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Original article

Comparative microarray analyses of adult female midgut tissues from feeding *Rhipicephalus* species☆

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ABSTRACT

The cattle tick, *Rhipicephalus microplus*, has a debilitating effect on the livestock industry worldwide, owing to its being a vector of the causative agents of bovine babesiosis and anaplasmosis. In South Africa, co-infestation with *R. microplus* and *R. decoloratus*, a common vector species on local livestock, occurs widely in the northern and eastern parts of the country. An alternative to chemical control methods is sought in the form of a tick vaccine to control these tick species. However, sequence information and transcriptional data for *R. decoloratus* is currently lacking. Therefore, this study aimed at identifying genes that are shared between midgut tissues of feeding adult female *R. microplus* and *R. decoloratus* ticks. In this regard, a custom oligonucleotide microarray comprising of 13,477 *R. microplus* sequences was used for transcriptional profiling and 2476 genes were found to be shared between these *Rhipicephalus* species. In addition, 136 transcripts were found to be more abundantly expressed in *R. decoloratus* and 1084 in *R. microplus*. Chi-square analysis revealed that genes involved in lipid transport and metabolism are significantly overrepresented in *R. microplus* and *R. decoloratus*. This study is the first transcriptional profiling of *R. decoloratus* and is an additional resource that can be evaluated further in future studies for possible tick control.

Introduction

The cattle tick, *Rhipicephalus microplus* is one of the most economically important tick species that affect global cattle production to date. This tick affects cattle indirectly by transmitting the protozoan (*Babesia bovis* and *B. bigemina*) and prokaryotic (*Anaplasma marginale*) pathogens, causing babesiosis and anaplasmosis, resulting in losses of milk and beef production (de la Fuente and Kocan, 2006; Wang et al., 2007). The cattle tick mostly occurs in tropical and subtropical regions between the 32° S and 40° N longitudes (Pipano et al., 2003), preferring a warm and humid climate. Furthermore, these ticks are best suited to cultivated land and woodlands where they are protected from desiccation. Due to the preference for a warmer climate, it can be speculated that the occurrence of *R. microplus* would spread to non-endemic areas due to climate change, leading to an even more widespread occurrence of disease causing pathogens such as *Babesia bovis*. This has been supported by reports that have indicated that *R. microplus* is displacing endemic *R. decoloratus* in South Africa (Tønnesen et al., 2004), spreading into areas that were previously unoccupied (Lynen et al., 2008). This is due to the fact that *R. microplus* has a higher reproductive rate than *R. decoloratus* and is less susceptible to host resistance (Lynen et al., 2008; Zeman and Lynen, 2010). This is a concern as *R. decoloratus* ticks transmit the less virulent *B. bigemina* strain, whereas *R. microplus* transmits both *B. bigemina* and the more virulent *B. bovis* strain (Homer et al., 2000; Jongejan and Uilenberg, 2004). Finally, chemical acaricides are losing their efficacy and a growing resistance to virtually all classes of acaricides is becoming a global problem (Rajput et al., 2006). This highlights the need for an alternative method to chemical control such as tick vaccines.

Immunological control of *R. microplus* was reported in the late 1980s when a low-abundance membrane-bound glycoprotein, Bm86, was used to confer protection against ticks in cattle (Willadsen et al., 1989; Willadsen, 2006). Consequently, two Bm86-based vaccines, GAVAC™ and TickGARD™, were commercialized

☆ Supplementary material associated with this article. Microarray data has been submitted to the Gene Expression Omnibus and can be accessed via the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=uzupceegdjqvrwz&acc=GSE55826.

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and used for cattle vaccination (Willadsen et al., 1995; Canales et al., 1997; de la Fuente et al., 1998). Initial reports on the efficacy of Bm86 vaccines were highly promising, indicating that three doses of purified antigen were sufficient to cause a 92% reduction in subsequent larval progeny (Willadsen et al., 1989). As part of an integrated pest management program, the vaccine led to a two-third reduction in the number of acaricide treatments necessary to maintain acceptable levels of tick infestation (de la Fuente et al., 1998). However, due to the reported variability in its efficacy and other industrial considerations, TickGARD is no longer commercially available. In contrast, GAVAC™ is still marketed in North and South America despite its reported shortcomings and a lack of widespread public acceptance of this vaccine (Guerrero et al., 2012). Vaccines do, however, still provide a more environmentally-friendly alternative to acaricides and alleviate the selective pressure for acaricide-resistant ticks (de la Fuente et al., 2007). Given the shortcomings of the Bm86 vaccines, novel vaccines need to be discovered. To date, the rate-limiting step in vaccine production has been the identification of effective antigens (Mulenga et al., 2000; Willadsen, 2001), but studies involving functional genomics, combined with reverse vaccinology, have led to the identification of numerous potential vaccine candidates (Maritz-Olivier et al., 2012).

Microarray technology is typically limited to model organisms, unless a substantial amount of representative sequence data is available (Naidoo et al., 2005). For this reason, studying the *R. microplus* transcriptome on a large scale has only recently been made possible due to the availability of a partially assembled genome and large EST databases (Lee et al., 2005; Wang et al., 2007; Bellgard et al., 2012). The BmiGI website is one such database and was launched in 2005 (Guerrero et al., 2005). It has subsequently been used for the design of custom microarrays to identify genes that are differentially expressed in larvae as a result of exposure to acaricides (Saldivar et al., 2008). DNA microarrays have also been used, among other studies, to identify *R. microplus* genes that are involved with host sensing and feeding (Rodriguez-Valle et al., 2010). Unfortunately, studies that focus on the transcriptome of ticks are severely impeded by the fact that most *R. microplus* nucleic acid sequences cannot be annotated (Wang et al., 2007; Bellgard et al., 2012). Up to 60% of the genome lacks similarity with sequences from other organisms (Wang et al., 2007), thereby negatively affecting the confidence of results for downstream analyses, such as the prediction of subcellular localization.

Both *R. microplus* and *R. decoloratus* (African blue tick) infest livestock in South Africa (Terkawi et al., 2011), making a vaccine targeting both ticks ideal. Compared to *R. microplus*, virtually no sequence data is currently available for *R. decoloratus*. However, based on phylogenetic analyses with mitochondrial 12S and cytochrome c oxidase I sequences, high sequence identity (93% and 88%, respectively) was obtained showing that these two ticks species are presumably closely related (Murrell et al., 2001). Previously, cross-species transcriptomic analysis of non-model organisms, using established array platforms for related species, have been performed successfully for other parasites including *Anopheles stephensi* and *Ancylostoma caninum* (Vontas et al., 2007; Cantacessi et al., 2009). It was therefore hypothesized that cross-species microarray technology could be employed to identify genes (and their encoded proteins) that are conserved or unique to the midgut tissues of feeding adult females of these two economically important tick species.

In this study, a previously designed custom oligonucleotide microarray, specific for *R. microplus* sequences, was used to identify genes that are shared between the midgut of *R. microplus* and *R. decoloratus* adult female ticks. Some 2476 genes were found to be shared, while 1084 were more highly expressed in *R. microplus* and 136 in *R. decoloratus*. Furthermore, Chi-square analysis revealed an up-regulation of genes involved in lipid transport and metabolism in both these ticks, perhaps suggesting a potential class of genes to target for tick-control.

Materials and methods

Tick rearing and sample collection

Pathogen-free *R. decoloratus* and *R. microplus* (Mozambique strain) larvae were obtained from ClinVet International (South Africa). These were stored at 25 °C (75–85% humidity) and fed on Holstein-Friesian (*Bos taurus*) cattle at the University of Pretoria Biomedical Research Center (UPBRC), Onderstepoort veterinary campus (South Africa). Engorged female ticks (3 biological replicates, 10 individuals per replicate) were collected 20 days post-infestation and dissected as per method outlined by Nijhof et al. (2010). Only the midgut tissues were used for this study and these were snap-frozen in TRI REAGENT® (Molecular Research Center, Inc.) and stored at −70 °C. Ethical clearance was obtained from the South African Dept. of Agriculture, Forestry and Fisheries as well as the University of Pretoria's Animal Use and Care Committee (Project approval number EC022-10).

Isolation of total RNA and cDNA synthesis

Midgut tissues were homogenized using 16G, 18G and 23G needles prior to RNA isolation following the manufacturer's guidelines for TRI REAGENT® (Molecular Research center Inc., USA). This was followed by an additional purification step using the RNeasy Mini kit, followed by on-column DNase digestion using the RNase-free DNase set (Qiagen, USA). Finally, RNA purity and integrity were assessed using the Experion™ RNA StdSens Analysis Kit (Bio-RAD, USA). cDNA synthesis was performed using Superscript™ III (Invitrogen™ Life Technologies, USA), poly-dT (5′-(T)$_{-25}$VN-3′; N = ATGC; V = AGC), random nonamer oligonucleotides and aminoallyl dUTP (Fermentas, USA). The cDNA concentration was determined using the Nanodrop-1000 (Thermo Fisher Scientific, USA). Samples were labeled with Cy3 and Cy5 by incubating the cDNA samples with the respective dye in a desiccator at room temperature for 2 h, followed by purification of the labeled cDNA using the QIAquick® PCR Purification Kit (Qiagen, USA).

Microarray

Design of a custom oligonucleotide microarray for *R. microplus* representing 13,477 contigs was reported previously (Maritz-Olivier et al., 2012). This database comprised all available EST data from GenBank (http://www.ncbi.nlm.nih.gov/nucest) and release 2.1 of the *R. microplus* gene index (Wang et al., 2007), which was then used to create an Agilent 8 × 15k 60-mer microarray. A balanced block microarray design incorporating a dye-swap was performed using three biological replicates and 20 picomoles of each Cy3- and Cy5-labeled cDNA were hybridized per array (four arrays in total) for 17 h at 65 °C. Slides were washed and rinsed in the Gene Expression Wash Buffer kit (Agilent Technologies, USA) and scanned using the GenePix™ 4000B scanner (Molecular Devices Inc., USA).

Microarray data analysis and functional annotation

Spot finding was performed using the GenePix Pro 6.0 feature extraction software (Molecular Devices Inc., USA) using default parameters, as well as manual inspection of all identified features. Using the *limma* package in the R environment (http://CRAN.R-project.org), within slide normalization was performed using locally weighted scatterplot smoothing (LOWESS),