovery

The potential for an economic return on investment in the heartworm disease area will certainly continue to drive anti-filarial drug discovery and development. Novel non-ML compounds suitable for preventive treatment are needed in order to control or delay the development of ML-resistant *D. immitis* populations. A comprehensive discovery approach includes phenotypic screening, mode of action and molecular target assessment, target based screening, medicinal chemistry, and advanced *in vitro* and *in vivo* profiling (Selzer and Epe, 2021). The availability of automated phenotypic screening assays with multiple endpoint readings enables a substantial increase of the number of compounds to be evaluated (Preston et al., 2015; Cornaglia et al., 2017; Partridge et al., 2018). In addition, rapid technological improvements in molecular biology, biochemistry, and related technologies allow detailed insight in the molecular mechanisms of drug action. The combination of phenotypic and molecular studies brings anti-filarial drug discovery to an innovative new level (Selzer and Epe, 2021). The introduction of advanced models to assess compounds against the larval stages of *Dirofilaria in vitro* and moreover *in vivo* will accelerate that approach and overcome the

### Table 2

APIS and products approved in the USA for prevention of heartworm disease or treatment of heartworm infections.

| Heartworm active API     | Species       | Route of application | Trade names                                      | Combination product with | Company                  |
|--------------------------|---------------|----------------------|-------------------------------------------------|--------------------------|--------------------------|
| Diethylcarbamazine citrate | dog, cat     | oral                 | Carban                                          |                          | Bimeda AH                |
|                          |               |                      | Diro-Form                                       |                          | Lloyd                    |
|                          |               |                      | Diroicide/Filarbits/Pet-Dec                      |                          | Zoetis                   |
|                          |               |                      | Filban                                          |                          | Intervet                 |
|                          |               |                      | Nemacide                                        |                          | Crenus Pharma            |
|                          | dog          | oral                 | Filarbits Plus                                  |                          | Zoetis                   |
|                          | cat          | oral                 | Centragard                                      |                          | Boehringer Ingelheim AH  |
|                          | dog          | topical              | Advantage DUO                                    |                          | Elanco                   |
|                          | dog          | oral                 | Heartgard Plus                                  |                          | Boehringer Ingelheim AH  |
|                          | dog          | oral                 | Iverhart Plu                                      |                          | Virbac AH                |
|                          | dog          | oral                 | Panacur Plus                                    |                          | Virbac AH                |
|                          | dog          | oral                 | Iverhart Max                                    |                          | Intervet                 |
|                          | dog          | oral                 | Interceptor Plus                                 |                          | Elanco                   |
|                          | dog          | oral                 | Sentinel                                        |                          | Elanco                   |
|                          | dog          | oral                 | Sentinel Spectrum                                |                          | Intervet                 |
|                          | dog          | oral                 | Interceptor Plus                                 |                          | Intervet                 |
|                          | dog          | oral                 | Trifexis                                        |                          | Elanco                   |
|                          | dog          | oral                 | ProHeart                                        |                          | Zoetis                   |
|                          | dog, ferret  | topical              | Advantage Multi                                 |                          | Zoetis                   |
|                          | cat          | topical              | Bravecto Plus                                    |                          | Zoetis                   |
|                          | dog          | oral                 | Simparica Trio                                   |                          | Zoetis                   |
|                          | cat          | topical              | Revolution                                       |                          | Zoetis                   |
|                          | cat          | i.v.                 | Revolution Plus                                  |                          | Zoetis                   |
|                          | dog          | i.v.                 | Caparsolate Sodium not marketed anymore           |                          | Zoetis                   |
|                          | dog          | i.v.                 | Immiticide                                       |                          | Zoetis                   |
|                          | cat          | i.e.                 | Revolution Plus                                  |                          | Zoetis                   |
|                          | dog          | i.e.                 | Caparsolate Sodium not marketed anymore           |                          | Zoetis                   |
|                          | dog          | i.e.                 | Immiticide                                       |                          | Zoetis                   |

Preventives dominate the market; only three products are approved for adulticidal treatment (see last two rows of table). Data retrieved from: U.S. Food & Drug Administration [https://animaldrugsatfda.fda.gov/adafda/views/#/search](https://animaldrugsatfda.fda.gov/adafda/views/#/search), accessed November 30th, 2020; AH – Animal Health.
genomics, transcriptomics or gene manipulation in helminths and medicine is possible today (Selzer et al., 2018; Johnson et al., 2020). One cades have been so innovative that personalized or precision human

beyond this scientific and technical challenges for a new heartworm preventive, the mechanism of action and the potential of derivatives of known an cycle of D. immitis.

Preventives dominate the market; only one product is approved for adulticidal treatment (see last row of table). Data retrieved from: EMA Europa Veterinary https://www.ema.europa.eu/en/medicines/field_ema_web_categories%3Aname_field/Veterinaria r

Table 3

| Heartworm active API | Species | Route of application | Trade names | Combination product with | Company |
|----------------------|---------|----------------------|-------------|--------------------------|---------|
| Ivermectin           | dog     | s.c.                 | Guardian inj. | Pyrantel                  | Elanco  |
| dog                  | oral    | Heartgard/Cardotek plus | Praziquantel | Boehringer Ingelheim AH  |
| Milbemycin oxime     | dog     | oral                 | Program plus | Lufenuron                 | Elanco  |
| dog, cat             | oral    | Milbemax generics thereof: Milbactor, Miliprazon, Milquantel Milpro | Praziquantel | Elanco Krka Virbac       |
| dog                  | oral    | Nexgard Spectra      | Afoxolane    | Boehringer Ingelheim AH  |
| dog                  | oral    | Trifexis             | Spinossad    | Elanco                   |
| Moxidectin           | dog     | s.c.                 | Aflaria      | Support Pharma, Fatto     |
| dog, cat, ferret     | topical | Advocate/Prinovox    | Imidacloprid | Bayer AH                 |
| cat                  | topical | Bravecto Plus        | Fluralaner   | Intervet                 |
| dog                  | oral    | Simparica Trio       | Sarolaner, Pyrantel embonate | Zoetis |
| Selamectin           | dog, cat| topical               | Stronghold generic thereof: Chanhold, Evicto | Zoetis Zoelittelle Pharmaceuticals, Virbac AH |
| cat                  | topical | Stronghold Plus      | Sarolaner    | Zoetis                   |
| Melarsomine dihydrochloride | dog | i.m.     | Imiticide     | Boehringer Ingelheim AH  |

Preventives dominate the market; only one product is approved for adulticidal treatment (see last row of table). Data retrieved from: EMA Europa Veterinary https://www.ema.europa.eu/en/medicines/field_ema_web_categories%3Aname_field/Veterinaria y, HMA Heads of Medicines Agencies https://mri.cts-mp.eu/veterinary/and CIMAVET https://cimavet.aemps.es/cimavet/ publico/home.html accessed November 30th, 2020; AH – Animal Health.

The scientific and technical challenges for a new heartworm preventive, the mechanism of action and the potential of derivatives of known anthelmintics such as emodepside and monopantel (Harter and von Samson-Himmelstjerna, 2002; Hess et al., 2016; Kuesel, 2016). Beyond the scientific and technical challenges for a new heartworm preventive, FDA registration has traditionally required a new drug to reach 100% efficacy. However, it has been suggested that the statistical and scientific requirements for registration need to be re-evaluated (Vidyashankar et al., 2017).

5.2. Genomics

Technology advancements of the life sciences in the last three decades have been so innovative that personalized or precision human medicine is possible today (Selzer et al., 2018; Johnson et al., 2020). One example of this breakthrough technology is seen in human cancer treatment, where CRISPR-Cas has positively impacted genetic research and gene therapy (Uddin et al., 2020; Zhang, 2020). In strong contrast, genomics, transcriptomics or gene manipulation in helminths and especially in filarial parasites is still in its infancy. The currently incomplete mapping of the D. immitis genome does not support rapid advancement of target-based approaches to drug discovery but applying advanced omics approaches may allow discovery of D. immitis or filariae specific drug targets in the coming years (Grote et al., 2017; Wheeler et al., 2020). The first draft genome sequence of a filarial nematode parasite – representing the first helminth parasite genome – was that of B. malayi in 2007 (Ghedin et al., 2007) nine years after the elucidation of the C. elegans genome (C. elegans Sequencing Consortium, 1998). Like many other parasites, B. malayi has undergone a genomic reduction in the course of evolution. While C. elegans has around 19,000 protein-coding genes (C. elegans Sequencing Consortium, 1998), B. malayi has only about 11,000 protein-coding genes in the draft genome (Ghedin et al., 2007), nearly completed by Tracey et al. (2020). Most interestingly, with the deciphering of the draft genome of B. malayi it became obvious that many filarial parasites harbor the endosymbiont Wolbachia (Scott et al., 2012). The first genome sequence of a filarial nematode important in animal health was that of D. immitis (Godel et al., 2012). As with most filarial parasites, D. immitis also harbors the endosymbiont Wolbachia, as analyzed throughout for the different life cycle stages and by tissue specific transcriptomics (Lack et al., 2014, 2015). Currently, many helmint genomes have been sequenced (Zarowiecki and Berriman, 2015) and most data are provided at the ‘WormBase ParaSite’ database (http://parasite.wormbase.org) (Howe et al., 2017).
More recently, genome sequencing and stage specific transcriptomic analysis of *D. repens* has been provided (Cafarelli et al., 2019). Additionally, extensive comparative genomics analyses have been performed for a total of 81 roundworms and flatworms, elucidating gene families relevant to parasitism including host parasite interaction, modulation of immune response or parasite migration, to name only a few (Coghlan et al., 2019). In this regard, the draft, unassembled state of the *D. immitis* genome has presented a major hurdle for target-based drug discovery. One particular approach that may offer the chance to accelerate maturation/assembly of the genome is long-read RNA sequencing technology (Doyle et al., 2020; Wheeler et al., 2020). Longer sequence reads offer the ability to better define the organization of the genome and illuminate isoform diversity (Doyle et al., 2020). The latter attribute in particular will greatly inform target-based drug discovery in *D. immitis* with high confidence gene models. Regardless of the genome assembly mechanism, better quality genomes will also facilitate identification and characterization of small RNAs that play a role in host-pathogen interactions. These interactions may offer new landscapes in which novel preventive targets present themselves.

### 5.3. Anti-Wolbachia drugs

As elucidated above, the symbiotic gram-negative *Wolbachia* is considered a valid drug target to control filariae due to the essential metabolic contributions of *Wolbachia* to *D. immitis* (Slatko et al., 2010; Landmann, 2019). The viability of that approach was shown by screening of the predicted *Wolbachia* proteome of *D. immitis* which revealed antibiotic drug targets such as Fts proteins essential for cell division (Fts - filamentous temperature-sensitive mutant), and Sec proteins involved in directed transport of proteins across membranes (Sec - protein-secreting ATPase complex) (Godel et al., 2012). This was demonstrated experimentally in studies using the tetracycline antibiotic doxycycline, which cleared *Wolbachia* from *D. immitis* and led to gradual reduction of microfilariae and adult nematodes (Bandi et al., 1999; McCall et al., 2014b). Doxycycline was also successfully employed in humans as an anti-*Wolbachia* therapy against *O. volvulus* in combination with ivermectin (Hoerauf et al., 2003). However, the required long-treatment periods represent a major draw-back of this approach for veterinary filariosis (Savadelis et al., 2018). While the positive effects of anti-*Wolbachia* drugs in adulcidical therapy have been demonstrated, the necessity of a long administration period makes them unsuitable as preventive drugs for dogs. Novel anti-*Wolbachia* drugs with shorter treatment regimens would be desired as outlined by the “anti-*Wolbachia* (A-WOL) Consortium” (Taylor et al., 2014). More recently, other anti-*Wolbachia* drugs like minocycline (Papich, 2017) and next-generation anti-*Wolbachia* candidates discovered through phenotypic screening (Turner et al., 2020) were evaluated for efficacy against *D. immitis*. Various novel anti-*Wolbachia* candidates are currently under investigation. Advanced high throughput cell-based phenotypic screens have revealed a number of potent anti-*Wolbachia* small molecules with unknown mode of action (Bakowski and McNamara, 2019). Moreover, a tyllosin analog microfilaricide showed a potent in vitro anti-*Wolbachia* activity in insect cells, performed well in preclinical safety studies, and showed efficacy in in vivo mouse and gerbil models (Taylor et al., 2019).

**Table 4**

APIS and products approved in Japan for prevention of heartworm disease or treatment of heartworm infections.

| Heartworm active API | Species | Route of application | Trade name | Combination product with | Company |
|----------------------|---------|----------------------|------------|--------------------------|---------|
| Ivermectin           | dog, cat| oral, topical         | Broadline  | Fipronil, Praziquantel, (S)-Methoprene | Boehringer Ingelheim AH |
|                      |         |                      | Cardosanec |                          | Meiji Seika Pharma |
|                      |         |                      | generics thereof: Azavanusca |              | Boehringer Ingelheim AH |
|                      |         |                      | Heartmectin |                          | Nissin Pharmaceutical |
|                      |         |                      | Pananectin |                          | ASKA AH |
| Milbemycin oxime     | dog      | oral                 | Milbemycin A |                          | Elanco |
|                      |         |                      | generics thereof: Milbeguard |              | SAMPO Pharm |
|                      |         |                      | Milbejelly  |                          | Meiji Seika Pharma |
|                      |         |                      | Milbemycin |                          | Fujita Pharmaceutical |
|                      | dog      | oral                 | Interceptor S |                          | Elanco |
|                      |         |                      | Praziquantel |                          | Meiji Seika Pharma |
|                      | dog, cat| oral                 | Milbamex   |                          | Elanco |
|                      |         |                      | Praziquantel |                          | Fujita Pharmaceutical |
|                      | dog      | oral                 | Nexgard Spectra |                          | Boehringer Ingelheim AH |
|                      |         |                      | Afoxoliner  |                          | Elanco |
|                      | dog      | oral                 | Panamectin |                          | Elanco |
|                      |         |                      | Spinossad  |                          | Fujita Pharmaceutical |
|                      | dog      | oral                 | Systec     |                          | Boehringer Ingelheim AH |
|                      |         |                      | Lufenuron  |                          | Elanco |
| Moxidectin           | dog      | oral                 | Moxidectin |                          | Zoetis |
|                      |         |                      | generics thereof: Moxguards |              | ASKA AH |
|                      |         |                      | Moxheart   |                          | Fujita Pharmaceutical |
|                      | dog      | s.c.                 | Proheart-12 |                        | Zoetis |
| Selamectin           | dog, cat| topical              | Advocate   |                          | Bayer Yakuhin |
|                      |         |                      | Imidacloprid |                          | Boehringer Ingelheim AH |
|                      | cat      | topical              | Revolution |                          | Zoetis |
|                      |         |                      | Sarolaner  |                          | Zoetis |
| Melarsomine dihydrochloride | dog | l.m. | Immiticide |                          | Boehringer Ingelheim AH |

Preventives dominate the market; only one product is approved for adulcidical treatment (see last row of table). Data retrieved from: MAFF National Veterinary Assay Laboratory [http://www.maff.go.jp/nval/accessed November 30th, 2020; AH – Animal Health.](http://www.maff.go.jp/nval/accessed November 30th, 2020)
5.4. Other approaches

The application of insecticides to block transmission of *D. immitis* to dogs was demonstrated in a study with topically applied imidacloprid and permethrin (Hayasaki and Saeki, 2009). The principle of using insecticides to prevent pathogen transmission including nematodes was reviewed by Beugnet and Franc (2012). More recently, dinotefuran-permethrin-pyriproxyfen (DPP) in a topical formulation was 95% effective in blocking transmission of *D. immitis* in two controlled laboratory studies (McCall et al., 2017a). If translatable to field conditions, that would

Table 5

| Heartworm active API | Species | Route of application | Trade names | Combination product with | Company |
|----------------------|---------|----------------------|-------------|--------------------------|---------|
| Abamectin            | dog     | oral                 | Virbac Canimax, Purina Total Care Heartwormer and Allwormer | Praziquantel, Oxibendazole | Virbac, Nestle Purina Petcare |
| Diethylcarbamazine citrate | dog | oral | Aristopet/Vitapet Heartworm, Dimmitrol | | Aristopet, Mavlab |
| Ivermectin           | dog     | oral                 | Exelpet EZY - Heartworm, Heartgard 30, Heartworm Soluble, I Love My Pet Heartworm Chewables, Nuheart, Saint Bernard Petcare Soluble Heartworm, Valuheart, Vetafarm Heart Gold | Exelpet EZY - Heartwormer + intestinal All-Wormer, Guardian Complete, Popantel Allwormer Plus Heartworm | Exelpet Products/Mars, Boehringer Ingelheim AH, Arkolette, My Pet Products Australia, Bocks/Flexsky in partnership, Australian Pharmavet Contract Manufacturing, Jurox & Zoo Pets, Vetafarm |
|                      | cat     | oral                 | Heartgard 30 FX, Startgard Plus for Kittens (Heartgard 30 FX + Frontline Plus combi pack) | Heartgard 30 Plus for Puppies (Heartgard 30 Plus + Frontline Plus combi pack) | Boehringer Ingelheim AH |
| Milbemycin oxime     | dog     | oral                 | NexGard Spectra, Interceptor Spectrum, Purina Total Care Heartwormer & Allwormer, Purina Total Care Heartwormer, Allwormer & Flea Control, Sentinel Spectrum | Praziquantel, Pyrantel embonate | Boehringer Ingelheim AH, Elanco |
|                      | dog, cat| oral                 | Milbemax, Milpro | Praziquantel, Spinosad | Elanco |
| Moxidectin           | dog     | oral                 | Proheart | | Zoetis |
|                      | dog     | s.c.                 | Proheart SR-12 Injection | | Zoetis |
|                      | dog     | topical              | Vets Choice for Fleas, Heartworm and Worms | Imidacloprid | Elanco |
|                      | dog, cat, ferret | topical | Advantage Advocate Exelpet Vet Series Flea, Intestinal & Heartworm, Exi-Flea Plus, Moxiclear | Imidacloprid | Elanco, Abbey Laboratories, Norbrook Laboratories |
|                      | dog, cat | topical | Aristopet All fleas, heartworm and worms | Imidacloprid | Aristopet, Shanghai Neway AH, Avet Health |
|                      | cat     | topical              | Bravecto Plus | Fluralaner | Intervet |
| Selamectin           | dog     | oral                 | Simparica Trio | Sarolaner, Praziquantel embonate | Zoetis |
| Selamectin           | dog, cat, rabbit | topical | Evico, Neoeco, Purevet/Revolution, Selapro, Wagg & Purr Fleas & Heartworm | | \| Voerbac, Shanghai Neway AH, Zoetis, Avet Health, Norbrook Laboratories, Avet Health |
| Melarsomine dihydrochloride | cat     | topical              | Revolution Plus | Sarolaner | Zoetis |
| Preventives dominate the market; only one product is approved for adulticidal treatment (see last row of table). Data retrieved from: Australian Pesticides and Veterinary Medicines Authority https://apvma.gov.au accessed November 30th, 2020; AH – Animal Health.
most likely lead to effective population control of the mosquitoes, resulting in a reduction of the infection pressure. In addition, it was demonstrated that a combination treatment with DPP and milbemycin oxime was 100% efficacious against the ML-resistant *Dirofilaria* JYD-34 isolate while neither the insecticide nor the milbemycin oxime alone showed a 100% efficacy (McCall et al., 2017b). Thus, the authors argued for a double defense protocol to provide effective prevention of heartworm disease in dogs. While repellents or insecticides would reduce transmission of *D. immitis*, they might not be completely effective as a monotherapy for prevention, but the synergistic activity in combination with a ML could provide a more complete prevention. However, these findings need to be verified under field conditions. Deltamethrin or imidacloprid and flumethrin containing collars (Scalibor®, Seresto®) claim to prevent predominantly ticks and fleas, and possibly also heartworm transmitting mosquitoes, but are not recommended for a monotherapy for prevention. The more recently introduced isoxazolines are not effective until ingested, thus do not act quickly enough to prevent transmission of *D. immitis* (Schorderet-Weber et al., 2017).

A safe and efficacious vaccine against *D. immitis* has the potential to substantially impact the established heartworm market. It would provide veterinarians and pet owners with an alternative to the current use of regularly administered MLs. However, at present there are only two anthelmintic vaccines commercialized: The lungworm vaccine Dictol® has been on the market for more than 50 years to protect cattle against *Dictyocaulus viviparous* infections (Jarrett et al., 1960). This attenuated vaccine is still used despite the threefold booster process and the requirement for a cold chain distribution process. More recently, a recombinant subunit vaccine demonstrated protective capability in cattle but does not provide a sterilizing efficacy (Strube et al., 2015). The Barberry® vaccine for sheep against *H. contortus* (also known as barber-pole worm) utilizes biochemically purified gut proteins from harvested worms from infected sheep (Scarff et al., 2020). The vaccine lasts for only 6 weeks after the sheep have been primed by a series of 3–6 vaccinations but aids in the control of *H. contortus* under high infection pressure (Bassetto et al., 2018) and has been commercialized in Australia since 2014 (Maxwell, 2015).

A vaccine against heartworm would need to fulfill a number of stronger requirements than those necessary for vaccines against ruminal GI-nematodes. Because the FDA set efficacy requirements for chemical preventives to 100%, it can be assumed that the efficacy of a heartworm vaccine would also need to approach or be 100%, which would be difficult to achieve. Possible alternatives to a mono vaccine prevention approach could be a combination of a vaccine with a ML. The vaccine would add an alternative mode of prevention and thus, such combination may be superior to protect against ML resistant heartworm isolates than MLs alone. However, beside the difficulties to develop an effective vaccine, acceptance of a vaccine-drug combination by dog owners would depend on additional parameters like the frequency of immunization boosters and the period of protection. A vaccine would only provide an advantage over current preventives if it protects dogs and potentially cats for an entire heartworm season, which in many areas would mean year-round. A genetically or even biochemically engineered vaccine would be superior to an attenuated *D. immitis* based product. The further advancement of the ‘omics’, and together with the rise of deep data analyses in multiple fields and other technologically advanced tools like mRNA or microRNA technologies (Britton et al., 2014; Sahin et al., 2014; Eis et al., 2019; Tombácz et al., 2021), may enable next-generation vaccine development against *D. immitis* in the future. Current success examples for an RNA-based vaccine approach are seen with the RNA vaccine candidates for global SARS-CoV-2 pandemic (Bloom et al., 2020).

6. Conclusion

Heartworm disease is a serious threat for dogs and cats in many parts of the world. Because it is unlikely that heartworm prevention can be achieved solely by improved vector control, preventative treatment based on actives of the ML class remains the mainstay of control. However, ML-resistant populations have been reported from the Mississippi River areas in the USA and have been characterized for their sensitivity status. Fortunately, for the majority of regions, todays available drugs are still of high value. With confirmed resistance to all MLs it is evident that new control methods are urgently needed. Continuous progression in technology and science enables new innovative approaches to search for novel drugs affecting filarial-specific targets. For example, focused assays have been developed to discover anti-*Wolbachia* compounds with an indirect but finally lethal effect on *D. immitis*. Apart from the search for novel APIs, the time and technology seem also ripe to discover highly effective vaccines. Anti-filarial vaccines would have the potential to change heartworm control substantially. Such a vaccine may become part of a more holistic heartworm control when combined with preventive drugs like MLs or yet to be discovered actives. Finally, it is important to emphasize compliance with veterinarians and pet owners in order to provide sufficient protection of the animals and to delay development of drug resistant *D. immitis* populations. Therefore, ideal novel products should be sustainable and highly effective, and should possess a convenient route of administration to encourage owner compliance with required dosing regimens. Covering a broader range of endo- and ectoparasites within one product is a desired add-on.

Declaration of competing interest

SN, JH, DSC and PMS are employees of Boehringer Ingelheim Animal Health, an organization with commercial interest in the animal health market. RK is an independent consultant of Boehringer Ingelheim Animal Health.

Acknowledgement

We are very grateful to Hiroki Maeda, Charles Q. Meng and Cara A. Noack for their technical support in preparing tables and figures. We thank Frédéric Beugnet and Steffen Rebbein for helpful and constructive discussions.

References

Abraham, D., Hess, J., Bondesen, B.A., Harrington, J.M., 2018. In Vivo Model for Parasitic Worm Infection Uses Immunocompromized Rodent and Methods for Evaluating Antiparasitic Compounds, Including Compounds Active against Canine Heartworm. WO201814892. World Intellectual Property Organization.

Akao, N., 2011. Human dífröfarinæsins in Japan. Trop. Med. Health 39, 65–71.

Althoff, T., Hibbs, R.E., Banerjee, S., Gouaux, E., 2014. X-ray structures of Gloc in apo reveal a gating mechanism of Cys-loop receptors. Nature 512, 333–337.

American Heartworm Society. 2020. Current canine guidelines for the prevention, diagnosis, and management of heartworm (*Dirofilaria immitis*) infection in dogs. American Heartworm Society accessed November 25th, 2020. https://d3h8c8khnqm2.cloudfront.net/images/pdf/2020_AHS_Canine_Guidelines_Summary_11_12.pdf?1605556516.

Ames, M.K., Atkins, C.E., 2020. Treatment of dogs with severe heartworm disease. Vet. Parasitol. 283, 109131.

Ames, M.K., VanVranken, P., Evans, C., Atkins, C.E., 2020. Non-Arsenical heartworm adulticidal therapy using topical moxidectin-imidacloprid and doxycycline: a prospective case series. Vet. Parasitol. 282, 109099.

Arena, J.P., Liu, K.K., Parisi, P.S., Scheaffer, J.M., Cully, D.F., 1992. Expression of a glutamate-activated chloride current in *Xenopus* oocytes injected with *Camarobothoides elegans* RNA evidence for modulation by avermectin. Brain research. Mol. Brain Res. 15, 339–348.

Arena, J.P., Liu, K.K., Parisi, P.S., Frazier, E.G., Cully, D.F., Mrozik, H., Scheaffer, J.M., 1995. The mechanism of action of avermectins in *Camarobothoides elegans*: correlation between activation of glutamate-sensitive chloride current, membrane binding, and biological activity. J. Parasitol. 81, 286–294.

Ashford, R.W., 2001. Current usage of nomenclature for parasitic diseases, with special reference to those involving arthropods. J. Med. Entomol. 15, 121–122.

Ashour, D.S., 2019. Ivermectin: from theory to clinical application. Int. J. Antimicrob. Agents 54, 134–142.

Atif, M., Smith, J.J., Estrada-Mondragon, A., Xiao, X., Salm, A.A., Capon, R.J., Lynch, J., 2019. *GluCl*-mediated inhibitory postsynaptic currents reveal targets for ivermectin and potential mechanisms of ivermectin resistance. PLoS Pathog. 15, e1007570.

Atkins, C.E., 2003. Comparison of results of three commercial heartworm antigen test kits in dogs with low heartworm burdens. J. Am. Vet. Med. Assoc. 222, 1221–1223.
Atkins, C.E., Lister, A.L., 2006. Heartworm disease. In: August, J.R. (Ed.), Consultations in Feline Internal Medicine. Elsevier Saunders, Philadelphia, PA, pp. 323–330.

Atkins, C.E., Murray, S.M., Whitby, D.R., Bland, J.L., Bushwall, J.W., Brooks, C.C., 2014. Heartworm ‘lack of effectiveness’ claims in the Mississippi delta: computerized analysis of provider-complaint 2004–2011. Vet. Parasitol. 202, 49–53.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.

Bakowski, M.A., McNamara, C.W., 2019. Advances in antiviral drug discovery for treatment of parasitic viral infections. Antivir. Res. 167, 104515.
McTier, T.L., Six, R.H., Pullins, A., Chapin, S., McCall, J.W., Rugg, D., Maeder, S.J., 1994. Use of doxycycline on early infections of Dirofilaria immitis in dogs. Vet. Parasitol. 206, 86–92.

McTier, T.L., Kramer, L., Gay, J.M., Cantor, G.H., 2001. Ivermectin sensitivity in associated with multidrug sensitivity in a sample of herding breed dogs living in Australia. Vet. Parasitol. 131, 193–209.

McKellar, Q.A., Benchaoui, H.A., 1996. Avermectins and milbemycins. J. Vet. Pharmacol. Therap. 19, 301–306.

Mealey, K.L., Bentjen, S.A., 2005. Frequency of the mutant MDR1 allele within the mosquito pathogen Wolbachia. Front. Physiol. 3, 196.

Mealey, K.L., Munyard, K.A., Bentjen, S.A., 2020. Dirofilaria immitis infections in dogs in clinical trials. Vet. Med. Small Anim. Clin. 63, 691–693.

Mealey, K.L., Varloud, M., Hodgkins, E., Mansour, A., DiCosty, U., McCall, S., Prats, C., 2016. Ivermectin sensitivity in dogs. Parasites Vectors 9, 535.

Mealey, K.L., Y., Falcón, Y., Falcón, E., 2010. Zoonotic parasites from South India belong to zoonotic species in Russian Federation: a big disease threat. Adv. Parasitol. 72, 133–179.

Mellado-Hernández, A., Bonnefont, A., Søgaard, K., 2012. Disrupting the function of the excretory-secretory apparatus in microfilariae of Dirofilaria immitis: lessons from secretomes meeting anthelmintics. Front. Physiol. doi.org/10.3389/fphys.2011.00007 1.

Moroni, B., Rossi, L., Meneguz, P.G., Orusa, R., Zoppi, S., Robetto, S., Marocco, F., Zitzen, P., 2020. Dirofilaria immitis in wolves recognizing northern Italy: are wolves competent hosts? Parasites Vectors 13, 205.

Moskvina, T.V., Ermolenskov, A.V., 2018. Dirofilaria immitis in Russian Federation: a big problem with large distribution. Russ. Open. Med. J. 7, e0102.

Nambara, S., Masuda, T., Nishio, M., Kuriyam, M., Takahashi, A., Ochiai, M., Iguchi, T., Kuroda, Y., Kato, S., Iguchi, H., Sogami, K., Saki, H., Oku, S., Maehara, Y., Suzuki, A., Mimori, K., 2017. Antimicrobial effect of the antiparasitic agent ivermectin via inhibition of Yes-associated protein 1 expression in gastric cancer. Oncotarget 8, 10766–10776.

Nodz-Djurgans, H.C., Bourgain, C., Piro, S., G. Dopa, J. K., Qone, O.A., Njikou, F., Pichard, R., Wanji, S., Kamgno, J., Bousinsequ, M., 2011. Reproductive status of Onchocerca volvulus after ivermectin treatment in an ivermectin-naïve and a frequently treated population from Cameroon. PLoS Neglected Trop. Dis. 5, e998.

Nelson, C.T., Myrick, E.S., Nelson, T.A., 2017. Clinical benefits of incorporating doxycycline into a canine heartworm treatment protocol. Parasites Vectors 10, 515.

Nguyen, C., Koh, W.L., Casteriano, A., Bejerink, N., Godfrey, C., Brown, G., Emery, D., Stupey, J., 2016. Mosquito-borne heartworm Dirofilaria immitis in dogs from Australia. Parasites Vectors 9, 535.

Ngo, M., Yoshiwaka, S., Ichikawa, Y., Nakagai, K., Matsumoto, J., Nogami, S., 2014. Prevalence of Dirofilaria immitis among shelter dogs in Tokyo, Japan, after a decade: comparison of 1999–2000 and 2009–2011. Parasite 21, 10.

Osei-Atweneboana, M.Y., Awadzi, K., Attah, S.K., Boakye, D.A., Gyapong, J.O., 2010. Phenotypic evidence of emerging ivermectin resistance in Onchocerca volvulus. PLoS Neglected Trop. Dis. 5, e998.

Oksanen, A., 2017. Heartworm prophylaxis. Aust. Vet. J. 54, 404–405.

Pantsar, P., R., G., Reblin, J., R. Neve, M., G., Forman, R., 1997. Chemotherapeutic heartworm control—the use of diethylcarbamazine in the control of Dirofilaria immitis infection in dogs. Clin. Microbiol. Rev. 10, 21–45.

Partridge, F.A., Brown, A.E., Buckingham, S.D., Willis, N.J., Wymes, D.M., Faram, R., H., E., 2018. Determining the efficacy of diethylcarbamazine for treating canine heartworm disease. Vet. Parasitol. 252, 162–167.

Pennisi, M.G., Tasker, S., Hartmann, K., 2008. Heartworm prophylaxis. Aust. Vet. J. 54, 404–405.

Pietikäinen, R., Falcón, S., Simón, A., Melcer, M., Morroni, B., Rossi, L., Meneguz, P.G., Orusa, R., Zoppi, S., Robetto, S., Marocco, F., Zitzen, P., 2020. Dirofilaria immitis infections in wolves recognizing northern Italy: are wolves competent hosts? Parasites Vectors 13, 205.

Pinto, V., Barbosa, M., Falcón, E., 2012. Heartworm disease (Dirofilaria immitis) and their vectors in Europe - new distribution trends. Front. Physiol. 3, 196.

Poirier, K., N., R., Bargues, M., L., Melero-Alcaraz, R., Pou-Barreto, C., Mas-Coma, S., S., Simon, F., 2007. Haplotype III of Culex pipiens implicated as natural vector of Dirofilaria immitis in an endemic area of Western Spain. Vector Borne Zoonotic Dis. 7, 653–658.

Poirier, K., Way, L., Cordeiro-Neto, J., Monroy, M.N., Simon, F., 2010. Zoonotic Dirofilaria immitis infections in a province of Northern Spain. Epidemiol. Infect. 138, 119–125.

Poirier, K., Cordeiro-Neto, E., Cordeiro-Neto, J., J., Perez, J., R., Hoffmann, A., Roman, N., R., A., 2015. Zoonotic Dirofilaria immitis infections in dogs in a province of Northern Spain. Epidemiol. Infect. 138, 119–125.

Poirier, K., Cordeiro-Neto, J., Monroy, M.N., Simon, F., 2010. Zoonotic Dirofilaria immitis infections in a province of Northern Spain. Epidemiol. Infect. 138, 119–125.
Taylor, M.J., Voronin, D., Johnston, K.L., Ford, L., 2013. Wolbachia filarial interactions. Cell Microbiol. 15, 520–526.
Taylor, M.J., Hoerauf, A., Townsend, S., Slattery, B.E., Ward, S.A., 2014. Anti-Wolbachia drug discovery and development: safe macrolactamides for onchocerciasis and lymphatic filariasis. Parasitology 141, 119–127.
Taylor, M.J., von Geldern, T.W., Ford, L., Hübner, M.P., Marsh, K., Johnston, K.L., Sjöberg, H.T., Spech, S., Pinnion, N., Tyer, H.E., Clare, R.H., Cook, D.A.N., Murphy, E., Steven, A., Archer, J., Bloemken, D., Lent, F., Koschel, M., Ehrens, A., Metuge, H.M., Chunda, V.C., NdongoMouhoua, P.W., Njououenou, A.J., Fombad, F., Carr, R., Morton, H.E., Alajayouuni, G., Hoerauf, A., Wanji, S., Kempf, D.J., Turner, J.D., Ward, S.A., 2019. Preclinical development of an oral anti-Wolbachia macrolide drug for the treatment of lymphatic filariasis and onchocerciasis. Sci. Transl. Med. 11.
Tombića, L., Weisman, D., Pardi, N., 2021. Vaccination with messenger RNA: a promising alternative to DNA vaccination. In: Sousa, A. (Ed.), DNA Vaccines: Methods and Protocols. Springer US, New York, NY, pp. 13–31.
Tracey, A., Foster, J.M., Paulini, M., Grote, A., Mattick, J., Tsai, Y.-C., Chung, M., Cotton, J.A., Clark, T.A., Geber, A., Holroyd, N., Korchl, J., Libro, S., Lustgarten, S., Michalski, M.L., Rogers, M.B., Twaddle, A., Dunning Hotopp, J.C., Berriman, M., Ghenin, E., 2020. Nearly complete genome sequence of Brugia malayi strain FR3. Microbiol Resour Announc 9, e00154-20. https://doi.org/10.1128/MRA.00154-20.
Traversa, D., Aze, G., Millillo, P., Capelli, G., Pampurini, F., Tunesi, C., Santori, D., Paololetti, B., Boari, A., 2010. Autochthonous foci of canine and feline infections by Dirofilaria immitis and Dirofilaria repens in central Italy. Vet. Parasitol. 169, 128–132.
Tritten, L., Geary, D.T., 2018. Helmuth extracellular vesicles in host-parasite interactions. Curr. Opin. Microbiol. 46, 73–79.
Turner, J.D., Marriott, A.E., Hong, D., N.P.O., Ward, S.A., Taylor, M.J., 2020. Novel anti-Wolbachia drugs, a new approach in the treatment and prevention of veterinary filariasis? Vet. Parasitol. 279, 109057.
Uddin, F., Rudin, C.M., Sen, T., 2020. CRISPR gene therapy: applications, limitations, and implications for the future. Front. Oncol. 10, 1387.
Venco, L., Genchi, M., Genchi, C., Gatti, D., Kramer, L., 2011. Can heartworm prevalence in dogs be used as provisional data for assessing the prevalence of the infection in wildlife? Vet. Parasitol. 279, 109057.
Ventrica, E., Fritzenwanker, M., Pantchev, N., Otranto, D., Kroidl, I., Mennebaum, M., Le, T.H., Anh Le, T., Ramänke, S., Schaper, R., von Samson-Himmelstjerna, G., Poppert, S., Krücken, J., 2016. The mitochondrial genomes of the zoonotic canine filarial parasites Dirofilaria immitis and Candidatus Dirofilaria (nochtiiella) Honkongensis provide evidence for presence of cryptic species. PLoS Neglected Trop. Dis. 10, e0005028.
Yilmaz, E., Wongkamchai, S., Rahman, S., Koutsovoulos, G.D., Blaxter, M.L., Poppert, S., Schaper, R., von Samson-Himmelstjerna, G., Krücken, J., 2019. High genetic diversity in the Dirofilaria repens species complex revealed by mitochondrial genomes of feline microfilaria samples from Harathawil, Thailand. Transbound. Emerg. Dis. 66, 389–399.
Yin, J., Park, G., Lee, J.E., Choi, E.Y., Park, J.Y., Kim, T.-H., Park, N., Jin, X., Jung, J.-E., Shin, D., Hong, J.H., Kim, H., Yoo, H., Lee, S.-H., Kim, Y.-J., Park, J.B., Kim, J.H., 2015. DEAD-box RNA helicase DDX23 modulates glioma malignancy via elevating miR-21 biogenesis. Brain 138, 2553–2570.
Zarowiecki, M., Berriman, M., 2015. What helminth genomes have taught us about parasite evolution. Parasitology 142 (Suppl. l), S85–S97.
Zheng, B., 2020. CRISPR/Cas gene therapy. J. Cell. Physiol. 236, 2459–2481.
Zhang, D., Zhang, Y., Liu, K., Liu, B., Xu, W., Gao, J., Ding, L., Tao, L., 2019. Ivermectin induces cell cycle arrest and apoptosis of HeLa cells via mitochondrial pathway. Cell Prolif 52, e12543.
Zhang, P., Zhang, Y., Liu, K., Liu, B., Xu, W., Gao, J., Ding, L., Tao, L., 2019. Ivermectin induces cell cycle arrest and apoptosis of HeLa cells via mitochondrial pathway. Cell Prolif 52, e12543.
Ömura, S., 2008. Ivermectin: 25 years and still going strong. Int. J. Antimicrob. Agents. 31, 91–98.
Ömura, S., Crump, A., 2014. Ivermectin: panacea for resource-poor communities? Trends Parasitol. 30, 445–455.