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Review

Wolbachia pipientis: Endosymbiont Bacteria of *Dirofilaria immitis* and its Role in Feline Dirofilariasis

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Abstract. Despite intense research on Feline and Canine Dirofilariosis, *Wolbachia pipientis* has been ignored for years. *W. pipientis* is an endosymbiotic bacterium that hosts certain nematodes, including *Dirofilaria immitis* known as heartworm disease. Feline dirofilariosis is of reserved prognosis due to the nature of the disease; its diagnosis is not exact with conventional methods but the implementation of molecular biology gives a vision for future research on the symbiotic relationship between *Wolbachia* and *Dirofilaria immitis* representing an advance in the diagnostic and therapeutic approach. This review discusses the biology of the bacterium, its relationship with dirofilariasis, the current aspects of the role in the pathogenesis of the disease and the molecular mechanisms of detection.

Key Words: Cats, Endosymbiont, Treatment, PCR, HARD.

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Description and taxonomy

W. pipientis belongs to the sub-group of the species α-proteobacteria (Diakou et al 2019) of the *rickettsial* order of the *Anaplasmataceae* group (Diakou et al 2017). *Wolbachia* is an endosymbiotic bacterium found in more than 20% of all insects (Wang et al 2019), arthropods (Khanmohammadi et al 2019), mosquitoes (Shaikevich et al. 2019), nematodes (Wang et al 2019) and arachnids (Gerth et al, 2017). First described by Venco et al. (2015) in the ovaries and testes of the *Culex pipiens* mosquito, it was recognized as a *Rickettsia* until proposed the name of *W. pipientis* due to its presence in the mosquito *C. pipiens* and in honor of his collaborator Wolbach.

It is a bacteria with similar characteristics to a *rickettsiae*, dimorphic, with small irregular shapes formed in rods (0.5-1.3 µm in length) and coccoid forms (0.25-0.5 µm in diameter) that exist near large forms (1-1.8 µm diameter), in turn, it presents a level of pleomorphism that increases with the age of the host cell (Satjawongvanit et al 2019). Its cytoplasm consists of ribosomes and nucleic acid fibers. *W. pipientis* is presented in a three-layer vacuolar envelope. The outermost layer is native to the host followed by the bacterial outer wall and the innermost cell layer, consisting of the plasma membrane of the bacterium (He et al 2018). The type containing *W. pipientis* is divided into 6 subgroups, subgroups A, B and E containing many of the *Wolbachia* found so far in arthropods (Vieira et al 2015), while subgroups C and D contain the *Wolbachia* of the Filarial nematodes. Members of subgroup F tend to be found in arthropods, such as termites, and in the nematode *Mansonella ozzardi*, the causative agent of filariasis in humans (Konecka et al 2019).

It is known that *W. pipientis* manipulates the reproduction of arthropods causing parthenogenesis, cytoplasmic incompatibility, feminization, and finishing with the death of males (Fitzpatrick et al 2019); in filarial nematodes they act as a compulsory mutualism bacterium (Savola et al 2019). This intracellular bacterium is observed in several species of the *Onchocercidae* family (Momčilović et al 2019), and there is a relationship within the different species of filaria (Savola et al 2019). Within the filarias, they can be found in *D. immitis* (Marcos et al 2017), *D. repens* (Savola et al 2019), *O. volvulus* (de Pinho Mixao et al 2016), *B. malyi* and *B. pahangi*. However, some filarial species do not contain it. 18 Species were analyzed showing that 10 of them harbored this endosymbiotic bacterium (Konecka et al 2019). The *Wolbachia* has also been detected in the different larval stages of *D. immitis*, the nematode that causes heartworm disease in dogs and cats. There is evidence that this bacterium is transmitted transovarrically to its offspring (Sato et al 2017), since it is observed in oocytes, developing eggs (Diakou et al 2017) and microfilariae of *D. immitis* (Satranarakun et al 2016) The treatment with antibiotics intervenes with the change of the

larval stages and reproduction of adult parasites, being consistent with the distribution in the tissues, reproductive organs and lateral cords (McTier et al 2019).
The *Wolbachia* presents two ways to stay in their hosts, one is by genetic lateral transfer, also called genetic horizontal transfer. This occurs because the bacterium is located in the host in the germline, which is required to be transmitted to the next generation (de la Puente et al 2018). This form of transmission has been described in several natural hosts, among them, various species of filarial nematodes (de la Puente et al 2018). Recent studies show that the host *Drosophila ananassae* has two genomes: an infective one ($wAna^{INF}$) and an integral one ($wAna^{ITG}$) (Turelli et al 2018). The second form of transmission involves a vertical transmission, passing from the host to his progeny (Werren et al 1995). This heritable symbiosis is transmitted from one cytoplasm to another due to its presence in the reproductive organs of the female, in order to ensure maximum transmission in the offspring of their hosts (Turelli et al 2018).

Feline heartworm disease

D. immitis is the main filaria that affects domestic and wild dogs and cats, domestic, ferrets and humans, causing heartworm disease. Dirofilariosis is reported worldwide in tropical climates, its prevalence is variable and depends on the dog population, the presence of vectors and climate (Álvarez-Fernández et al 2018). The prevalence in felines is not well known because the antemortem diagnosis is difficult, but it is generally considered to be equivalent of 9 to 18% of the canine population of the same area (Venco et al 2015). Humans and cats are erroneous hosts and the disease behaves in a different way. The dog is the definitive host and serves as a natural reservoir. Because the cat is an imperfect host, it presents many migrations, involving body cavities, systemic arteries and central nervous system (Vieira et al 2015). Cats can be asymptomatic and be diagnosed accidentally by 28% (Genchi et al 2018); 25% of cats are naturally resistant to infection. Clinically affected cats have a wide range of clinical signs such as a chronic cough and dyspnea. Anorexia and weight loss occur in some individuals; a systolic murmur may also be present when the parasites reside in the right atrioventricular orifice. Other abnormalities, such as ascites, hydrothorax, chylothorax, pneumothorax, ataxia, and syncopes may also be present, although these are less common (Venco et al 2015).
In cats, the disease can develop in two ways: a strong vascular and inflammatory pulmonary response, where most of the immature parasites that reach the pulmonary artery die in a short time (Lee-Fowler et al 2018); the second occurs due to a thromboembolism and pulmonary inflammation, caused by the death of a few adult parasites that can live in the host from 2 to 4 years (Genchi et al 2018). The first phase is misdiagnosed as asthma or allergic bronchitis; however, it is currently known as respiratory disease associated with heartworm (HARD), where it coincides with the arrival of immature parasites to the pulmonary artery and arterioles of the lungs associated with an intense pulmonary reaction. causing an acute inflammatory response and pulmonary parenchyma with the subsequent death of many parasites. This reaction is hypothesized to be caused by the activity of pulmonary intravascular macrophages, which are a component of the reticuloendothelial system that cats possess but which dogs do not have.
Clinical signs associated with this phase disappear and decrease when adult parasites mature. In this stage, the patient presents clinical signs of a cough, dyspnea and intermittent vomiting at 3 months post infection (Lee-Fowler et al 2018). The second form of presentation refers to the death of adult parasites, developing their fragmentation resulting in pulmonary inflammation and thromboembolism, often leading to a severe lung injury and sudden or acute death in 20% of affected cats. This reaction can occur even with the death of a single adult parasite. Surviving cats have alveolar cell type II hyperplasia that replaces type I alveolar cells, cause permanent lung dysfunction and chronic respiratory disease even in the absence of parasites. Although the adult parasites harbor the endoarteritis in the pulmonary artery, some cats show no clinical signs (Lee-Fowler et al 2018). In this cycle, there is dyspnea, cough, hemoptysis, collapse, vomiting, neurological signs, heart failure, and sudden death (Lee-Fowler et al 2018). The life cycle of *D. immitis* in cats is similar to that in dogs, a mosquito-infested with larvae L3 that feeds on a cat deposits the infective phase L3 on the wound (Lee-Fowler et al 2018). The following molt from L3 to L4 occurs in the subcutaneous tissue and muscle within 3 days (Liu et al 2018).
Few cats compared to dogs can present microfilaremia of 7-9 months post infection; microfilaraemia occurs in 20% of cats with female parasites and mature males but it is transient (Liu et al 2018) persisting for 1 to 2 months (Lee-Fowler et al 2018). In these individuals, the total infestation is 2 to 4 adult parasites per animal and can live for 2-4 years, unlike dogs, where they can reach 7.5 years (Arzamani et al 2017).

Inflammatory response

Like many filarial parasites, *D. immitis* hosts an endosymbiotic bacterium of the genus *Wolbachia* (Carretón et al 2017). *W. pipientis* is not a vertebrate pathogen since the infection has never been documented, but it is likely to contribute to the inflammatory pathology of dirofilariosis; however, its role is still not clear (Pietikäinen et al 2017). After its identification, interest in understanding the role it plays in the pathogenesis and the immune response in heartworm infection has increased. There is substantial evidence that *Wolbachia* comes into contact with the host organism of *D. immitis*. *Wolbachia* has been identified with the use of immunohistochemical techniques using polyclonal antibodies against surface proteins in many organs and cells in dogs, such as renal tubules, glomeruli, liver and pulmonary inflammatory cells (Spitzen & Takken, 2018).

In addition, antibodies to *Wolbachia* and *Wolbachia* surface protein inflammatory antigens deposited in tissues can circulate in these animals. In cats, IgG antibodies against *Wolbachia* surface proteins can be detected in experimental and natural infections. Currently, it is known that *Wolbachia* induces an inflammatory response in the host by activating the TLR2 and TLR4 receptors, with a MyD88 signaling protein in *D. immitis* infections (Bennuru et al 2016), in *Onchocerca volvulus* infections which activates the TLR2 receptors and TLR4 in cornea of mice but not TLR4 and TLR9 in human corneas (Que et al 2019)

Although Specht et al. (2018) reported that *Onchocerca volvulus* and *Brujia malayi* stimulates the inflammatory process through the activation of TLR2 and TLR6 receptors, and partial activation of TLR1, where MyD88 stimulates a cascade of kinases through TRIF and TRAM (Karadzovska et al 2017). The authors described the molecular mechanism of the immunological hyporesponse in humans mediated by a heat shock protein hsp60, which interacts with TLR4 receptors, and showed that ROS and mROS act as second messengers destroying the mitochondrial membrane potential, regulated and controlled by the expression of Bax and Bid genes, and decrease of Bad, with the subsequent activation of caspases through NF-κB and the activation of proinflammatory cytokines TNF-α and IL-6.
The molecular mechanisms of the inflammatory process caused by *Wolbachia* in dogs and cats infected by *D. immitis* have not been described, but it is evident that the respiratory disease associated with the heartworm (HARD) coincides with the arrival of immature parasites to the pulmonary artery and arterioles of the lungs and develops an intense pulmonary reaction (Lin et al 2017); this reaction was particularly attributed to the release of *Wolbachia* antigens after the disintegration of adult parasites (Brown, 2018).

Diagnostic approach

The diagnosis of heartworm disease or heartworm infection in cats is very difficult to determine due to its low parasitic load and low levels of circulating antigens (Mohammed et al 2018). Currently, there is no simple test for diagnosis; confirmation usually requires a combination of the following: chest x-rays and antibody tests that allows supposing the disease, echocardiography and antigen test confirm the infection (Venco et al 2015). Echocardiography, in particular, is very sensitive for diagnosis (Genchi et al 2018). Muñoz-Caro et al. (2018) reported the presence of IgG antibodies against *Wolbachia* in cats infected with *D. immitis*. The authors also studied the response of antibodies against *D. immitis* and *Wolbachia* in cats infected naturally and experimentally, with and without larvicidal treatment. They described an increase of IgG antibodies against the WSP antigen in experimentally infected cats without treatment; however, in experimentally infected cats treated with larvicidal drug one-month post-infection, the response was a continuous increase of IgG antibodies against WSP until the end of the experiment. This shows that cats have a response to the endosymbiotic bacterium *Wolbachia*, which occurs due to the massive death of larvae or pre-adult worms. These results lead to the hypothesis that *Wolbachia* surface proteins and probably other molecules stimulate the host's immune system (Bakowski et al 2019).
With the availability of molecular techniques, such as the polymerase chain reaction (PCR), the investigation into this matter is promising. In recent years it has become evident that this bacterium is very common and has important effects on its hosts (Hotopp, 2018). There is a large number of publications that describe the phylogeny of this bacterium, using several genes. The first used *Wolbachia* gene was the 16S rDNA (O'Neill et al 1992), followed by the cell division gene, the ftsZ gene (Werren et al 1995), the heat shock protein gene groE and the cell surface protein gene Wsp (Masui et al. 1997).
The Wsp gene synthesizes a homologous protein from the outside of the membrane (Bakowski et al 2019) and obtains an immunological response in the host that is infected with the filarial nematodes that harbor *Wolbachia* (Ghosh et al 2019). The use of PCR for the detection of *Wolbachia* has been carried out in tissues, nematodes (Satjawongvanit et al 2019), and blood from dogs infected with filarias (Hotopp, 2018), not for diagnostic purposes, but for the demonstration of the pathophysiology of the disease. A study in blood of cats infected with filariae and healthy individuals, using real-time and conventional PCR as a diagnostic opportunity, found no differences between these two tests, presenting the same sensitivity and specificity, but reported that the presentation of dirofilariosis was of 0.3-1.8%, an underestimated value with respect to *Wolbachia's* presentation that was 11.1-15.1%. These results would be related to the pathobiology of filaria, where cats harbor few parasites with a short period of life. The researchers propose the following hypothesis about the discrepancy of the results:
1. *D. immitis* and *Wolbachia* are present and these, in turn, are found in greater quantity and are detected more easily.
2. The massive release of *Wolbachias* occurs within 90 days post-infection by migration of L5 into the pulmonary blood vessels.
3. The *Wolbachia* bacterium enters the host with the nematode but remains to infect the host cells after the disappearance of the adult parasites.
4. Finally, *Wolbachia* is released by an external source that is not *D. immitis* (Martin et al 2019).

Therapeutic approach

Treatment is limited to symptomatic therapy because adulticide therapy is risky and does not increase the survival time (Cafarelli et al 2019). Surgical removal treatment in patients can be performed, but it is high risk and is only done in some cases where immediate curative treatment is necessary (Que et al 2019). Some cats experience a spontaneous cure due to the natural death of the parasite, without presenting symptoms. The filarial nematodes depend on the *Wolbachia* for fecundity, growth, and survival (Pedram et al 2019), making it interesting as a therapeutic target and disease control (Shaikevich & Ganushkina, 2018). It has been shown that *Wolbachia* and the molecules released by this bacterium can produce an inflammatory response in vitro. *Wolbachia* is found in all stages of parasite development and is abundant in the hypodermis of males and females, as well as in the reproductive organs of the female. They are released in considerable quantities from the body of the parasites during molting, through the production of microfilariae and when pre-adult and adult parasites die (Savola et al 2019). It is not known how long the *Wolbachia* can persist outside its host nematode, or whether vertebrates can act as an intermediate host. *Wolbachia* represents one of the great pandemics in the history of life, infecting at least 106 insect species only (Werren et al 1995). Tetracyclines such as doxycycline are known to be efficient against *Rickettsiae*. These act by inhibiting the protein synthesis of the bacteria (Carvajal et al 2019). Currently, it is known that tetracyclines have biological consequences in the life cycle of filariae, interrupting embryogenesis and the molting process of larval stages, resulting in the sterility of parasites and inhibiting larval development (Tejedor-Junco et al 2018). It has been documented that in canines experimentally infected, with treatment against *Wolbachia* before using the adulticide drug, the pulmonary side effects associated with

parasite death decrease. At present, there are no studies on the use of tetracyclines in felines, for this reason, there are disadvantages when establishing therapies because the optimal time is unknown in its initiation, what is the effective dose for the destruction of *Wolbachia* and the time of treatment for its eradication in felines.

Conclusion

The clinical significance of heartworm infection in cats has certainly grown in recent years. Due to its small size and its innate resistance to *D. immitis*, the prognosis of the infection in cats is considered reserved. The mutualism between *D. immitis* and *Wolbachia* has been a new objective for the development of antibiotic therapy in Feline Dirofilariosis; the identification of key molecules and pathways of pathophysiology are currently under investigation. Some authors tend to speculate that the diagnosis of dirofilariasis in felines could be performed serologically with the detection of anti-Wolbachia antibodies, but these antibodies are produced 2 months after infection; in turn, these antibodies begin to disappear after 8 months (Sinha et al 2019; de la Puente et al 2018). With the current techniques of molecular biology, such as the use of PCR in the diagnosis of *Wolbachia*, parasitic infection can be detected secondarily by heartworm. In addition, knowing that the pathophysiology of the disease is attributable to the lesions caused by this bacterium in the host would be a great tool in the diagnosis of the disease. The treatment with antibiotic therapy eliminates the *Wolbachia*, being at this moment a field of extensive investigation, which can provide the control of the symptoms of the patient, break the life cycle of the parasite or help in its elimination. Advances in molecular and genomic biology allow us to identify therapeutic objectives or tools for treatment.

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