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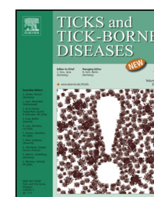

Original article

Rhipicephalus (*Boophilus*) *microplus* tick in vitro feeding methods for functional (dsRNA) and vaccine candidate (antibody) screening

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ABSTRACT

Rhipicephalus (*Boophilus*) *microplus* (Acari: Ixodidae) ticks cause economic losses for cattle industries throughout tropical and subtropical regions of the world estimated at $US2.5 billion annually. Lack of access to efficacious long-lasting vaccination regimes and increases in tick acaricide resistance have led to the investigation of targets for the development of novel tick vaccines and treatments. In vitro tick feeding has been used for many tick species to study the effect of new acaricides on the transmission of tick-borne pathogens. Few studies have reported the use of in vitro feeding for functional genomic studies using RNA interference and/or the effect of specific anti-tick antibodies. In particular, in vitro feeding reports for the cattle tick are limited due to its relatively short hypostome. Previously published methods were further modified to broaden optimal tick sizes/weights, feeding sources including bovine and ovine serum, optimisation of commercially available blood anti-coagulant tubes, and IgG concentrations for effective antibody delivery. Ticks are fed overnight and monitored for ∼5–6 weeks to determine egg output and success of larval emergence using a humidified incubator. Lithium-heparin blood tubes provided the most reliable anti-coagulant for bovine blood feeding compared with commercial citrated (CPDA) and EDTA tubes. Although >30 mg semi-engorged ticks fed more reliably, ticks as small as 15 mg also fed to repletion to lay viable eggs. Ticks which gained less than ∼10 mg during in vitro feeding typically did not lay eggs. One mg/ml IgG from Bm86-vaccinated cattle produced a potent anti-tick effect in vitro (83% efficacy) similar to that observed in vivo. Alternatively, feeding of dsRNA targeting Bm86 did not demonstrate anti-tick effects (11% efficacy) compared with the potent effects of ubiquitin dsRNA. This study optimises *R. microplus* tick in vitro feeding methods which support the development of cattle tick vaccines and treatments.

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Introduction

Rhipicephalus (*Boophilus*) *microplus* (cattle tick, Acari: Ixodidae) is a one-host tick species associated with losses in cattle production in tropical and subtropical regions of the world with a recently reported spread to West Africa (Jonsson et al., 2001; Madder et al., 2011; Perez de Leon et al., 2012). The cattle tick is one of the most economically important ectoparasites and vectors of potentially fatal tick-borne diseases, anaplasmosis and babesiosis (Jonsson et al., 2008). Ticks are currently mostly controlled through the application of chemical acaricides with widespread reports of ticks developing resistance to several classes of chemicals (Lovis et al., 2013). Vaccinations based on the Bm86 tick gut antigen have been successfully applied in countries such as Cuba (Valle et al., 2004; Vargas et al., 2010), while failing in terms of adoption and/or success in other countries potentially due to sequence variation (Cunha et al., 2012; de la Fuente et al., 2007a; Garcia-Garcia et al., 1999; Sossai et al., 2005). Researchers have thus focussed on the identification of potential acaricide targets or drugs and also on the search for alternative immunogenic antigens (Concepcion et al., 2013; Cossio-Bayugar et al., 2012; Stutzer et al., 2013).

For both drug and vaccine development, research can be limited by access to host animals with tick challenge trials being both time-consuming and cost-prohibitive. Non-host laboratory animals can be used for antigen testing, however, responses do not always correlate to those of the natural host, which is particularly evident for the host-specific cattle tick, *R. microplus*. Rather than using artificial

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feeding methods, several groups have relied upon RNA interference (RNAi) dsRNA knockdown techniques to demonstrate either potential drug or vaccine targets (Almazán et al., 2010; Barnard et al., 2012). Issues relying upon RNAi include the lack of association with immunity (host antibody responses to the target are not examined), most treatments are not delivered by the natural process of feeding, and studies do not demonstrate the specificity of the dsRNA treatments. The latter is due to the lack of a genome sequence for most tick species, thus researchers are unable to screen dsRNA treatments for target specificity (Lew-Tabor et al., 2011). Indeed, it has been demonstrated that off-target effects occur using long dsRNA treatments in ticks which leads to non-specific silencing of non-target mRNAs, a phenomenon well reported in other arthropod species (Lew-Tabor et al., 2011; Ma et al., 2006). Nonetheless, the use of short dsRNAs (~100 bp) decreases the risk of non-specific silencing (Lew-Tabor et al., 2011) and is a useful tool for the identification of drug targets. Specific antibody treatments, however, are the treatment of choice for screening of vaccine candidates. Data from such functional (RNAi) and antibody studies can complement each other when used under robust in vitro feeding conditions. This would provide researchers with in vitro options for screening potential antigens or drug targets prior to in vivo large-animal trials.

Several conditions for in vitro feeding have been established by different researchers using *Rhipicephalus* spp. and other tick species. These conditions include: tube types, position of mouthparts, anti-coagulant preferences, serum versus whole blood, host blood species, and also the use of mated vs. unmated females. A glass capillary tube feeding technique was first developed by Chabaud (1950) to feed *Hyalomma excavatum*, *H. dromedarii*, *Dermacentor reticulatus (=pictus)*, and *Rhipicephalus sanguineus* ticks (Chabaud, 1950). In these early experiments, ticks did not feed to engorgement, and palps were spread with only the hypostome inserted into capillary tube (Gregson, 1957). In the 1960s, using *R. appendiculatus*, it was demonstrated that the palps do not need to be spread and that ticks fed equally well with the entire capitulum inserted into the capillary tube (Joyner and Purnell, 1968; Purnell and Joyner, 1967). During their study of *Theileria parva* transmission, Joyner and Purnell (1968) demonstrated that greater volumes of serum or plasma were consumed compared with heparinised or defibrinated blood, but that secretion of infective forms of *T. parva* was only achieved when *R. appendiculatus* was fed whole blood. In vitro feeding of *R. appendiculatus* nymphs showed that heparinised blood was most efficacious, followed by defibrinated blood with nymphs reaching engorgement weights comparable to host-fed ticks and subsequently also moulting to adult ticks (Waladde et al., 1993). Nymphs fed using blood treated with either acid citrate dextrose (ACD) or ethylenediamine-tetracetate (EDTA) did not consume blood or gain weight (Waladde et al., 1993). It has long been known that mated ticks feed more actively than those unmated and that the rate of engorgement is also related to mating (Gregson, 1943). The first method developed for feeding *R. microplus* pre-engorged adult females demonstrated heparin as the preferred anti-coagulant, and mouthparts were separated with the palps glued on the outside with the hypostome inserted into a pasteur-pipette-modified glass tube rather than a capillary tube (Willadsen et al., 1984). In addition, to demonstrate host specificity a higher proportion of ticks laid viable eggs when fed bovine blood compared to those fed rat, rabbit, or guinea pig blood (Willadsen et al., 1984). This method was subsequently used to study in vitro *Babesia* spp. transmission (Agbede et al., 1986; Inokuma and Kemp, 1998). Two papers have since been published describing *R. microplus* in vitro feeding protocols which diverged from this original protocol by using capillary tubes and larger and smaller ticks, respectively (de la Vega et al., 2000; Gonsioroski et al., 2012). In vitro tick feeding has thus mainly been exploited as a tool to study tick-borne disease transmission (Joyner and Parnell, 1968; Inokuma and Kemp, 1998). Weight gain has most often been used as a measure of successful feeding (Bennett, 1974a, 1974b), however, researchers have also incorporated radioactive labelling, xenodiagnosis, and molecular methods to monitor tick-borne disease transmission in ticks in vitro (Broadwater et al., 2002; Macaluso et al., 2001). Further developments include the use of membrane feeding which enables the completion of a whole tick life cycle in vitro and has been applied to study the toxic effects of commercial acaricides as well as disease transmission of tick-borne pathogens including *T. parva* vectored by *R. appendiculatus* (Bonnet et al., 2007; Kröber and Guerin, 2007a; Kuhnert et al., 1995; Musyoki et al., 2004). Perhaps due to the short hypostome of the adult female *R. microplus* (300 μm), membrane feeding methods have been suggested but not extensively reported (Kröber and Guerin, 2007b).

There are very few studies reporting the effects of feeding-specific antibodies or antiserum in vitro for all tick species. In vitro experiments feeding serum from naive and tick (*R. appendiculatus*)-exposed rabbits and bovines showed that with increased exposure to ticks, rabbit serum appeared to inhibit feeding by either physical gut disruption or altered feeding behaviour (Losel et al., 1993). The same observations had been already previously made in vivo for this tick species (Walker and Fletcher, 1987). It was suggested that perhaps antibodies would deter feeding if the gustatory sensilla on the tick's cheliceral denticles 'sense' factors in resistant serum (Losel et al., 1992). It thus seems feasible that antibodies could have an effect on the gut or feeding of ticks in vitro which could be exploited as a method for screening potential vaccine antigens. A recent *R. microplus* study fed 2 different monoclonal antibodies targeting 2 potential vaccine candidates, however, monoclonal antibody concentrations were not divulged, no egg viability data was reported, and only a slight decrease in total egg weight was observed from one monoclonal antibody treatment (Gonsioroski et al., 2012). The effects of these novel antigens in vivo have not been reported, thus it is difficult to determine if the in vitro studies correlate to potentially protective tick vaccine candidates. Similarly, as mentioned above, it is common for RNAi studies to inject dsRNA into fully engorged females to examine the phenotypic effects of silencing particular genes (reviewed by de la Fuente et al., 2007b). A more natural delivery method of dsRNAs would be the in vitro feeding of semi-engorged females which can be monitored for successful oviposition and larval eclosion. In a previous study, *Ixodes scapularis* nymphs were fed dsRNA in vitro, however, they were subsequently placed onto hosts to examine changes in nymphal development (Soares et al., 2005). The in vitro feeding of specific antibodies or dsRNAs has not been extensively exploited using semi-engorged female ticks to provide preliminary data for the selection of putative vaccine candidates. The aim of this study was to revisit the standardisation of in vitro feeding methods for *R. microplus* by examining palp positioning, commercial blood anti-coagulation tubes, and the potential use of bovine or ovine serum. These modified in vitro tube feeding protocols for *R. microplus* semi-engorged females were subsequently validated using dsRNA and/or antibody treatments against the gut antigen Bm86 (Rand et al., 1989) and ubiquitin (Kurscheid et al., 2009) as model targets.

Material and methods

Tick collection and preparation

R. microplus female ticks (non-resistant field strain, NRFS) (Stewart et al., 1982) were collected from Hereford cattle at the Biosecurity Tick Colony (Queensland Department of Agriculture, Fisheries and Forestry, DAFF) at the Queensland Animal Science

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