Ticks and Tick-borne Diseases 8 (2017) 391–395

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Ixodes ricinus immunogenic saliva protein, homologue to *Amblyomma americanum* AV422: Determining its potential for use in tick bite confirmation

Darko Mihaljica[a,*], Dragana Marković[b], Željko Radulović[a,c], Albert Mulenga[c], Sanja Ćakić[a], Ratko Sukara[a], Jelena Samardžić[d], Snežana Tomanović[a]

[a] University of Belgrade, Institute for Medical Research, Centre of Excellence for Food and Vector-Borne Zoonoses, Laboratory for Medical Entomology, Dr Subotića 4, PO Box 102, 11129 Belgrade, Serbia
[b] University of Belgrade, Institute for Medical Research, Department of Immunology, Dr Subotića 4, PO Box 102, 11129 Belgrade, Serbia
[c] Texas A&M University, College of Veterinary Medicine, Department of Veterinary Pathobiology, USA
[d] University of Belgrade, Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a, 11010 Belgrade, Serbia

ARTICLE INFO

Article history:
Received 13 October 2016
Received in revised form
30 December 2016
Accepted 4 January 2017
Available online 10 January 2017

Keywords:
Tick bite markers
Salivary proteins
Exposure to ticks
Ixodes ricinus

ABSTRACT

Tick bites often go unnoticed, so specific reliable tests are needed to confirm them for prompt diagnosis and treatment of tick-borne diseases. One of the promising candidates for developing such a test is AV422, a tick saliva protein that has been conserved across tick genera. In this study, we demonstrate the potential of the AV422 homologue from *Ixodes ricinus* to be used for tick bite detection for both Prostriata and Metastriata. We expressed recombinant (r) *I. ricinus* (*Ir*) AV422 in *E. coli* and subjected it to Western blot analysis using rat antibodies to saliva proteins of both *I. ricinus* (Prostriata) and *Dermacentor reticulatus* (Metastriata) larvae. Our data demonstrate that r*Ir*AV422 specifically bound to antibodies from sera of rats used for both *I. ricinus* and *D. reticulatus* larvae feeding, but not to antibodies from control serum, emphasizing its specificity since tick bites were the sole cause of sera reactivity.

1. Introduction

Ticks are obligate hematophagous ectoparasites of amphibians, reptiles, birds and mammals and globally distributed from the tropics to the polar regions. Estrada-Peña and Jongejan (1999) listed 12 argasid and more than 20 ixodid tick species that are often found feeding on humans. In addition to the direct pathogenic effects that they can cause during the feeding process, ticks are medically more important as vectors of pathogens. With regards to the diversity of pathogens they can transmit, ticks stand out more than any other hematophagous arthropods (Dennis and Piesman, 2005). Approximately 900 tick species have been described (Guglielmone et al., 2010), of which nearly 10% have an impact on human and animal health (Jongejan and Uilenberg, 2004).

Ixodes ricinus is the most common tick species parasitizing humans throughout Europe (Bartosik et al., 2011; Briciu et al., 2011; Jameson and Medlock, 2011; Otranto et al., 2014), which presents a significant risk for transmission of tick-borne diseases (TBD). It is a known vector of various human pathogens, such as tick-borne encephalitis virus (Hubalek and Rudolf, 2012), *Borrelia burgdorferi* sensu lato complex (Rizzoli et al., 2011; Vu Hai et al., 2014), *Anaplasma phagocytophilum* (Parola, 2004), causative agents of human rickettsiosis (Medlock et al., 2013) and *Babesia divergens* (Hildebrandt et al., 2013; Vu Hai et al., 2014).

The majority of TBD has nonspecific clinical manifestations, similar to the common flu and can easily be misdiagnosed, especially in cases where a record of the tick bite is missing (Briant and Marshall, 2000). Additionally, the number of co-infections, when two or more pathogens are present in a single tick, have been recorded frequently (Belongia, 2002; Milutinović et al., 2008; Stańczak et al., 2004; Swanson et al., 2006; Tomanović et al., 2013). The bite of

* Corresponding author.
E-mail addresses: darko.mihaljica@imi.bg.ac.rs, dbmzbgd@yahoo.com (D. Mihaljica), draganac@imi.bg.ac.rs (D. Marković), zradulovic@cvm.tamu.edu (Ž. Radulović), amulenga@cvm.tamu.edu (A. Mulenga), sanja.cakic@imi.bg.ac.rs (S. Ćakić), ratko.sukara@imi.bg.ac.rs (R. Sukara), jelena.samardzic@imgge.bg.ac.rs (J. Samardžić), snezanat@imi.bg.ac.rs (S. Tomanović).

http://dx.doi.org/10.1016/j.ttbdis.2017.01.001
1877-959X/

such a tick could potentially transfer several tick-borne pathogens, which additionally may complicate the clinical picture of infected patients. Consequently, confirmed tick attachment is important information in the diagnosis of TBD. One of the reasons why tick bites often remain unnoticed is that components of tick saliva have anesthetic properties (Francischetti et al., 2009). Additionally, the predominant developmental stage of *I. ricinus* found to feed on humans are nymphs (Wilhelmsson et al., 2013), which are difficult to detect due to their small size. Thus, for patients suspicious of having TBD without a record of the tick bite, it is necessary to develop a diagnostic test as a tick bite history check. In addition to its use in diagnostics of human TBD, these tests could help in veterinary medicine in a similar manner, as well as in epidemiological studies of TBD to check the exposure of different possible hosts to tick bites and assess the tick control measures undertaken.

Detection of specific antibodies against tick saliva proteins (TSP) in sera of suspected hosts is an objective marker of tick bite exposure (Schwartz et al., 1991). One of the proteins studied for this purpose in humans is calreticulin (Alarcon-Chaidez et al., 2006). However, there are concerns about the specificity of antibodies to tick calreticulin, due to possible cross-reactions in individuals with endoparasitic infections (Jaworski et al., 1996; Kim et al., 2015; Xu et al., 2005). An alternative approach involves the use of proteins specific for ticks which are not present in other organisms. Tick saliva AV422 protein was previously described in *A. americanum* species (Mulenga et al., 2013b). The gene for AV422 protein was found to be upregulated by feeding of the tick (Mulenga et al., 2007), and a following study confirmed that *A. americanum* females start to inject this protein into hosts within 24 h after attachment (Mulenga et al., 2013b). Bioinformatics analysis revealed that AV422 was conserved across tick species, with no mammalian or other arthropod homologues, which makes it a unique tick saliva protein (Mulenga et al., 2013b, 2007). Recently, AV422 and its homologues were reported in saliva proteomes of *A. americanum* and *I. scapularis* (Kim et al., 2016a; Lewis et al., 2015; Radulović et al., 2014), *Rhipicephalus microplus* (Tirloni et al., 2014) and *Haemaphysalis longicornis* (Tirloni et al., 2015). Therefore, the aim of this study was to investigate the *I. ricinus* (*Ir*) homologue of tick specific AV422 saliva protein in the context of developing a universal tick bite confirmation test, using recombinant (r) *Ir*AV422.

2. Materials and methods

2.1. Cloning of the IrAV422 coding sequence and expression of rIrAV422

Aligned nucleotide sequences of 17 AV422 homologues from different tick species of genera *Ixodes, Rhipicephalus* and *Amblyomma* (XM002406216, GADI01002126, GACK01004441, BK007204, JO842177, XM002410237, GADI01001646, JO844573, BK007127, XM002414928, XM002401121, XM002414494, XM002414488, XM002414490, XM002414491, JO844187, and XM002414929) were used to design primers (5′-ggatccCAGGCTTGCCACCTTCG-3′ and 5′-gaattcTTAGTTGCCCAGGTAGGCGGAG-3′) for the amplification of the *Ir*AV422 coding sequence. As a template for amplification we used *I. ricinus* cDNA synthesized by RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Vilnius, Lithuania) from total RNA extracted using TRIzol Reagent (Ambion, USA) according to the manufacturers' protocol from unfed *I. ricinus* females collected from vegetation in the vicinity of Belgrade, Serbia. Expression of r*Ir*AV422 saliva protein was performed following standard protocols and manufacturers' instructions, using BL21(DE3)pLysS *E. coli* cells (Invitrogen, Carlsbad, USA) transformed with pRSET A expression vector (Invitrogen, Carlsbad, USA), that contained the obtained *Ir*AV422 coding sequence. As a feature of expression using pRSET A, the obtained protein product contained the N-terminal 6xHis tag. Transformed cells were grown in super optimal broth (SOB) and after routine lysis of bacteria, r*Ir*AV422 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to the PVDF (polyvinylidene difluoride) membrane (Thermo Fisher Scientific, Rockford, USA), and eluted from it as previously described (Kim et al., 2016b). Specific reactivity was checked by Western blot analysis using mouse Anti-HisG-HRP antibody (Invitrogen, Carlsbad, USA) against the 6xHis tag. The band of purified r*Ir*AV422 was detected using a chromogenic Metal Enhanced DAB substrate kit (Thermo Fisher Scientific, Rockford, USA).

2.2. Generating antibodies to larval tick saliva proteins

To obtain tick larvae for feeding on rats to generate antibodies to tick saliva proteins, engorged *I. ricinus* and *D. reticulatus* females were collected from domesticated animals, and maintained (at a tick rearing facility of Institute for Medical Research, Belgrade, Serbia) under controlled conditions at 22 °C in 97% relative humidity with 16:8 h light-dark cycle until eggs were laid. Eggs were separated from the female and kept at the same conditions until larvae hatched. Prior to use in the experiment, larvae were kept at controlled conditions for at least 15 days to mature and prepare for feeding.

Repeated larval tick infestation of one-month old male Wistar rats (bred at the Institute for Medical Research, Belgrade, Serbia) were performed, in order to generate two types of antibodies to *I. ricinus* and to *D. reticulatus* larval tick saliva proteins, as previously described by Mulenga et al. (2013a). Larvae were placed on the head, mostly on the ears, and were allowed to feed to repletion. Four rounds of infestation with larvae (ca. 50 larvae per round per rat) were performed, resulting in continuous larvae feeding on rats for 33 days on average. Two weeks after the last larvae infestation rat blood was collected from the abdominal aorta under sodium pentobarbital (35 mg/kg intraperitoneally (i.p.)) anesthesia. The blood was allowed to clot and after centrifugation sera were stored at −80 °C until use. For collection of control serum, rats which were not exposed to tick larvae were used. The experimental protocol was approved by the Ethic Committee of the Institute for Medical Research, University of Belgrade, Serbia and Veterinary Directorate, Ministry of Agriculture and Environmental Protection, Republic of Serbia (No 323-07-04717/2015-05). The protocol was in accordance with the National Law on Animal Welfare ("Sl.gl.RS" No6/10) which is consistent with guidelines for animal research and principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes (Official Daily N. L 358/1-358/6, 18. December 1986) and the Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU of the European Parliament and of the Council, 22 September 2010).

Validation of produced antibodies was performed by checking rat sera reactivity with larval tick proteins. Spontaneously detached engorged *I. ricinus* larvae were collected, pooled, and homogenized in TRIzol Reagent, followed by total protein extraction according to the manufacturers' instructions. For the serum reactivity check, protein extracts were subjected to SDS-PAGE, transferred to nitrocellulose membrane (Amersham Bioscience, UK) and immunoblotted with rat sera, followed by horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (Thermo Fisher Scientific, Rockford, USA) as a secondary antibody. Reactivity was