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IgM-antibody responses of chickens to salivary antigens of *Triatoma infestans* as early biomarkers for low-level infestation of triatomines

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ABSTRACT

The recombinant form of a highly immunogenic 14.6 kDa protein in *Triatoma infestans* saliva (r*Ti*SP14.6) is a potential epidemiological marker for the detection of triatomine bug populations using IgG responses in peridomestic chickens. However, the persistence of the IgG response prevents it being of value for several months in areas where triatomine control programmes have been implemented. In this investigation, IgM-antibody reactions to crude salivary antigens or r*Ti*SP14.6 decayed rapidly after exposure of chickens and were measurable for only 18 days after a single challenge with *T. infestans*. In serial exposure experiments, chickens from low and high exposure groups showed no significant differences in anti-saliva and anti-r*Ti*SP14.6 IgM-antibody titres. Highly immunogenic salivary antigens of 12 and 14 kDa were recognised by all chicken sera. Sera from peridomestic chickens from sites of known *T. infestans* infestation in Bolivia also recognised these two antigens and no differences in the IgM responses of sera from chickens from low and high infestation households were detected. IgM responses were specific to infested households and could not be detected in sera from non-infested households. Cross-reactivity studies showed that at least four other triatomine species share the 14.6 kDa salivary antigen. No IgM responses were detected against salivary proteins of mosquitoes and sandflies. Thus, we believe that r*Ti*SP14.6 represents a promising epidemiological marker for the detection of low numbers of triatomines in peridomestic habitats, and the comparison of IgM and IgG responses can be used to detect re-infestation soon after insecticide-based control programmes.

1. Introduction

Current methods to assess the prevalence and intensity of triatomine bug infestations in domestic and peridomestic sites involve timed manual collections using an irritant spray or artificial shelter units (Garcia-Zapata and Marsden, 1993; Gürtler et al., 2001). These methods are costly, require skilled staff, and usually lack the sensitivity and precision necessary for detection of low-density populations especially after insecticide spraying in vector control programmes. Additionally, current methods are too expensive for large-scale surveillance campaigns and are not easily adaptable to many peridomestic settings (Rabinovich et al., 1995; Gürtler et al., 2001; Vazquez-Prokopec et al., 2002). Thus, new methodologies are required to detect residual or re-emerging *Triatoma infestans* populations and for long-term monitoring of previously endemic regions for Chagas disease (Dias, 2007).

Previous investigations have demonstrated the usefulness of IgG antibody responses in chickens and guinea pigs to salivary proteins of *T. infestans*, the most effective vector of Chagas disease, to detect low-level infestations of triatomines (Schwarz et al., 2009a). We identified and synthesised a highly immunogenic recombinant salivary protein of *T. infestans* (r*Ti*SP14.6) which was very effective in detecting differences in household-infestation levels of *T. infestans* in Bolivia using chicken sera from these habitats (Schwarz et al., 2009b). IgG antibodies against salivary antigens of other haematophagous insects did not cross-react with r*Ti*SP14.6, but this recombinant protein reacted with those of four species of triatomines, and thus appears to be triatomine-specific (Schwarz et al., 2009b). Therefore, r*Ti*SP14.6 is a promising epidemiological marker for detecting the presence of small numbers of different species of triatomines and the development of immune-based Chagas disease

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risk surveillance. IgG antibodies to saliva and r*Ti*SP14.6 remained detectable in chickens up to 5 months after triatomine exposure, thus limiting their value for the detection and monitoring of re-infestation shortly after control measures (Schwarz et al., 2009a).

In addition to IgG, two further immunoglobulin classes, IgA and IgM occur in chickens (Sharma, 1999; Ratcliffe, 2006). Whereas IgG is generated as the predominant secondary antibody response, IgM is produced as the first antibody isotype in the primary antibody response, and it is the major immunoglobulin class expressed on the surface of B lymphocytes of chickens (Leslie and Clem, 1969; Ratcliffe, 2006). IgM can be used as an indicator of recent exposure to foreign macromolecules from infectious agents and to vector salivary proteins (Orlandi-Pradines et al., 2007). Whilst IgM responses to pathogens have been studied extensively (e.g. Garraud et al., 2003; Charrel and de Lamballerie, 2004; Sambri et al., 2004; Barbazan et al., 2009), only a few studies have examined IgM responses to salivary proteins of haematophagous arthropods (Wikel, 1985; Cross et al., 1993; Astigarraga et al., 1997; Das et al., 2000; Montero-Solis et al., 2004; Nebreda Mayoral et al., 2004; Waitayakul et al., 2006; Orlandi-Pradines et al., 2007; Menten-Dedoyart et al., 2008). For example, IgM responses to tick salivary proteins enabled identification of the main vectors of spotted fever in a region of Spain (Nebreda Mayoral et al., 2004) and genus-specific IgM and IgG responses to salivary proteins of *Anopheles dirus* and *Aedes aegypti* have been identified (Waitayakul et al., 2006). The short persistence of IgM responses have been demonstrated in sera from travellers who had no detectable IgM antibodies to *Anopheles gambiae* salivary gland proteins 3 months after leaving endemic areas of these mosquitoes in Africa, however specific IgG antibodies were still present (Orlandi-Pradines et al., 2007).

In the present study, we describe the IgM-antibody responses of chickens to saliva of *T. infestans* using both experimental challenge sera and field samples from Bolivia, and evaluate r*Ti*SP14.6 as an exposure marker for future use in epidemiological surveys.

2. Materials and methods

2.1. Antigens

All experiments were performed either with pooled saliva from *T. infestans* or with r*Ti*SP14.6, a recombinant form of the 14.6 kDa salivary protein of *T. infestans* (GenBank Accession No. ABR27885). *T. infestans* originated from a domestic population from Northern Chile, the Cachiyuyo village (29°1′48.90″S, 70°53′55.53″W, 808 m), at the border of the provinces Atacama and Coquimbo (Schaub, 1989; Kollien and Schaub, 1998). Typically volumes of 0.5–1 μl saliva/triatomine were obtained from approximately 300 fifth instars and adults using capillary pipettes (Amino et al., 2001). The protein concentration was determined using a BCA Protein Assay Kit (Perbio Science, Germany) according to the manufacturer's instructions. Aliquots of saliva, containing 30 μg protein/μl, were stored at −80 °C. The 14.6 kDa salivary protein of *T. infestans* was produced as recombinant protein (r*Ti*SP14.6) in a mammalian cell expression system and purified as previously described (Schwarz et al., 2009b)..

2.2. Immune sera

Sera from chickens exposed only once to five starved adult *T. infestans* and bled daily for 5 days were used to determine the earliest IgM response to salivary proteins of triatomines (Schwarz et al., 2009a). To determine the duration of the IgM-antibody response in chickens to triatomine bites, three chickens were exposed to five starved adult *T. infestans* for 1 h. Animals were bled every 2 days until no IgM was detectable by ELISA (see Section 2.4). Blood (0.5 ml) was taken from the brachial vein and centrifuged at 10,000*g* for 10 min at room temperature. Sera were stored at −20 °C. Prior to the first triatomine feeding, pre-exposure serum was taken from each animal as a negative control.

Sera from chickens exposed regularly to a low or high number of *T. infestans* for a period of 24 weeks in a previous study and from the subsequent post-challenge period were used (Schwarz et al., 2009a). To measure cross-reacting serum IgM responses, chickens were challenged with the triatomines *Triatoma brasiliensis, Triatoma sordida, Rhodnius prolixus* and *Panstrongylus megistus*; and the mosquitoes *Anopheles freeborni, Ae. aegypti* and *Culex quinquefasciatus* as described by Schwarz et al. (2009a). Additionally, sera from mice exposed to *Lutzomyia longipalpis* was obtained as described by Schwarz et al. (2009b). *Aedes aegypti* occurs in Bolivia (van der Stuyft et al., 1999), whilst the other insects are representative members from genera of vectors found in Bolivia.

Pre-exposure chicken sera were collected as negative controls in this study. For the positive controls, sera were pooled from chickens which had been used for routine maintenance of triatomines for at least 6 months.

From September to November 2007, *T. infestans* were collected at peridomestic sampling sites in Bolivia. Animals from these sites were classified into three exposure groups according to the number of bugs collected by a survey team of three to five persons in a period of 30–60 min. The groups were no exposure (no bugs), low exposure (1–12 bugs) or high exposure ($\geqslant$100 bugs). Blood samples from 61 chickens were collected from the following 12 rural villages in the Department of Cochabamba: Sipe Sipe (17°27′2.78″S, 66°21′38.91″W, 2555 m), Tamborada (17°26′22.249″S, 66°9′18.772″W, 2565 m), Lipez (17°33′47.12″S, 66°15′27.643″W, 2542 m), Llallagua (17°47′57.122′S, 65°15′43.997″W, 2688 m), Chajra Corral (18°1′18.30″S, 64°55′25.157″W, 1796 m), Molle Molle (18°3′7.152″S, 64°54′46.459″W, 1729 m), Chojtama (17°32′47.178″S, 66°16′2.841″W, 2558 m), Pampas (18°3′26.812″S, 64°54′35.01″W, 1708 m), Peña Colorada (18°10′5.288″S, 64°52′0.309″W, 1583 m), Chulla (17°22′27.275″S, 66°18′19.558″W, 2576 m), Cala Caja (17°33′27.941″S, 66°8′36.118″W, 2516 m) and Arpita (17°33′51.62″S, 66°4′15.049″W, 718 m). The blood was processed as described above.

All animal procedures at the Ruhr-Universität Bochum were licensed by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany. In Bolivia the blood was taken by veterinarians of the Faculty of Veterinary Medicine, Universidad Mayor de San Simón, Cochabamba, Bolivia.

2.3. SDS–PAGE and Western blotting

Salivary proteins of *T. infestans* (70 μg protein) were separated by 15% SDS–PAGE under reducing conditions. The proteins were transferred onto nitrocellulose membrane and processed as previously described (Schwarz et al., 2009a). Western blots were either incubated with pooled sera from chickens collected after the last exposure to a low or high number of triatomines in the long-term study (as described in Section 2.2), or with individual chicken serum samples from Bolivia (dilutions 1:10 in PBST [PBS/0.1% Tween 20]/5% dried skimmed milk). Peroxidase-conjugated goat anti-chicken IgM (GeneTex, Inc., USA) diluted 1:500 in PBST/5% dried skimmed milk was used as a secondary antibody.

2.4. ELISAs

Concentrations of IgM in chicken sera were measured by ELISA using either 0.5 μg *T. infestans* total salivary protein or 0.5 μg r*Ti*SP14.6 coated per well as previously described (Schwarz et al., 2009a,b). The plates were either incubated with (i) individual serum samples from chickens exposed once to *T. infestans* and bled daily for 5 days, (ii) individual samples from chickens exposed once to *T. infestans* collected every 2 days until no IgM response was measured, (iii) pooled sera of the last exposure from chickens exposed to a