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ABC transporter efflux pumps: A defense mechanism against ivermectin in *Rhipicephalus* (*Boophilus*) *microplus* ☆

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ABSTRACT

ATP-binding cassette (ABC) transporters are efflux transporters found in all organisms. These proteins are responsible for pumping xenobiotic and endogenous metabolites through extra- and intracellular membranes, thereby reducing cellular concentrations of toxic compounds. ABC transporters have been associated with drug resistance in several nematodes and parasitic arthropods. Here, the ability of ABC transporter inhibitors to enhance ivermectin (IVM) sensitivity was tested in larvae and adult females of *Rhipicephalus* (*Boophilus*) *microplus*. Larvae of susceptible and IVM-resistant tick populations were pre-exposed to sub-lethal doses of the ABC transporter inhibitors Cyclosporin A (CsA) and MK571, and subsequently treated with IVM in a Larval Packet Test (LPT). ABC transporter inhibition by both drugs significantly reduced the concentration for 50% lethality (LC_{50}) values of four IVM-resistant populations but IVM sensitivity of a susceptible population remained unchanged. IVM sensitivity in adults was assessed through an artificial feeding assay. The addition of CsA to a blood meal substantially affected IVM toxicity in adult female ticks from a resistant population by reducing oviposition and egg viability, although it did not alter IVM toxicity in susceptible females. Three partial nucleotide sequences with similarity to ABC transporters were retrieved from the DFCI *Boophilus microplus* Gene Index (http://compbio.dfci.harvard.edu/index.html). Their transcriptional levels in the midgut of resistant and susceptible females were determined by quantitative PCR, showing that one of these sequences was significantly up-regulated in IVM-resistant females and suggesting its participation in IVM detoxification. We believe this work reports the first known evidence for the participation of ABC transporters in IVM resistance in *R. microplus*.

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1. Introduction

The selection of pesticide resistance in arthropod populations is one of the main obstacles to the chemical control of important vector species (Rosario-Cruz et al., 2009). During the last 20 years, almost one century after the first report of arthropod resistance (Melander, 1914), an increase in new cases of resistance has been reported for various parasite species (FAO, 2004).

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodide) is one of the most important ectoparasites in tropical and sub-tropical areas of the world, being responsible for severe economic losses to cattle production manifested as blood loss and hide damage (Jonsson, 2006; Seixas et al., in press). Moreover, it is the most important vector of cattle disease agents such as *Babesia* spp. and *Anaplasma* spp. (Kocan et al., 2000; Jonsson et al., 2008). In several countries its resistance to most acaricides has been confirmed, which represents a worldwide drawback for successful tick control (Martins and Furlong, 2001; Klafke et al., 2006; Dos Santos et al., 2009; Castro-Janer et al., 2010). Defining molecular mechanisms behind acaricide resistance in *R. microplus* is crucial for parasite control efforts, since more detailed knowledge of this problem could provide a fresh framework for the development of new acaricides, fostering the progress of faster and more sensitive resistance detection methods (Rosario-Cruz et al., 2009).

☆ *Note:* Nucleotide sequences data reported in this paper are available in the GenBank™ database under the Accession Nos. JN098446, JN098447 and JN098448.

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Macrocyclic lactones (MLs) are among the most successful classes of anti-parasitic drugs. They are widely used for the control of endo- and ectoparasites, including *R. microplus* (reviewed by Geary, 2005; Fox, 2006). MLs activate glutamate-gated (GluCl) and/or gamma-aminobutyric acid-gated chloride ion channels in nerve and muscle cells in arthropods and nematodes, leading to paralysis of peripheral motor function, inhibition of feeding and reproduction and, ultimately, death (Wolstenholme and Rogers, 2005; Fox, 2006). Despite the positive results MLs have afforded in terms of parasite control, the selective pressure caused by the massive application of these drugs during the past few years has triggered the development of resistance in a number of these parasites, including the nematodes *Onchocerca volvulus* (Townson et al., 1994), *Cooperia* spp. (Coles et al., 2001) and *Haemonchus contortus* (Kaplan, 2004; Coles et al., 2005), as well as the mites *Tetranychus urticae* (Kwon et al., 2010) and *Sarcoptes scabiei* (Currie et al., 2004). Currently, ivermectin (IVM) is one of the ML most used for tick control (Perez-Cogollo et al., 2010; Mendes et al., 2011). As a result, cattle tick populations resistant to IVM have been reported in Brazil since 2001 (Martins and Furlong, 2001; Klafke et al., 2006) and, more recently, in Mexico (Perez-Cogollo et al., 2010) and Uruguay (Castro-Janer et al., 2011).

At present, the molecular basis of resistance to MLs is not well understood. Insensitivity of the GluCl receptor, which prevents drug binding to its target site, has been associated with IVM resistance in some nematodes and arthropods (Dent et al., 2000; McCavera et al., 2009; Kwon et al., 2010). In arthropods, ML resistance is also associated with an increase in oxidative metabolism (Scott, 1989; Argentine et al., 1992) and a decrease in drug penetration (Scott, 1989). Although multiple factors can influence drug resistance, the final concentration of a drug in the parasite is a key determinant for it efficacy and is dictated by drug absorption, distribution and elimination parameters. Recently, it has become evident from molecular, biochemical and pharmacokinetic studies that the most important molecules involved in all of these processes are ATP-binding cassette (ABC) transporter proteins (Lespine et al., 2008; Bourguinat et al., 2011).

ABC transporters comprise a superfamily of membrane-integrated proteins expressed in all organisms, from bacteria to humans. They are essential to several physiological processes, since they are responsible for the translocation of a wide variety of substrates including amino acids, metal ions, peptides, metabolites and toxins (Holland and Blight, 1999). One of their most studied functions is cellular defense (Holland and Blight, 1999; Leslie et al., 2005), when these transporters actively pump a broad range of structurally and chemically different compounds (as multidrug transporters) out of the cell against their concentration gradients in an ATP-dependent process, mediating multidrug resistance (MDR) mechanisms, some of which have been well characterised in cancer cells and pathogens (reviewed by Lage, 2003; Higgins, 2007).

Most ABC transporters share a fundamental structure composed of four domains: two multiple membrane-spanning domains that are poorly conserved between orthologues from different organisms and two well conserved nucleotide-binding domains (NBDs). In humans, 49 ABC transporters have been identified, based on sequence homology and protein organisation, and are divided into seven subfamilies, designated ABCA to ABCG (Dean et al., 2001). Only members of the families ABCB (ABCB1, P-glycoprotein, P-gp), ABCC (ABCC1–5, MDR-associated proteins, MRPs) and ABCG (ABCG2, breast cancer resistance proteins, BCRP) appear to be associated with MDR (Leslie et al., 2005). The over-expression of these genes has been associated with drug resistance in cancer cells and constitutes one of the molecular mechanisms responsible for treatment failure (Kuo, 2007). Also, over-expression of human homologues ABC transporters has been associated with drug resistance in other organisms. IVM resistance has been associated with over-expression of the genes *mrp-1* and *pgp-1* in the nematode *Caenorhabditis elegans* (James and Davey, 2009) and with over-expression of *pgp-1* in the mite *S. scabiei* (Mounsey et al., 2010).

Taking into account the importance of ABC transporters, the aim of the present study was to investigate their involvement in IVM resistance in *R. microplus*. Inhibitors of ABC transporters were used to assess their potential to enhance the susceptibility of resistant tick populations to IVM in two life stages: larvae, analyzed through a Larval Packet Test (LPT), and adult females, by an artificial capillary feeding assay. To establish the possible molecular mechanisms of IVM resistance, we also determined the transcriptional levels of three ABC transporter genes belonging to the ABCB and ABCC subfamilies. To our knowledge, the results reported here show the first known evidence of the participation of ABC transporters in acaricide detoxification in *R. microplus*.

2. Materials and methods

2.1. Cattle

Six-month-old Hereford steers were obtained from a tick-free area and housed in individual tick-proof pens on slatted floors at the Faculdade de Veterinária of the Universidade Federal do Rio Grande do Sul, Brazil. The animals were infested with 15-day-old tick larvae (Parizi et al., 2011). Twenty-one days post-infestation, partially engorged female ticks were manually removed from cattle and detached fully engorged female ticks were collected from the floor. All experiments were conducted following the guidelines of the Ethics Committee on Animal Experimentation of the same university.

2.2. Tick strains

The Porto Alegre tick strain (POA), obtained by experimental infestation on bovines, was used as a susceptible control. This strain was originally collected in the district of Porto Alegre, state of Rio Grande do Sul (Brazil), from a farm without a history of acaricide use and was established in our laboratory. It has been maintained under standard laboratory conditions in the absence of acaricide exposure for multiple generations. This strain has been used as a susceptible reference strain to cypermethrin, deltamethrin and flumethrin (Martins et al., 1995), IVM (Klafke et al., 2006) and fipronil (Castro-Janer et al., 2010). Engorged female ticks of field populations were collected from cattle on farms located in the municipality of Jacareí, State of São Paulo, Brazil (JUA population) and in the municipalities of Pântano Grande, São Gabriel and Alegrete, State of Rio Grande do Sul, Brazil (PNO, SGA and CAV populations, respectively). On all farms, MLs were used for tick control. Detached engorged females were maintained at 27–28 °C and 80–90% relative humidity. Following oviposition, eggs were transferred to 3 mL glass vials which were then plugged with a cotton cap. Larval hatching occurred approximately 30 days after collection of engorged females. Bioassays were performed with 14–21 days old larvae.

2.3. In vitro selection of resistant larvae and colony maintenance

Twenty engorged females of the JUA strain (generations F1 and F2) were placed in a 100 mL plastic container with 20 mL of 1% ethanol containing IVM at 100 ppm (technical grade IVM, Sigma–Aldrich, USA), as this concentration allowed the oviposition of treated females. Ticks were kept in solution for 30 min at room temperature with gentle agitation. Afterwards, females were dried on paper towels and glued dorsally onto double-sided sticky tape

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