FEMALE TICK *HYALOMMA MARGINATUM MARGINATUM* SALIVARY GLANDS: PRELIMINARY STUDY ON PROTEIN CHANGES DURING FEEDING PROCESS AND ANTIGENS RECOGNIZED BY REPEATEDLY INFESTED CATTLE

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Summary :

Proteins extracted from salivary glands of unfed, three days and five days fed adult *Hyalomma marginatum marginatum* were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We have noticed changes during the three feeding steps. Some proteins disappeared during feeding process (23,38,39,40 to 50, 95 and 112 kDa), they might be proteins which were converted in other substances and are secreted. Other antigens (13 to 14, 20, 25, 29, 165 and 210 kDa) were synthesized as a result of tick attachment and feeding. They may be related to growth and development or are the ciment which fixed the adult. Also, three Holstein calves were infested five times with 100 pairs of adult ticks of the same species. The five infestations were performed two weeks from the previous infestation. The sera before infestations and after each infestation were used in western-blot analyses to identify antigens from five days salivary gland extracts of the primary infestation of ticks. Three antigens (18.7, 50 and 80 kDa) were revealed weakly after the first and the second infestations by sera samples but not at infestation onward. Others (13.5, 17 to 18.5, 25, 30, 70, 133, 176 and 193 kDa) were revealed only by sera taken after manifestation of resistance (third infestation). A 13.5 kDa antigen was particularly revealed when resistance had appeared and became more evident after the fourth and fifth infestations. The late antigens recognized might be associated with establishment of calves resistance against ticks.

KEY WORDS : *Hyalomma marginatum marginatum*, tick, salivary glands, antigens, SDS-PAGE, resistance.

Résumé : GLANDES SALIVAIRES DE LA TIQUE FEMELLE *HYALOMMA MARGINATUM MARGINATUM* : ÉTUDE PRÉLIMINAIRE SUR LES MODIFICATIONS PROTÉIQUES LIÉES À L'ENGORGEMENT ET LES ANTIGÈNES RECONNUS PAR LES BOVINS RÉGULIÈREMENT INFESTÉS

Des extraits de glandes salivaires de tiques adultes Hyalomma marginatum marginatum *ont été analysés par SDS-PAGE. Trois stades de tiques ont été utilisés, des tiques à jeun ou des tiques après trois ou cinq jours de gorgement. Certains antigènes (23, 38, 39, 40 à 50, 95 et 112 kDa) disparaissent tôt durant le processus de gorgement. Ces antigènes sont probablement convertis et sécrétés en d'autres substances. D'autres antigènes (13 à 14, 20, 25, 29, 165 et 210 kDa) sont synthétisés après fixation de la tique et le début du gorgement. Ces antigènes doivent êtres liés à la croissance et au développement de la tique ou aux mécanismes de fixations de cette dernière. En outre, trois bovins de race Holstein ont fait l'objet de cinq infestations massives (100 paires de tiques par infestation) à intervalles de 15 jours et cela après gorgement de la dernière tique de l'infestation précédente. L'extrait des glandes salivaires des tiques de la première infestation après cinq jours de gorgement a été analysé par la technique de western-blot en utilisant les sérums des bovins expérimentaux avant et après chaque infestation. Les bovins produisent des anticorps dès la première infestation. Trois antigènes (18,7, 50 et 80 kDa) ont été révélés dès la première et la deuxième infestations, après ils ne sont plus détectés. D'autres antigènes (13,5, de17 à 18,7, 133 et 193 kDa) n'ont été détectés qu'après la manifestation de la résistance (troisième infestation). L'antigène 13,5 kDa est particulièrement révélé dès l'apparition de la résistance et devient plus marqué à la quatrième et cinquième infestations. Les antigènes (25, 30, 70, 176 kDa) n'ont été détectés qu'après la quatrième et cinquième infestations. Ces antigènes tardivement reconnus pourraient être associés à l'établissement de la résistance des bovins vis-à-vis des tiques.*

MOTS CLÉS : Hyalomma marginatum marginatum, *tiques, glandes salivaires, antigènes, gel SDS-PAGE, résistance.*

A vast amount of work remains to be done in the ongoing battle against ticks and tick-borne diseases. The vectors and pathogens can be most effectively studied by interactive, multidisciplinary research teams. The development of acaricide resistance (Nolan, 1990) calls for alternative control strategies for ticks and tick-borne diseases. The most promising of these alternatives, anti-tick vaccines, have advanced from a possibility to a reality in the past ten years (Wikel, 1996). Several studies tried to give a clear picture of the immunology of acquired resistance (Brossard *et al.*, 1991; De Castro & Newson, 1993; Sahibi *et al.*, 1997a,b,c). Information regarding immunogens responsible for specific responses is estimated. Indeed, it is already known that tick salivary glands perform numerous vital functions. They secrete cement which anchors mouth parts to the skin of the host (Kemp *et al.*,1982), and serve as primary osmoregulatory organs by which ions and water are eliminated in the host during feeding process (Kaufman & Sauer, 1982). The salivary components maintain an intimate association between the parasite and the host (Kaufman,

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1989; Ribeiro, 1989; Gordon & Allen, 1991; Ribeiro *et al.*, 1992; Sauer *et al.*, 1995). The pharmacological proprieties of saliva have more than one biologic activity. Salivary gland derived molecules have anti-hemostatic, vasodilatory, anti-inflammatory and immunosuppressive proprieties (Wikel *et al.*, 1994; Wikel, 1996). Tick salivary glands contain apyrase which inhibits platelets aggregation by hydrolyzing adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP) and orthophosphate (Ribeiro, 1987; Titus & Ribeiro,1985). Prostaglandin E_2 (PGE_2), which is produced by tick salivary glands, inhibits platelet aggregation and causes vasodilatation (Champagne, 1994; Ribeiro *et al.*, 1985). Salivary apyrase may prevent aggregation of neutrophils and mast cell degranulation (Ribeiro *et al.*,1990). Salivary gland extracts of *Rhipicephalus appendiculatus* contain a 65 kDa anti-coagulant that inhibits the activity of factor X_a or other components of the prothrombinase complex (Limo *et al.*, 1991). *Ixodes dammini* saliva contains a kininase able to destroy bradykinin (Ribeiro *et al.*, 1988). Salivary glands are the focus of all medical and veterinary studies associated with ticks. It is widely believed that many diseases are caused by organisms inoculated into host body via tick saliva (Sauer, 1977; Ribeiro *et al.*, 1987; Champagne, 1994). Therefore, salivary glands have been a subject of intensive studies, by semi-thin sectioning coupled with histochemistry (Binnington, 1978; Gill & Walker, 1984) and electron microscopy (Krolak *et al.*, 1982; Walker *et al.*, 1985). These studies showed that the structure of ixodidae salivary glands is more complex than it was originally thought. Also, acinus types showed morphologic changes during attachment and feeding, and appeared to synthesize and secrete their products throughout the feeding period (Binnington, 1978; Gill & Walker, 1987; Kaufman, 1989).

In a previous paper (Sahibi *et al.*,1998), it was reported that three calves infested five times with adults *Hyalomma marginatum marginatum* ticks exhibited manifestation of suppression of humoral responses to homologous salivary gland antigens followed by manifestation of immuno-resistance. The calves produced high titer of antibodies against antigen of tick salivary glands five days after the first infestation, but the titers declined thereafter. We observed that feeding and fertility of the ticks were severely inhibited by the fourth infestation, showing evidence of a resistance of calves. However, lower antibody titers were observed. On these observations, we investigate a preliminary study of salivary gland antigens to which calves reacted after each of the five heavy infestations and their relation with the manifestations of a resistance. The protein changes during tick feeding course were also investigated in this paper.

MATERIALS AND METHODS

Ticks

A colony of *H. marginatum marginatum* (Koch, 1844) ticks was maintained at the Institut Agronomique et Vétérinaire Hassan II, Rabat, Morocco, at 85 ± 1 relative humidity and 28°C. Larvae and nymphs were confined to ears of rabbits using cloth-bags. Adult ticks used for salivary gland antigen preparation were fed on sheep using the same method. In all experiments, four- to five-week old adult ticks were used to infest cattle.

Hosts

Three Holstein (males), eight to twelve months old weighing 70-80 kg, were used. The animals were provided from application farm where animals were well-kept. They had a regular treatment against ticks, so spontaneous infestation is not possible. During the experiment, they were kept on a small plot with bare dry ground which prevented spontaneous new tick infestation. The calves were fed artificially with cattle pellets and fresh alfalfa, and provided fresh water ad libitum.

Experiment

Our experiment consisted of infesting calves five times with 100 pairs (100 males and 100 females) of *Hyalomma marginatum marginatum* (Koch, 1844). Ticks were deposited in a cloth sack and confined to both ears of calves. Sacks were examined daily and the number of engorged ticks was recorded until all ticks which engorged had detached. Each female was weighed and incubated in individual container (8/2.5) until the end of the oviposition period, or discarded after 30 days if it failed to oviposit. The weight of the egg mass was also recorded. The five infestations were done at two-week intervals, after the last tick from the previous infestation had detached. The results of these part were published by Sahibi *et al.* (1998). In this report we studied the antigens recognized by antibodies in serum samples taken before and after each infestation. We also studied the salivary gland antigens of fed and unfed ticks.

Preparation of antigens

Antigens were extracted from unfed, three and five days fed females of *H. marginatum marginatum* (Koch, 1844) reared under laboratory conditions. The ticks were washed in several solutions of 70 % alcohol and in Phosphate Buffer Saline (PBS). The female ticks were fixed in wax and their salivary glands removed and placed in PBS (10 salivary glands/50 µl).

The samples were sonicated (10 times) at full power, 30 seconds each time, in ice. Homogenates were then centrifuged at 10,000 g at 4°C for 15 min, and supernatants stored at – 20°C. The protein content was determined using Lowry *et al.* (1951) method.

Electrophoresis

Electrophoresis of salivary gland extracts was performed essentially as described by Laemmli (1970). Salivary gland extracts were boiled for 5 min in sample loading buffer Tris-Hcl 0.5 M PH 6.8 with SDS and mercaptoethanol. Ten µg of tick proteins were loaded per gel lane. Extracts were run on 5 % to 20 % acrylamide gradient gel prepared in Tris-Hcl buffer at 1.5 M, pH 8.8. Polypeptides were stained by silver staining.

Western blot

Electrophoretic transfer and immunodetection of tick antigens was performed essentially as described by Tsang *et al.* (1983), using Tris-Buffer Saline [pH 7.5] (TBS), 2 % Tween in TBS as a blocking buffer and Bio-rad horseradish peroxidase (HRPO) color developer containing 4-chloro 1-naphtol. Extracts from salivary glands were run on 5 to 20 % gradient polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane, for one hour at 100 V in electrode solution (Tris 25 mM, glycine 11'2 mM at SDS 0.1 %, pH 8.3). After transfer, nitrocellulose strips were incubated at room temperature for one hour in TBS-Tween 2 %. Membranes were incubated individually in bovine sera diluted at 1/100 in TBS-Tween 0.2 %, for two hours at 37°C. After washing, strips were incubated one hour at 37° C with Rabbit anti-bovine IgG (heavy and light chains specific) conjugated to HRPO (1/2000). After three further washings with TBS-Tween 0.05 %, strips were colored with 4-chloro-1-naphtol and H_2O_2. The strips were rinsed in distilled water when purple bands appeared.

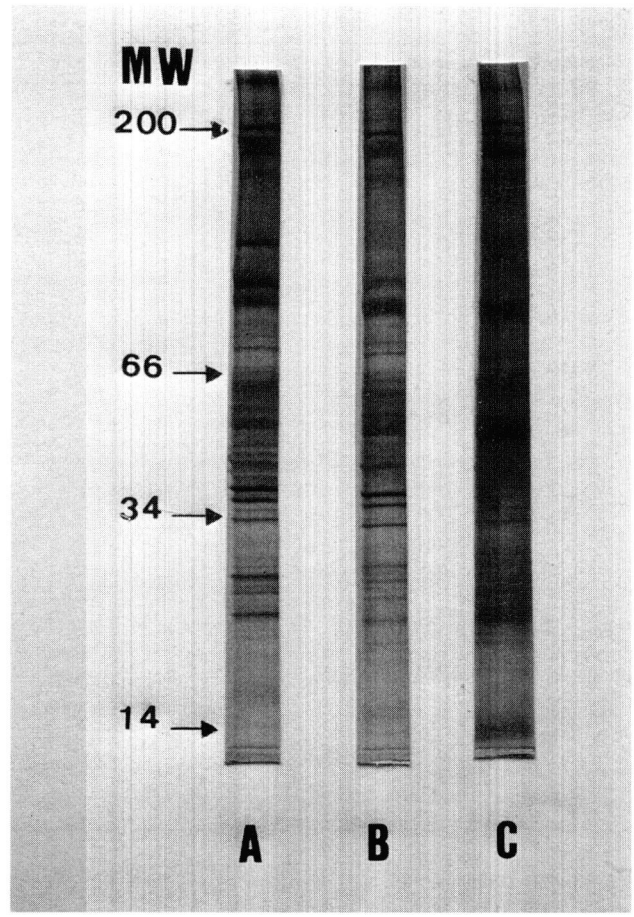

Fig. 1. – SDS-polyacrylamide gel electrophoresis pattern showing protein changes in salivary glands during feeding of *Hyalomma marginatum* female ticks.

A: Unfed ticks;
B: 3-days fed ticks;
C: 5-days fed ticks.

(Adjacent numbers represent relative molecular weight).

RESULTS

Salivary gland proteins were analyzed by SDS-PAGE (Fig. 1) at three feeding steps. Silver staining of proteins allowed us to detect differences in the number and concentration of proteins revealed. In unfed ticks, 34 proteins were detected. After three days of feeding, we observed 37 proteins. Finally after five days of feeding 40 proteins were seen. Table I represents the relevant proteins. Most of these proteins were classified into eight groups.

Group 1: these proteins were revealed during the three steps of feeding (34, 42, 50, 72, 80, 165, 193, 200, 220 and 229 kDa).

Group 2: (23, 26, 38 and 39 kDa) these proteins were present in salivary glands of unfed ticks and were seen to diminish as the tick feeding process progressed.

Group 3: (95 kDa) this protein was present in unfed and three days fed ticks and disappeared in five days fed ticks.

Group 4: (40, 46, 48 and 112 kDa) these proteins were present only in unfed ticks.

Group 5 : (20, 25 and 210 kDa) these proteins were present shortly in unfed ticks and became more strong thereafter.

Group 6 : (176 kDa) this protein was present in unfed ticks, decreased during early stages of feeding and increased during subsequent stages.

Group 7: (29 kDa) this protein was obvious for a brief period in unfed ticks and increased after 72 hours of feeding to disappear at five days of feeding.

Proteins	Salivary gland present in ticks		
	Unfed	3-days fed	5-days fed
229	++	++	++
220	+	+	+
210	–	(+)	++
200	++	++	++
193	++	++	++
176	–	(+)	++
165	+	+	+
112	++	(+)	–
95	+++	+	–
80	++	++	+
72	++	++	++
72	++	++	+
50	++	++	++
48	+	–	–
46	+	–	–
42	++	++	+
40	+	–	–
39	+++	++	(+)
38	+++	+	(+)
34	++	++	+
29	(+)	+	–
26	++	(+)	(+)
25	(+)	+	++
23	++	+	+
20	(+)	(+)	+
18.7	–	–	+
17	–	–	(+)
13-14	–	–	+++

– : protein not visible.
(+) : lightly visible.
+, ++, +++ : relative amounts of protein visible.

Table I. – Major protein changes in salivary glands of *Hyalomma marginatum marginatum* females during three phases of feeding.

Group 8: (17, 18.7, around 13 and 14 and 133 kDa) these proteins became obvious only after five days of feeding.

A pool of sera of three calves at each infestation were used to characterize antigens from salivary gland extracts of five days fed ticks. Table II represents the relevant proteins revealed by sera of calves before and after each infestation with *Hyalomma marginatum marginatum*. We observed that no proteins were recognized by sera before infestations. Early after the first infestation the calves produced antibodies against tick antigens, and were present at subsequent infestations (17 kDa). This protein became stronger since the third infestation. Some antigens were revealed after the first and the second infestations (18.7, 50 and 80 kDa) but very weakly or not at all by sera of the third infestation onward. After the third infestation, other antigens were revealed by the sera samples (13.5, from 17 to 18.7, 133 and 193 kDa). Interestingly, at the fourth and fifth infestations, the 13.5 kDa antigen was more evident. Other proteins were recognized by sera of calves (25, 30, 70, 113 and 176 kDa).

Proteins	Infestations				
	1 th	2 nd	3 th	4 th	5 th
193	–	–	+++	–	–
176		–	–	–	+++
133	–	–	++	–	–
113	–	–	–	–	++
80	–	+	++	–	–
70	–	–	–	+++	+++
50	–	+	++	–	–
30	–	–	–	–	++
25	–	–	–	–	++
18.7	–	+	++	–	–
17	+	++	++	+++	+++
13.5	–	–	++	+++	+++

– : proteins not recognized.
+, ++, +++ : relative amounts of protein recognized.

Table II. – Salivary gland proteins detected by calve sera after each infestation.

DISCUSSION

Repeated feeding by ixodid ticks on a host may produce a resistance to further tick feeding. Acquired host resistance to tick feeding can result in reduced blood-meal volume, decreased engorgement weight, prolonged duration of feeding, diminished production of ova, reduced viability of ova, inhibited molting, and death of engorged ticks (Wikel, 1996). Many studies have shown resistance of hosts to various species of ticks (Wikel *et al.*, 1994; Rechav & Filden, 1995; Moran *et al.*, 1996). Advances in studies of tick salivary glands showing their importance during ticks feeding provide a better understanding of the interrelated elements in acquisition and expression of host immunity to ticks. For the last decades many authors have tried to identify materials that could be used for vaccination against ticks. Shapiro *et al.*, 1987, showed that a 90 kDa molecule derived from *Rhipicephalus appendiculatus* salivary glands was implicated in induction of acquired resistance by rabbits. A 20 kDa salivary gland polypeptide induces resistance to *Amblyomma americanum* (Brown & Askenase, 1986). Our results show that attachment and short feeding were the primary stimuli for the synthesis of new salivary gland proteins. Major protein changes were observed, in the proteins of groups 2, 3, 4 which are present in the glands of unfed ticks and were absent or diminished in glands during later stages of feeding. These proteins might be secreted in the initial phase of feeding process or converted into other substances during the feeding process (Fawcett *et al.*, 1981). Proteins of groups 5, 6, 7 and 8 appear to be synthesized as a result of tick attachment and feeding. These proteins may be related to growth and development of acinus types of salivary glands (Binnington, 1978; Kaufman, 1989). Comparable

observations were made with *Amblyomma americanum* (Mc Swain *et al.*, 1982; Jaworski *et al.*, 1990) and *Rhipicephalus appendiculatus* (Shapiro *et al.*, 1986).

These changes may have physiological significance from engorgement of the ticks. Advances in studies of tick salivary gland physiology allow new insights into biological properties of tick salivas (Kaufman, 1989; Sauer *et al.*, 1995), and may help to understand a mechanism that make possible persistence of ticks in the field.

The study of salivary gland protein changes occurring during attachment and feeding may be a key for identification of vaccine candidates for efficient immune stimulation of the host. However, several investigations related to tick infestations (Moran *et al.*, 1996; Sahibi *et al.*, 1997a), and immunization (Wikel, 1996; Sahibi *et al.*, 1997b,c) with tick extracts have highlighted the existence of host resistance. In attempt to identify antigens related to resistance, we have analyzed sera from calves after several infestations. The antigen used for the western blot was prepared from salivary glands of five days fed ticks. We have used this stage of feeding because the ultrastructure and functional development were completed (Binnigton, 1978; Gill & walker, 1987; Kaufman, 1989). We noticed that during heavy repeated infestations a variety of antibodies are produced (Sahibi *et al.*, 1998). Some antigens (18.7, 50, 80 kDa) were revealed with sera from first and second infestations and not by sera samples obtained after the third infestation. Other antigens, 13.5, 17 to 18.7, 133 and 193 kDa, were revealed for the first time only by sera taken when manifestation of anti-ticks resistance (third infestation) had appeared. This resistance was manifested by an inhibition of tick feeding and fertility. The percentage of engorged females significantly affected dropped to 55 %. Female weight was also affected by 68 %. Likewise, egg mass weight dropped to 66 % (Sahibi *et al.*, 1998). Because of consistent association between the time of revelation of these antigens and the manifestation of a resistance, we believe that they may be responsible for anti-tick protection. We also observed that a 13.5 kDa protein may be particularly relevant for protection because it was revealed by calves sera after the third infestation and became more evident at fourth and fifth infestation. This antigen was only observed in salivary gland extracts when ticks had five days fed, so it is synthesized as a result of tick attachment. Perhaps it may be a factor which appears to have biologic activity which facilitates ticks engorgement. However, at the last infestations (fourth and fifth) other antigens were revealed (25, 30, 70 and 176 kDa). These lately recognized antigens might be associated with calves resistance against ticks. These possibly protection-associated antigens were observed in salivary gland extracts after three or five days feeding. However, it will be more interesting to identify an antigen in unfed salivary glands which induce early antibody production, and may be able to inhibit tick attachment and might be a good vaccine candidate. However, we have identified a 50 kDa antigen present in unfed salivary glands. This antigen was revealed weakly by the first infestation sera and became very prominent after the second infestation. Association of this protein (50 kDa) and antigens revealed after expression of a resistance of calves (thirth infestation) (13.5, 25 and 30 kDa), and immunization with this complex of molecules might be efficient for establishment of protection against *Hyalomma marginatum marginatum.*

Nevertheless, the previous studies on antigens associated with protection were essentially proteins with low molecular weight of 20 or 25 kDa as reported by Brown (1988) or 29 kDa as reported by Barriga *et al.* (1992) induce a resistance against *Amblyomma americanum.* Rutti & Brossard (1992) reported vaccination against *Rhipicephalus appendiculatus* with a 20 kDa molecule which induced a protection in different host species against different ticks. In our results, we also observed that antigens with low molecular weight (13.5, 25 and 30 kDa) seem to be related to protection. The 13.5 kDa protein may be particularly relevant to protection because it was revealed with the same strength by the sera of the third to fifth infestations when calves were resistant to tick infestations (Sahibi *et al.*, 1998). Indeed, the role of this molecule in acquisition and expression of anti-tick protection should be further evaluated. The antigen 25 kDa where present in the three feeding steps, this protein can also confer a protection against *Hyalomma marginatum marginatum* and act when tick was just attached to host. Our results demonstrated that production of several antigenic polypeptides was induced by the feeding process. Immuno-reactive polypeptides from the salivary glands of *Hyalomma marginatum marginatum* females have been identified. Certain polypeptides are apparently induced by attachment and feeding. Some hypothesis were made on importance of these antigens, however more proof must be given. Besides, antigens of similar molecular weight were found in other tick species suggesting that these proteins are conserved. Antigens identified through these studies may prove useful in efforts to artificially induce immunity to tick feeding. So more investigation must be done to characterize these antigens and perhaps find a cross reactivity with other *Hyalomma* species.

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