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ORIGINAL ARTICLE

Cloning and molecular analysis of *voraxin-α* gene of *Rhipicephalus* (*Boophilus*) *microplus*

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Abstract To identify suitable targets for development of cross-protective tick vaccine, in silico analysis was attempted and male tick derived molecule, voraxin-α was targeted. The *voraxin-α* homologue of *Rhipicephalus* (*Boophilus*) *microplus* was cloned, sequenced and analyzed employing standard methods. The deduced amino acids sequence analysis of the 419 bp cloned *voraxin-α* gene of *R.* (*B.*) *microplus* indicated very high (94.6 %) similarity with *voraxin-α* of the *R. appendiculatus* and moderate to low identity with *Amblyomma hebraeum, Dermacentor silvarum* and *Haemaphysalis longicornis*. The results suggest that recombinant voraxin-α might be a good candidate as cross-protective anti-tick vaccine.

Keywords Voraxin-α · Anti-tick vaccine · *Rhipicephalus* (*Boophilus*) *microplus*

Introduction

Ticks as veterinary parasite, cause deleterious effects in two ways, firstly, they suck significant quantities of blood, which itself, affect the weight gain and milk production of dairy animals (Graf et al. 2004). Secondly, the impact of tick borne diseases is immense on livestock health (de la Fuente and Kocan 2003). Amongst the different species of ticks, *Rhipicephalus* (*Boophilus*) *microplus* is a major one affecting livestock health, as a debilitating agent itself and as a vector of babesiosis and anaplasmosis (Sonenshine 1993). On global basis the losses incurred by livestock industry due to ticks and tick-borne diseases (TTBDs) was estimated in the range of 14,000–18,000 million US$/year (De Castro 1997). In India, the annual cost of control of TTBDs of cattle has been estimated as US$498.7 million (Minjauw and Mc Leod 2003).

The TTBDs control program is solely focused on repeated use of harmful chemicals which is not eco-friendly and pose serious threat to environment. There is also increasing evidence that present strategies based on acaricides are not cost effective (Pegram et al. 1993; Ostfeld et al. 2006). Regardless of the demonstrated success of acaricides, societal and scientific concerns over the exclusive dependency upon chemicals have emphasized the need for the development and introduction of alternatives to acaricides that are consistent with the principles of sustainable TTBDs control (Willadsen 2006; Madzimure et al. 2011).

Recent development of a generic approach for the control of ticks is the use of the anti-tick vaccine, which is designed to protect host animals from tick infestations (Ghosh et al. 2007; Kumar et al. 2012). Vaccine-controlled field trials in combination with acaricide treatments demonstrated that an integrated approach resulted in control of tick infestations while reducing the use of acaricides (de la Fuente and Kocan 2003; de la Fuente et al. 2007). These trials demonstrated that control of ticks by vaccination has the advantages of being cost-effective, reducing environmental contamination, and preventing the selection of drug resistant ticks that result from repeated acaricide application. In addition, these vaccines may also prevent or reduce

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transmission of pathogens by reducing tick populations and/or affecting vectorial capacity (de la Fuente et al. 2007; Rodriguez et al. Rodriguez Valle et al. 2004).

Though, the large number of antigens were investigated for their effects against ticks, the only antigen i.e., Bm86 has been transformed into the commercial vaccine. The Bm86 molecule derived from the *R.* (*B.*) *microplus* showed variable efficacy against different strains of the homologous and a few heterologous species (Garcia–Garcia et al. 1999; Sossai et al. 2005). There is an urgent need of identification of conserved vital tick molecules which may be exploited for the development of vaccine against multiple tick species. The vital organ and its secretory and regulatory molecules may be targeted for the development of eco-friendly sustainable cross protective anti-tick vaccine. Recent data indicated that the host immunoglobulin can cross the gut of the ticks to reach to hemolymph to cells (Vaughan et al. 2002; de la Fuente et al. 2011). The finding has increased the possibilities of targeting both intracellular and secretory molecules for vaccine development.

Since copulation of female *Ixodid* ticks is mandatory in order to achieve full engorgement and production of fertilized eggs, mating-related molecules are important in the tick feeding and reproductive behaviour (Kaufman 2007). Weiss and Kaufman (2004) cloned male testis/vas deferens-derived '*voraxin*' from *Amblyomma hebraeum* and first showed that injection of recombinant voraxin into virgin females stimulates blood feeding to engorge. In addition, mean tick weight was significantly reduced and 75 % of the mated female ticks tested did not engorge at all on immunized animals. Recently, Yamada et al. (2009) cloned and characterized complete cds of *voraxin*-α homologue of *R. appendiculatus* and following immunization 40 % reduction in engorgement and 50 % reduction in egg weight upon homologous challenge infestations was recorded. These results suggest the feasibility of voraxin-α as an useful anti-tick antigen. Until now, there is no information on voraxin-α homologues in *R.* (*B.*) *microplus*. In this study, we report the cloning of *voraxin*-α homologue gene of *R.* (*B.*) *microplus* and analysis of genetic similarity or dissimilarity with other *voraxin*-α homologue from different tick species. This in silico study will help in decision making process to consider this molecule as broad-spectrum anti-tick vaccine candidate before going for more expensive and complicated vaccination trial.

Materials and methods

Ticks

Rhipicephalus (*Boophilus*) *microplus* ticks (IVRI line I, registration number: NBAII/BM/1/1998) was used for the study. The reference tick line was maintained as reported by Ghosh and Azhahianambi (2007). Briefly, the ticks were fed on healthy calves of 4–6 months old which were maintained as per the guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), a statutory Indian body monitoring the use of animals in experimentation. Usually 2–3 calves were used for each feeding cycle. After each feeding cycle, the animals were kept free for 15–20 days. Fully engorged adults were kept in desiccators where 85–90 % relative humidity (RH) was maintained. The desiccators were kept at 28 °C for oviposition. After completion of oviposition, the dead female ticks were removed from the glass tubes in order to avoid the fungal growth. The freshly laid eggs were kept at 28 °C with 85–90 % RH for maturation and hatching to larvae. The homogeneity in the reference lines was periodically checked after cloning and sequencing of its 16S rRNA gene (Accession Nos. GU222462, GU323287, GU323288).

RNA extraction, cDNA synthesis and cloning of *voraxin*-α gene

Four days old partially fed male and female ticks were used for the isolation of total RNA using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to manufacture's protocol. Reverse transcription was performed using the first strand cDNA synthesis kit (Fermentas, USA).

For cloning of the gene, different sets of primers were designed from the conserved regions of *voraxin*-α homologue of *Amblyomma hebraeum* (AB488697), *Rhipicephalus appendiculatus* (AY442319), *Dermacentor silvarum* (HQ908089) and *Haemaphysalis longicornis* (HQ908090). Different permutations and combinations were attempted to optimize the reagents and PCR condition for amplification of *voraxin*-α homologue of *R.* (*B.*) *microplus*. PCR fragments, which are within the expected size for the *voraxin*-α, were purified and routinely cloned into TA-cloning vector (Ins TAclone™ PCR Cloning Kit, Fermentas, USA) and colony PCR positive clone was sequenced.

Sequence analysis

Nucleotide sequence identity analysis was performed using the BLAST program (NCBI). All nucleotides [open reading frame (orf) only] and its deduced amino acid sequences from the different species of ticks were analyzed using the program MegAlign (DNAstar, USA). The deduced amino acid sequences were used to seek for orthologs in the non-redundant GenBank protein sequence database by BLASTP analysis. The generated sequence of *R.* (*B.*) *microplus* and retrieved sequences of other hard ticks [*A. hebraeum* (AB488697), *R. appendiculatus* (AY442319), *D. silvarum* (HQ908089) and *H. longicornis* (HQ908090),

parentheses contain GenBank accession number] were aligned using ClustalW, neighbour-joining analysis was performed using the Mega4 package (Tamura et al. 2007). Gaps were treated as pair wise deletions, amino acid distances were calculated using Poisson model and branch supports were estimated using bootstraps analysis (10,000 bootstraps).

Results

Amplification of *voraxin-α* gene of *Rhipicephalus* (*Boophilus*) *microplus*

The *voraxin-α* gene was amplified using the primer set (forward primer: 5′ACGTCACCCATGTTGATC3′ and reverse primer: 5′GATGGCAGTCCCTTGCG3′) without any non-specific reaction using the optimized PCR condition: initial denaturation of 95 °C for 2 min and 32 cycle of 94 °C for 40 s, 48 °C for 30 s and 72 °C for 30 s and final extension at 72 °C for 15 min. The partial cds (incomplete 3′ ends) of the gene of *R.* (*B.*) *microplus* was amplified as a 419 bp product (Fig. 1A). The GenBank accession numbers of the cloned gene is JX502818. The nucleotide sequence was 419 in length, including nine nucleotides upstream of the initial ATG codon and the 3′ end of the gene was incomplete (Fig. 1B).

Sequence analysis

BLASTP analysis of the submitted *voraxin-α* sequence using non-redundant GenBank protein database retrieved the same protein. Alignments of deduce amino acid sequences of *voraxin-α* gene from different hard ticks were shown in Fig. 1C. The variation in amino acid sequences among the tick species which is highlighted (black shadow) and the percentage identity amongst ticks were in the range of 17.6–90.4 % (Table 1). The per cent identities of nucleotide sequence of *voraxin-α* gene of *R.* (*B.*) *microplus* were 90.2, 42.7, 39.5 and 42.9 % against *R. appendiculatus, D. silvarum, H. longicornius* and *A. hebraeum*, respectively (Table 1).

Phylogenetic analysis of the amino acid sequences is shown in Fig. 1D. On the basis of amino acid composition of *voraxin-α*, the *R.* (*B.*) *microplus* and *R. appendiculatus* comes within same clade which once again confirm the *Rhipicephalus* species were paraphyletic with respect to the species of *Boophilus*.

Discussion

The development of new methods to control tick infestations and reduce the incidence of tick borne diseases while minimizing acaricide applications is essential towards improving cattle health and production in tropical and subtropical regions of the world. Development of vaccine against tick species with broad spectrum activity require the discovery and characterization of new tick protective antigens, a process that constitutes the limiting steps for advancing this area of research (Canales et al. 2009). Anti-tick vaccines have been developed based on the molecular cloning, characterization and analysis of tick molecules that play an important role in tick physiology. Weiss and Kaufman (2004) reported that the transfer of voraxin-α and voraxin-β via copulation is essential for female engorgement and oviposition in *A. hebraeum*. He also speculated that male factor (MF) from male gonad of *A. hebaerum*, which can hasten the onset of salivary gland degeneration (Lomas and Kaufman 1992) and engorgement factor (EF) from *D. variabilis*, which are produced by the male and promotes female feeding to repletion (Pappas and Oliver 1972) are the same substance because both factors are found in testis of fed males. Fortunately, the recombinant voraxin-α showed both MF and EF activity. Yamada et al. (2009) cloned and characterized the complete cds of *voraxin-α* homologue of *R. appendiculatus* and reported that voraxin-α is not only expressed in male gonad but also ubiquitously expressed in virgin and mated females and engorging nymphs. But the level of expression of voraxin-α in nymphs and in females were very-very low (i.e. 1/1,000 or less per actin) compared to those in male testis. In the present study we amplified for the first time the *voraxin-α* gene from both male and females of *R.* (*B.*) *microplus.*

Analysis of nucleotide sequence (orf only) and the deduced amino acid sequence of the cloned *voraxin-α* cDNA showed high level of conservation compared to the *voraxin-α* sequence from *R. appendiculatus* whereas, the variation among the different other ticks were moderate to high. The predicted amino acid similarities give the hypothesis that voraxin-α is widely conserved in different tick genera. Moreover, amplification of *voraxin-α* from female tick RNA signaled towards possible role of voraxin-α in female tick physiology (Yamada et al. 2009).

The voraxin-α, which is classified as concealed antigen elicit protective anti-tick immune response in rabbit when immunized with recombinant voraxin-α. Immunization trial showed 40 % reduction in female tick weight and 50 % reduction in egg weight upon homologous challenge infestation of *R. appendiculatus* in rabbit (Yamada et al. 2009). Very high level of similarity (94.6 %) in amino acid sequence and sharing of common clade in phylogenetic analysis for voraxin-α homologue of *R. appendiculatus* and *R.* (*B.*) *microplus* creates a possibility of development of cross-protective vaccine against these two species. This preliminary work suggest that recombinant voraxin-α might be a good candidate as cross-protective anti-tick vaccine.

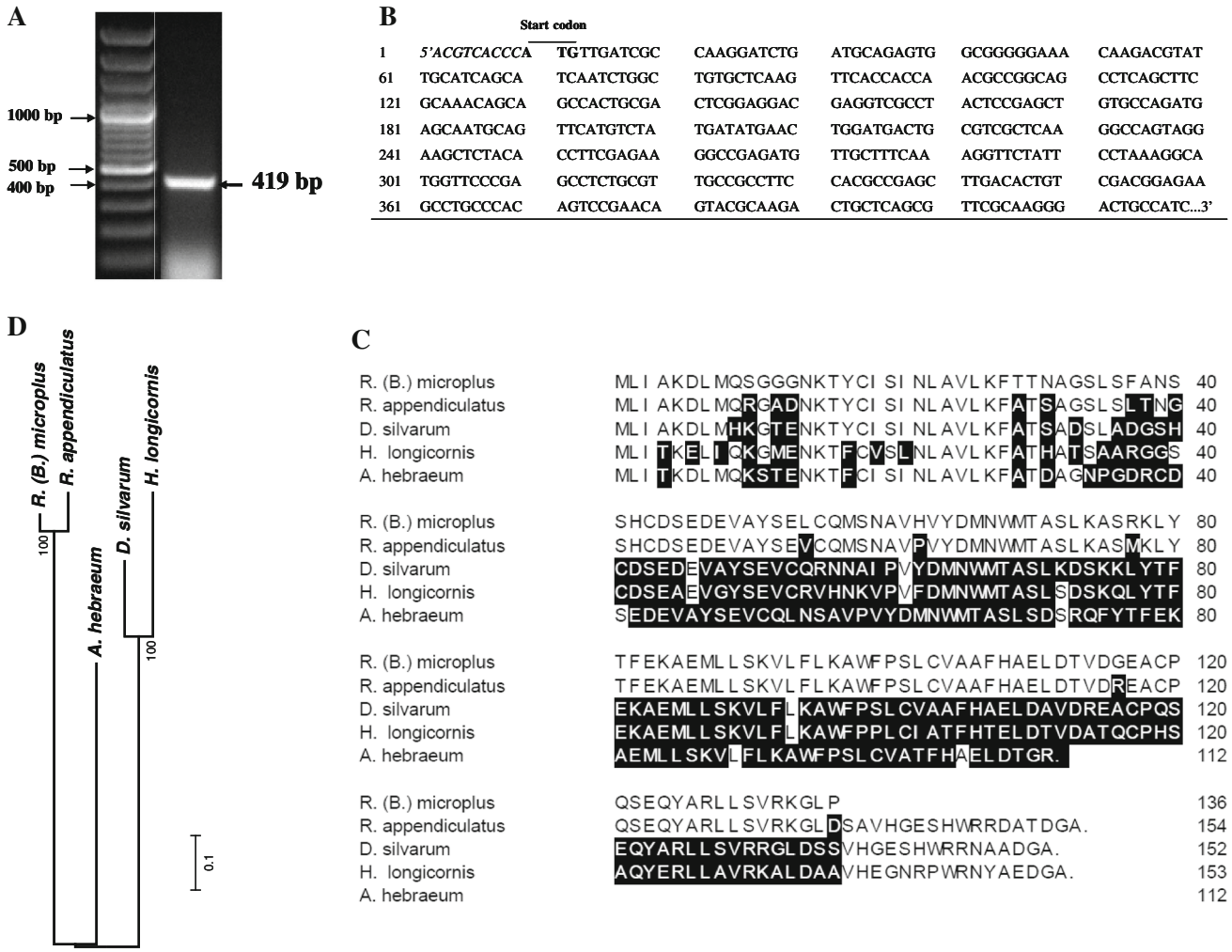

Fig. 1 Amplification and molecular analysis of *voraxin-α* gene. **A** and **B**: PCR amplification and nucleotide sequence of *voraxin-α* homologue of *R.* (*B.*) *microplus* (419 bp), respectively. DNA ladder: 100 bp plus DNA ladder (MBI, Fermentas, USA). **C** and **D**: Sequence alignment and neighbour-joining analysis of deduce amino acid sequences of *voraxin-α* (orf only) gene of different species of hard ticks, respectively. The amino acid residues that differ from *R.* (*B.*) *microplus* are highlighted by *black solid shade*. The evolutionary distances were computed using the Poisson correction method. Branch value indicated in 0.1 point scale

Table 1 The percentage identity of nucleotide (bold letter) and amino acid (italics letter) sequence of voraxin-α homologue (orf only) of different hard ticks

	R. (*B.*) *microplus*	*R. appendiculatus*	*D. silvarum*	*H. longicornis*	*A. hebraeum*
R. (*B.*) *microplus*	–	**90.2**	**42.7**	**39.5**	**42.9**
R. appendiculatus	*90.4*	–	**41.2**	**38.6**	**43.5**
D. silvarum	*22.8*	*21.7*	–	**71.7**	**44.6**
H. longicornis	*19.9*	*17.6*	*66.4*	–	**40.8**
A. hebraeum	*25.9*	*26.8*	*27.7*	*26.8*	–

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Conflict of interest We declare that we have no conflict of interest.

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