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In vitro establishment of ivermectin-resistant *Rhipicephalus microplus* cell line and the contribution of ABC transporters on the resistance mechanism

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ABSTRACT

The cattle tick *Rhipicephalus microplus* is one of the most economically damaging livestock ectoparasites, and its widespread resistance to acaricides is a considerable challenge to its control. In this scenario, the establishment of resistant cell lines is a useful approach to understand the mechanisms involved in the development of acaricide resistance, to identify drug resistance markers, and to develop new acaricides. This study describes the establishment of an ivermectin (IVM)-resistant *R. microplus* embryonic cell line, BME26-IVM. The resistant cells were obtained after the exposure of IVM-sensitive BME26 cells to increasing doses of IVM in a step-wise manner, starting from an initial non-toxic concentration of 0.5 μg/mL IVM, and reaching 6 μg/mL IVM after a 46-week period. BME26-IVM cell line was 4.5 times more resistant to IVM than the parental BME26 cell line (lethal concentration 50 (LC50) 15.1 ± 1.6 μg/mL and 3.35 ± 0.09 μg/mL, respectively). As an effort to determine the molecular mechanisms governing resistance, the contribution of ATP-binding cassette (ABC) transporter was investigated. Increased expression levels of ABC transporter genes were found in IVM-treated cells, and resistance to IVM was significantly reduced by co-incubation with 5 μM cyclosporine A (CsA), an ABC transporter inhibitor, suggesting the involvement of these proteins in IVM-resistance. These results are similar to those already described in IVM-resistant tick populations, and suggest that similar resistance mechanisms are involved *in vitro* and *in vivo*. They reinforce the hypothesis that ABC transporters are involved in IVM resistance and support the use of BME26-IVM as an *in vitro* approach to study acaricide resistance mechanisms.

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1. Introduction

Rhipicephalus microplus is one of the most deleterious cattle ectoparasites worldwide. In addition to the direct effects of *R. microplus* infestation on milk, meat, and leather production, this tick species is the vector for the infectious agents that cause bovine babesiosis and anaplasmosis (Jonsson, 2006; Jonsson et al., 2008).

The development of chemicals with acaricidal properties favored the adoption of these substances as the main method of cattle tick control (Guerrero et al., 2012). However, *R. microplus* has become resistant to most commercially available acaricides, thereby reducing the effectiveness of tick control (Klafke et al., 2006; Castro-Janer et al., 2011; Guerrero et al., 2012).

Macrocyclic lactones, such as ivermectin (IVM), are increasingly used to control endo- and ectoparasites, including *R. microplus* (Fox, 2006; Guerrero et al., 2012). IVM activates glutamate-gated chloride ion channels in invertebrate nerve and muscle cells, causing the paralysis of peripheral motor function and death of the organism (Fox, 2006). However, recent reports concerning IVM-resistant cattle tick populations in Brazil (Martins and Furlong, 2001; Klafke et al., 2006), in Mexico (Perez-Cogollo et al., 2010), and in Uruguay (Castro-Janer et al., 2011) have emphasized the importance of understanding the mechanisms of acaricide resistance to improve tick control methods.

Previous studies have associated ATP-binding cassette (ABC) transporters with drug resistance in nematodes (James and Davey, 2009), arthropods (Pohl et al., 2011; Atsumi et al., 2012), and cancer cells (Gillet et al., 2007). ABC transporters are integral membrane proteins expressed in all organisms, and are essential for several physiological processes (Holland and Blight, 1999). Certain ABC transporters are involved in the cellular detoxification of xenobiotics by pumping the drugs across membranes before they can reach their target site (Szakács et al., 2008). ABC transporters are able to transport drugs with diverse structures and functions, conferring a cellular phenotype termed "multidrug resistance" (Higgins, 2007).

Recently, we demonstrated the association of ABC transporters with macrocyclic lactones (IVM, abamectin and moxidectin) and organophosphate resistance (Pohl et al., 2011, 2012) in *R. microplus*. The results of these studies linked the participation of ABC transporters to drug resistance, *via* a multidrug detoxification mechanism.

Currently, the routine detection of acaricide resistance is based on bioassay techniques, such as the larval packet test and the adult immersion test; however, bioassays are time-consuming and laborious (Drummond et al., 1973; Klafke et al., 2006). The development of more sensitive diagnostic methods based on molecular biology methodologies is a convenient alternative, such as the allele-specific PCR assay to detect a mutation in the paratype sodium channel gene, which identifies pyrethroid resistance in ticks (Guerrero et al., 2001). However, the development of more sensitive methods relies on understanding the mechanisms involved in resistance, which in turn depends on studying tick populations in which acaricide resistance has already been established in the field.

In this sense, *in vitro* cell culture is an attractive alternative, because the experimental parameters can be more easily controlled, thereby reducing variability between experiments. Additionally, cell culture is a cost-effective approach to investigate drug resistance mechanisms, even before resistance has been established in field populations. Moreover, cell culture experiments allow the rapid screening of new drugs under development. For example, the selection of cancer cell lines for resistance to cytotoxic drugs has been a key element in the identification of mutations responsible for drug resistance (Edwards et al., 2008).

Considering the importance of understanding the mechanisms of drug resistance to improve the detection and prevention of acaricide-resistant tick populations, we questioned if an acaracide resistant tick cell line selected *in vitro* has similar resistance mechanisms to those governing acaricide resistance *in vivo*, which would be a useful approach to study acaricide resistance. In this study, we report the *in vitro* selection of an IVM-resistant *R. microplus* cell line derived from the BME26 cell line (Esteves et al., 2008), and demonstrate the involvement of ABC transporters in the process of IVM resistance in these cells.

2. Materials and methods

2.1. Cell lines and maintenance

An IVM-resistant cell line (BME26-IVM) was derived from drug-sensitive BME26 cells, which were originally isolated from *R. microplus* embryos (Esteves et al., 2008). The cell lines were grown as adherent monolayers in complete medium, as described by Esteves et al. (2008) (L-15B300 medium supplemented with 5% heat-inactivated Fetal Bovine Serum (FBS) (Gibco), 10% Tryptose Phosphate Broth (TPB) (BD), penicillin (100 U/mL), streptomycin (100 mg/mL) (Gibco), and 0.1% bovine lipoprotein concentrate (ICN), pH 7.2). For BME26-IVM cells, the complete medium was supplemented with IVM (6 μg/mL) (Sigma) diluted from a stock solution of 2 mg/mL IVM in 50% methanol. The cells were grown at 34 °C in 25 cm^2 plastic flasks (Falcon) in 5 mL of the medium, which was replaced every 7 days.

2.2. Establishment of IVM-resistant cell line

The BME26-IVM cells were derived from the parental BME26 cells (23rd passage) by continuous exposure to increasing concentrations of IVM in a step-wise manner. Every 4 weeks, the cells were harvested with a cell scraper and transferred into a new plastic flask containing the same concentration of IVM or into a flask containing a higher concentration of the drug. Starting from an initial non-toxic concentration of 0.5 μg/mL IVM, the cells were eventually able to grow in the presence of 6 μg/mL IVM after 46 weeks of selection.

2.3. Growth curve and doubling time determination

BME26 (40th passage) or BME26-IVM (38th passage) cells were seeded (5×10^5 cells) in 25 cm^2 plastic flasks in triplicate and cultured in 5 mL of complete medium.