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Macrocyclic lactone resistance in *Dirofilaria immitis*: Failure of heartworm preventives and investigation of genetic markers for resistance

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ABSTRACT

Macrocyclic lactone (ML) endectocides are used as chemoprophylaxis for heartworm infection (*Dirofilaria immitis*) in dogs and cats. Claims of loss of efficacy (LOE) of ML heartworm preventives have become common in some locations in the USA. We directly tested whether resistance to MLs exists in LOE isolates of *D. immitis* and identified genetic markers that are correlated with, and therefore can predict ML resistance. ML controlled studies showed that LOE strains of *D. immitis* established infections in dogs despite chemoprophylaxis with oral ivermectin or injectable moxidectin. A whole genome approach was used to search for loci associated with the resistance phenotype. Many loci showed highly significant differences between pools of susceptible and LOE *D. immitis*. Based on 186 potential marker loci, Sequenom® SNP frequency analyses were conducted on 663 individual parasites (adult worms and microfilariae) which were phenotypically characterized as susceptible (SUS), confirmed ML treatment survivors/resistant (RES), or suspected resistant/loss of efficacy

Abbreviations: ML, macrocyclic lactones; LOE, loss of efficacy; SUS, susceptible; RES, survivor/resistant; mf, microfilaria; IVM, ivermectin; MO, milbemycin oxime; MOX, moxidectin.

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(LOE) parasites. There was a subset of SNP loci which appears to be promising markers for predicting ML resistance, including SNPs in some genes that have been associated with ML resistance in other parasites. These data provide unequivocal proof of ML resistance in *D. immitis* and identify genetic markers that could be used to monitor for ML resistance in heartworms.

1. Introduction

Heartworm disease can be a devastating parasitic disease for companion animals, and wild canids can be also infected with the parasite (Bowman and Atkins, 2009; Carlson, 1985; Kreeger et al., 1990; Miller et al., 2007; Phillips and Scheck, 1991; Pratt et al., 1981). The mainstay of heartworm control for almost three decades has been the use of macrocyclic lactone (ML) preventives. The Companion Animal Parasite Council (CAPC) and the American Heartworm Society (AHS) both promote year-round heartworm prevention. However, some practitioners still place dogs on preventive therapy only during the period of the year when they consider the pet to be most at risk of infection. These heartworm preventives kill the L3–L4 larval stages, preventing the establishment of adult parasites. All available ML heartworm preventives were approved to be 100% effective, after testing under field and laboratory conditions, when originally registered with the United States of America, Food and Drug Administration (Hampshire, 2005).

Since the first report of ML LOE against *Dirofilaria immitis* in 2005 (Hampshire, 2005), ML resistance has been suspected (Blagburn et al., 2011; Bowman, 2012; Snyder et al., 2011a,b), and IVM resistance has been recently confirmed (Pulaski et al., 2014). A correlation between the LOE phenotype and a P-glycoprotein genotype was recently established (Bourguinat et al., 2011a,b). As owner non-compliance is an alternative explanation for these cases, it has been proposed that LOE cases due to ML resistance can be distinguished from cases arising from inadequate compliance with a 7-day microfilaria (mf) suppression test (Geary et al., 2011). However, the 7-day mf suppression test is not optimal for routine use for diagnosis of resistant heartworm cases, especially in epidemiological surveys. Determination of phenotypic ML resistance, under controlled experimental conditions, and evidence of significant genetic differences compared with known susceptible isolates, was required to unequivocally prove ML resistance. Development of a test based on molecular markers of the resistant phenotype would greatly facilitate more extensive studies into the geographical extent of ML resistance and guide the use of alternative options for heartworm prevention.

2. Materials and methods

2.1. Ethics statement

All animal work performed at Cornell University was approved by the Institutional Animal Care and Use Committee (protocol # 2011-0022) which follows all regulations of the Animal Welfare Act and is enforced by the United States Department of Agriculture. All animal work performed at Novartis Animal Health was approved by the Novartis Animal Health Animal Welfare Officer in St. Aubin, Switzerland, as well as the cantonal authorities (representing the federal Ethics Commission for Animal Experimentation) of the Canton of Fribourg, Switzerland. Animal Welfare Permit numbers: "FR 401/08 E" and "2010_46_FR". All animal work performed in Grand Canary was approved by the Ethics Committee of the Veterinary Medicine Service of the University of Las Palmas de Gran Canaria (Approval No. MV-2010/06) and was carried out in accordance to current European legislation on animal protection. The corresponding protocol followed the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Research conducted at Auburn University was reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (Approval No. 2011-1885) which follows all regulations of the Animal Welfare Act and is enforced by the United States Department of Agriculture.

2.2. Samples (Table 1 and Supplementary data S1)

2.2.1. Efficacy studies

Worms in the efficacy studies were derived from two isolates, one obtained from a dog identified as Td2008 from West Monroe, Louisiana, USA and one from a dog identified as Jd2009 from Earle, Arkansas, USA (summarized in Supplementary data S1). Those two isolates were not related to each other. Mf from Td2008 were classified as resistant to MLs in the *in vitro* migration assay (Blagburn, 2010; Bourguinat et al., 2011b). Mosquitoes were fed on infected blood from this dog and the resultant L3 larvae used to infect a recipient dog. Mf obtained from the recipient dog were referred to as Td2008-1. The original host dog for Td2008-1 had been treated orally with ivermectin (IVM) at weekly intervals, beginning with one dose of 3 μg/kg, followed by 11 doses of 6 μg/kg, 4 doses of 12 μg/kg, and finally 8 doses of 24 μg/kg (interrupted for one week after the 4th dose). During this period, the dog remained microfilaremic. L3s derived from mf harvested from Td2008-1 were used at Auburn University to infect a second dog, Td2008-2, which was subsequently, transferred to the Novartis research facility in Switzerland.

Jd2009 received monthly ML heartworm preventives of milbemycin oxime (MO) in 2004 and 2005, IVM/pyrantel in 2006 and 2007, and IVM/praziquantel/pyrantel in January 2008 until early July 2008. Jd2009 tested negative for HW antigen in 2005, 2006, and 2007. This dog was HW antigen positive and microfilaremic on April 11, 2008 despite a history of compliance with HW preventives. Mf