# Antigens from *Ornithodoros erraticus* gut membranes induce lethal, complement-mediated, anti-tick immune responses in pigs and mice

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**Abstract**

*Ornithodoros erraticus* is an argasid tick that can transmit severe diseases such as human relapsing fever and African swine fever. In Southern Europe *O. erraticus* lives in close association with swine on free-range pig farms. Application of acaricides for the eradication of *O. erraticus* from pig farms is inefficient. This is the reason why we tried to develop an anti-*O. erraticus* vaccine as alternative method of control. Accordingly, we were prompted to investigate the protective possibilities of a gut membrane extract from the parasite (GME) that has not been studied hitherto. Administration of the GME with Feund's adjuvants (FAs) to pigs and mice induced a Th1 protective response able to kill 80% of the immature forms of the parasite in the first 72 hours post-feeding and to reduce the fecundity of females by more than 50%. The action of the vaccine is the result of damage to the gut wall of the argasid and is mediated by activation of the complement system. The administration of GME with alum, instead of with FAs, reduced the degree of protection, presumably owing to the lowered complement fixing activity of the subclasses of antibody synthesised in the Th2 responses, which are enhanced by the alum. The protective antigens of the GME were expressed by all the developmental stages examined and are probably proteins from the luminal membrane of enterocytes. These antigens were seen to be more abundant in recently fed parasites than in fasting specimens, suggesting that their expression is induced after blood ingestion.

*Keywords*: *Ornithodoros erraticus*; tick, vaccine; complement; gut antigens

## 1. Introduction.

*Ornithodoros erraticus* is an argasid tick of medical and veterinary importance owing to the diseases it can transmit to humans (relapsing fever) and to swine (African swine fever). Accordingly, control of the tick would greatly improve the control of such diseases. In Southern Europe *O. erraticus* lives in close association with swine on free-range pig farms, hidden in holes, cracks and fissures inside and around the pig-pens (Oleaga-Pérez et al., 1990). This type of behaviour means that the eradication of *O. erraticus* from the farms through the use of acaricides is very difficult to achieve simply because it is not feasible to ensure that the acaricide will reach all places where the parasite hides (Astigarraga et al., 1995).

In light of the above, and to avoid other problems deriving from the use of acaricides such as environmental damage or increases in acaricide resistance in tick populations (Tellam et al., 1992; Opdebbeck et al., 1994; Mulenga et al., 2003; Xu et al., 2005), our team started to develop an anti-*O. erraticus* vaccine as an alternative method of control.

In view of the protection obtained, against hard ticks with the so-called "concealed" antigens or antigens that are not normally exposed to the host's immune system (Willadsen and Kemp, 1988; Willadsen and McKenna, 1991), our first aim was to assess the protective capacity of several extracts of concealed antigens from *O. erraticus*. With this in mind, we administered several antigenic extracts prepared from the tick gut as well as from the tick haemolymph, synganglion and coxal glands to pigs. We choose the gut as the target organ because it is immediately accessible to the host's immune effectors ingested with blood (complement, antibodies, leucocytes), and because the antigens from the digestive tract of *Boophilus microplus* (i.e., Bm86) were the basis for the development of the only anti-tick vaccine that has been commercialised to date (see reviews of Willadsen, 1997, 1999, 2001 and Vercruysse et al., 2004). We also investigated tick haemolymph, synganglion and coxal glands because in argasids -as in ixodids- host immunoglobulins pass intact from the gut to the haemolymph (Chinzei and Minoura, 1987; Ben Yakir, 1989) and hence all the organs bathed by this fluid can be accessed by antibodies and presumably be negatively affected.

However, all these extracts of concealed antigens from *O. erraticus* induced immune responses that were completely innocuous for the parasite, suggesting that the extracts did not contain protective antigens (Astigarraga et al., 1995). Owing to their manner of preparation, all the extracts essentially contained soluble proteins but not membrane proteins, which were concentrated in a fraction of insoluble material whose value as a vaccine was not examined in the work by Astigarraga et al. (loc. cit). Since the membrane proteins of gut cells, in particular those expressed by enterocytes on their luminal surface, may be of great interest in the field of vaccines, as was the case of *B. microplus* (Willadsen, 2001), it has become crucial to check the protective capacity of the fraction of insoluble material that was not analysed by Astigarraga et al. (loc. Cit.); i.e., the fraction containing the gut membranes from *O. erraticus*.

This was the aim of the present work, in which we report the procedure followed for the collection of *O. erraticus* gut membranes, the protective effect induced by that fraction when administered to pigs and mice, and the involvement of the complement system in the protective mechanism.

## 2. Material and Methods.

### *2.1. Animals.*

A total of 33 pigs and 20 mice were used as hosts in the vaccination experiments. All the pigs were Iberian breed females with an initial weight of 15 kg. They were from a farm free of ectoparasites. Along the experimental period they were fed on non-medicated commercial feed. The mice were 7-week-old BALB/c females from Charles River Laboratories (Barcelona, Spain). They were also fed on non-medicated commercial feed.

### *2.2. Parasites*

In all the experiments we used specimens of *O. erraticus* from a laboratory colony established from ticks captured in Salamanca, western Spain. The ticks were fed regularly on rabbits and were kept in an environmental chamber at 28 ºC, 85 % relative humidity and 16 h light / 8 h darkness. These ticks were used as the source of antigenic material and to test the action of the immune response induced by vaccination.

### *2.3. Preparation of gut membrane extracts of O. erraticus*

Extracts were obtained from fasting adult specimens (males and females in equal proportions); from fed adults taken at 6 and 72 hours post-feeding (p.f.), and from fed nymphs-1 collected at 6 h p.f. The specimens were processed in batches of 50 individuals per batch.

The procedure consisted in emptying and washing the gut, osmotic lysis of the enterocytes in order to remove their cytoplasmic contents and finally fragmentation by sonication of the cell membranes and basal membrane. All these operations were performed maintaining the ticks, guts and buffers in an ice bath.

The details of the procedure were as follows. Ticks were dissected in a Petri dish containing phosphate-buffered saline (PBS) and their guts were removed and transferred to a new dish with fresh PBS. Each gut was slit open with the aid of entomological pins, allowing their luminal content to be poured into PBS. Once they had been emptied, each batch of 50 guts were placed in a 15 ml test tube and washed with 10 ml of PBS. The guts were vigorously shaken in the PBS, after which they were allowed to sediment and the supernatant was discarded.

Following this, they were resuspended in 10 ml of ultrapure water (MilliQ water), changing the water every 10 min until all the enterocytes had been lysed. In general, lysis began to be appreciated -from the turbidity of the medium- by the seventh wash and was completed by the fourteenth, when the guts no longer released material to the medium.

The supernatant from the last wash was discarded and the tissue remains were resuspended in 2 mL of PBS and sonicated 5 times for 1 min at 60 watts. The resulting suspension was termed gut membrane extract (GME). The protein concentration of the different batches of GME was estimated by the method of Markwell et al. (1979).

126 *2.4. Vaccination procedure and collection of sera.*
127
128 Unless otherwise indicated, the vaccination protocol was as follows:
129 The pigs were vaccinated at 15-day intervals with three doses of GME administered subcutaneously
130 at four points on their abdomens. Each dose contained 150 μg of protein in 1 ml of PBS. The first dose
131 was administered emulsified in an equivalent volume of Freund's complete adjuvant (FCA); the second
132 with Freund's incomplete adjuvant (FIA) and the third dose with no adjuvant. Further booster doses, if
133 any, were given without adjuvant. The pigs were bled immediately before the administration of each dose
134 and immediately after each infestation with parasites.
135 The mice were vaccinated in a similar way to the pigs; that is, with three fortnightly doses of GME
136 administered subcutaneously in Freund's adjuvants (FAs). In the mice, the dose was 50 μg in a final
137 volume of 0.3 ml of emulsion. Mice were bled from the retro-orbital plexus before the first dose of
138 antigen and just before infestation with ticks.
139 Blood samples were allowed to clot and sera were removed and stored at -80ºC.
140
141 *2.5. Vaccination trials and parasite infestations (Table 1).*
142
143 Trial 1. The aim of this was to check whether GME induces a protective response in swine. Six pigs
144 were divided into two groups of 3 pigs each and treated as follows: Group 1, GME from adult ticks taken
145 at 6 hours p.f. (aGME6h); control group, adjuvant. Parasite infestations: the first four infestations were
146 carried out at 7, 14, 63 y 70 days after the third dose. Each pig was challenged with 15 females, 25 males
147 and 100 nymphs-3 per infestation. Immediately after the fourth infestation, the pigs received a booster
148 dose of antigen and 7 days later a fifth infestation, identical to the previous ones, was performed.
149 Trial 2. The aim of this was to compare the protective capacity of the GME from nymphs-1 with that
150 of the GME from adults. Nine pigs were divided into three groups of 3 pigs each: Group 1, aGME6h;
151 Group 2, GME from nymphs-1 taken at 6 hours p.f. (nGME6h); control group, adjuvant. Parasite
152 infestations: two infestations with 15 females, 25 males and 100 nymphs-1 per pig were carried out at 7
153 and 14 days after the third antigen dose.
154 Trial 3. The aim of this trial was to study the effect of adjuvant and of the physiological state of the
155 specimens from which the GME had been obtained on the immune response and its protective action.
156 Twelve pigs were divided into four groups of 3 pigs each: Group 1, GME from fasted adult ticks
157 (aGMEfa); Group 2 and Group 3, GME from adult ticks taken at 6 and 72 hours p.f. (respectively,
158 aGME6h and aGME72h); Group 4, aGME6h but administered with $Al(OH)_3$ (alum) instead of FAs. Two
159 control groups (one per adjuvant) of 3 pigs each were included in this trial; one group was given FAs and
160 the other was given alum. Parasite infestations: two infestations with 15 females, 25 males, 100 nymphs-2
161 and 100 larvae per pig were carried out on days 7 and 14 after the third antigen dose.
162 Trial 4. This experiment was carried out to check whether the GME induces protective responses in
163 mice and, if so, to check whether the complement system is involved in the protective mechanism.
164 Twenty mice were divided into two groups of 10 mice each: Group 1, aGME6h + FAs; control group,

adjuvant. At 7 days after the third antigen dose, each mouse was infested once with 50 nymph-2 and 50 larvae.

Twenty-one days after the infestation the mice were given a booster antigen dose and 7 days after this dose half of the mice from each group (5 mice/group) were decomplemented with 10 units/mouse of cobra venom anticomplementary protein (CVF) (Sigma) following the procedure described by Astigarraga et al. (1997). At 48 hours after treatment with CVF, all the mice (both treated and untreated) were bled -to assess their degree of decomplementation- and challenged with 50 nymphs-2. The degree of decomplementation of the mice was determined by measuring the level of complement C3 component in the mouse sera by radial immunodiffusion, according to the protocol described in Astigarraga et al. (1997).

*2.6. Evaluation of the protection induced by the GMEs and statistics.*

The degree of protection was determined by measuring the usual parameters in ticks fed on vaccinated animals and comparing the results with those from ticks fed on control animals. These parameters were feeding time, the amount of blood ingested, the number of egg laid by the females, egg viability, the rate of moult of immature stages and mortality rates at 48-72 hours, 15 days and 3 months post-feeding.

The values obtained for the parasites fed on the animals from each group were summarized as means ± standard errors. Statistical differences between the vaccinated groups and the corresponding controls in each trial were assessed by one-way ANOVA. Values of $P < 0.05$ were taken to be significant.

*2.7. Humoral response.*

The levels of pig IgG, IgG1 and IgG2 antibodies and the levels of mouse IgG, IgG1 and IgG2a antibodies against the GMEs were monitored by indirect ELISA according to the standard protocol described previously (Astigarraga et al., 1995, 1997). Briefly, polystyrene plates (Sigma) were coated with 1 µg of antigen per well in 100 µl of carbonate buffer, pH 9.6, and post-coated with 1% bovine serum albumin in PBS. The sera were analysed in duplicate at a dilution of 1/100 in TPBS (PBS containing 0.05% Tween 20). Peroxidase-conjugated anti-pig IgG (Sigma), anti-pig IgG1 (Serotec) and anti-pig IgG2 (Serotec) were used, respectively, at dilutions of 1/6000, 1/100 and 1/500 in TPBS. The peroxidase-conjugated anti-mouse IgG (Sigma), anti-mouse IgG1 (The Binding Site) and anti-mouse IgG2a (The Binding Site) were used, all of them at a dilution of 1/2000 in TPBS. *Ortho*-phenylene-diamine was used as chromogen substrate for peroxidase and the reactions were stopped with 3N sulphuric acid.

*2.8. SDS-PAGE and Western blots.*

SDS-PAGE and Western blots were carried out essentially as described elsewhere (Baranda et al.. 2000). The GMEs were subjected to SDS-PAGE in a Laemmli (1970) discontinuous gel system. The stacking gel was 3% and the resolving gel was a 5-20% gradient. The gels were loaded with 50 µg of protein per lane or each 5 mm of gel width. After running, the gels were either electrotransferred onto

nitrocellulose paper or stained with silver nitrate and Coomassie blue and dried. The electrotransfer step was carried out over 3 hr at 60 volts in a 2 mM TRIS/192 mM glycine buffer, pH 8. The sheets were cut into 5 mm strips, post-coated in 1.5% BSA and then incubated with different sera diluted 1/100. After washing, the strips were incubated with 1/2000 diluted peroxidase-conjugated anti-pig-IgG. Finally, the reactive bands were developed using 4-chloro-1-naphthol as chromogen. The dried gels and the nitrocellulose strips were scanned directly and processed digitally to compose the corresponding figures.

## 3. Results

### *3.1. Vaccination of pigs with aGME6h: humoral response, antigens recognized by the vaccinated animals, and effect of the response in O. erraticus.*

Vaccinated animals showed high serum levels of IgG anti-aGME6h, which peaked after the second antigen dose and remained detectable for at least 70 days after the last antigen dose. A booster antigen dose induced a new IgG peak (Fig. 1(A)). IgG1 and IgG2 were the only IgG subclasses measured in our pigs. Fig. 1(B) shows that both subclasses reached similar levels in all the vaccinated pigs and that although IgG1 was more abundant than IgG2 the differences between them were not significant.

The three vaccinated pigs recognised the same antigens on aGME6h: a band of 200 kDa, a group of numerous bands between 100 and 30 kDa and, very weakly, a group of bands between 12 and 8kDa (Fig. 1(D)). To check whether there was cross-reactivity between the GME and the saliva of the argasids, the aGME6h was challenged by Western blot against the serum of one pig infested with *O. erraticus* and against the serum of one pig vaccinated with *O. erraticus* salivary gland extract, both obtained in earlier works by Astigarraga et al. (1995). No bands were recognised by these two sera on the aGME6h (Fig. 1(D)).

The protective action of the response was assessed in 5 infestations with 25 males, 15 females and 100 nymphs-3 per pig and infestation (Table 2). No differences were observed in the feeding time of the specimens or between groups (vaccinated *vs.* controls) or between infestations. Neither were differences observed in the amount of blood ingested by the different developmental stages with respect to the control group. However, the males ingested similar amounts of blood in all five infestations while, in contrast, the females and nymphs-3 ingested decreasing amounts of blood from the first to the fifth infestation.

The number of eggs laid by the females decreased significantly in the specimens fed on the vaccinated group with respect to the controls in the first two infestations (respectively, 45% and 27.1%). In the remaining infestations there were no differences between groups as regards this parameter.

The mortality of the adults was negligible or zero in all infestations and groups. By contrast, the nymphs-3 fed on the vaccinated animals had high mortality rates: close to 50% in the first infestation, decreasing progressively from the second to the fourth infestation to 12%, while in the fifth infestation, after a new dose of aGME6h, the figure rose to almost 25%. In all the infestations, the affected nymphs died between 48-72 h p.f, all of them adopting a characteristic post-mortem aspect, with a swollen body, the legs completely straight and both body and legs pallid in colour (Fig. 2(A)). The nymphs that did not die in the first 72 hours survived with no apparent damage and moulted normally. Microscopic examination of the intestine of the dead specimens revealed that their intestinal epithelium was so degraded that it was scarcely visible (Fig. 2 (C)), while that of the surviving specimens had a normal

aspect and consistency (Fig. 2(D)). The mortality of the nymphs-3 fed on the control pigs was negligible or zero, and their post-mortem aspect was the usual one for specimens that have died from causes not attributable to the vaccine; that is, with the legs folded and generally dark reddish in colour (Fig. 2 (B)).

*3.2. Influence of the developmental stage from which the GME is obtained.*

The composition of the GME6h from adults and nymphs-1 was apparently identical in SDS-PAGE and both extracts induced similar humoral responses and the recognition of the same antigens, which were the same as those revealed in the previous experiment (Fig. 3).

The action of these responses was assessed in two infestations with 25 males, 15 females and 100 nymphs-1 per pig and infestation. Both extracts induced the same protective effect and with almost the same intensity; that is, a drop in the fecundity of the females of more than 50% and the death of up to 83.4% of immature forms (Table 3). The remaining parameters measured showed values similar to the control in both infestations (data not shown).

*3.3. Influence of adjuvant and the physiological state of the specimens used as a source of GME in the immune response and its protective action.*

As shown in Fig. 4(A), the aGMEs taken at 6 and 72 hours p.f. induced higher levels of IgG than the aGME from fasted specimens. Fig. 4 (A) also shows that of the two adjuvants administered with aGME6h the FAs induced IgG levels that were twice as high as those induced by alum. Additionally, the levels of anti-GME IgG1 and IgG2 in week five reached one half and one quarter, respectively, of the IgG levels in all the vaccinated animals (data not shown).

The three types of GME examined in this experiment (fasting, 6 hours and 72 hours) showed a similar composition in SDS-PAGE, although with some differences in the intensity of certain bands, such as those larger than 200 kDa and one of 45 kDa, which were more abundant in the aGMEs from 6 and 72 hours p.f. than in the GME from fasting specimens (Fig. 4(B)). The sera from the pigs vaccinated with the different aGMEs revealed very similar antigen patterns on their homologous extracts, although the animals vaccinated with the GME from fasting specimens recognised the antigens more weakly. In turn, the administration of aGME6h + alum induced the recognition of the same antigens as aGME6h + FAs, although at reduced intensity (Fig. 4(C)).

The action of the response was assessed in two infestations with 25 males, 15 females, 100 nymphs-2 and 100 larvae per pig and infestation. The results in the six control pigs were similar, such that in order to simplify the comparisons, all were grouped in a single control group (Table 4). As in the previous experiments, the protective action was reflected in a decrease in the fecundity of the females and a high mortality in the immature forms fed on the vaccinated animals with respect to the controls. The other parameters measured were similar to the controls in all groups and infestations (data not shown). All the aGMEs assayed in this experiment induced a protective response, although the aGME from 6 and 72 h p.f. induced more potent responses than the aGME from fasting specimens, except as regards larval mortality. Regarding the adjuvants, administration of the same extract (aGME6h) with FAs induced a protective response that was more potent than when administered with alum (Table 4).

*3.4. Vaccination of mice with aGME6h*

aGME6h + FAs induced a strong humoral response in the mice, characterised by high levels of IgG and IgG1 (ODs close to 2.5), but also by high IGg2a levels (ODs close to 1.7). The sera of the vaccinated mice recognized the same antigens as those of the pigs in the preceding experiments (Fig. 5(B)). The action of the response was assessed in a single infestation and as a function of a single parameter: the mortality of nymphs-2 and of larvae (Table 5). aGME6h induced a protective response in the mice, with an effectiveness comparable to that observed in the pigs. The nymphs-2 were the developmental stage most affected, with 67.4% mortality while the larvae showed a more reduced, although significant (28.7%), mortality.

*3.5 Decomplementation and effects on the mortality of immature forms.*

Before treatment with CVF, the serum C3 levels of all mice ranged between 602 and 958 µg/ml (753 ± 123 µg/ml). At 48 hours after the administration of CVF, the C3 serum levels of the treated mice fell to values of between 0 and 32 µg/ml (17.7 ± 12.9 µg/ml), while the C3 serum levels of the untreated mice remained above 600 µg/ml (720 ± 101 µg/ml).

At 48 h after CVF administration 50 nymphs-2 were fed on each mouse. The mortality of nymphs-2 fed on the control mice (decomplemented or not) was seen to be negligible or zero, while, that of the nymphs-2 fed on vaccinated mice remained significantly high (40.2%) in non CFV-treated mice and was zero in the decomplemented rodents (Table 5).

## 4. Discussion

As pointed out in the Introduction, our aim in this study was to check whether the membranes of the gut of *O. erraticus* contain protective antigens. To do so, we first prepared a crude extract of such membranes theoretically free of host blood remains and of soluble remains from the parasite gut. This extract was prepared from adult specimens of both sexes taken at 6 h p.f., and it was administered with Freund's adjuvants. The results from trial 1 clearly showed that the extract contained protective antigens and that the response induced by such antigens mainly affected the immature forms (nymphs-3), eliciting the death of almost 50% of them. This effect seemed to be very promising, and indeed striking, because we had as a reference the anti-*B. microplus* vaccine based on Bm86, whose effect is essentially a decrease in the fecundity of females (Rodríguez et al., 1995; Jittapalapong et al., 2004). Also striking were the speed of the effect (in the first 72 hours p.f.) and the characteristic aspect of the dead nymphs -with their legs completely straight- which was completely unlike that of nymphs that had died from causes not attributable to the vaccine (Fig. 2(A), (B)). Examination of the gut of the nymphs affected revealed that their death was associated with destruction of their intestinal epithelium (Fig. 2(C), (D)).

The protective effect of the vaccine lost intensity with time, but remained at significant levels up to at least 10 weeks post-vaccination, the time when a booster antigen dose raised the level of protection again. This evolution of the degree of protection was parallel to that of the levels of anti-GME IgG in the sera of

the vaccinated pigs, suggesting a direct relationship between the mortality rate of nymphs-3 and the levels of anti-GME IgG.

On the other hand, there seems to be no relationship between the mortality rate of the nymphs-3 and the levels of anti-aGME6h IgG1 and IgG2 since the mortality rate of nymphs-3 varied considerably from one pig to another (note the SEs in Table 2 for the first infestation), whereas the levels of both subclasses were similar in the tree vaccinated pigs (Fig. 1(B)). As a result, it is possible that the different mortality rates of nymphs-3 on each pig might depend on the level of other porcine IgG subclasses (Sánchez-Vizcaino, 2001) that were not analysed. This analysis could not be performed owing to the unavailability of the necessary anti-immunoglobulins.

In this first experiment we also observed -in the females and nymphs-3 but not in the males- a gradual decline in the amount of blood ingested from the first to the fifth infestation. This phenomenon is difficult to account for, but we suspect that it should not be attributed to the possibility that the anti-GME response might have affected tick feeding There are two reasons for this idea: first, because the aGME6h and saliva do not share antigens, as seen from the absence of cross-reactivity in the Western blot shown in Fig. 1(D) and the fact that the bites of the parasite do not act as booster antigen doses, raising protection in the ensuing infestation (see Table 2), and -second- because it also occurred in the females and nymphs-3 fed on the control pigs. Whichever the cause (perhaps use in the infestation of specimens immediately after oviposition or moulting), the fact is that despite the reduced ingestion, the vaccine affected the ticks up to at least 10 weeks post-vaccination (fourth infestation).

Finally, in trial 1 we also observed that vaccination with the GME6h obtained from adults did not affect the mortality of the adults whatsoever but did elicit a significant decrease in the fecundity of the females in the first two infestations (respectively 41.5% and 27.1%). We are unable to explain why the adults did not die but it could be assumed that this was perhaps due to the greater physical resistance of their gut since these organisms are more robust that nymphs-3. Were this the case, the damage induced by the vaccine to the intestinal epithelium of the adults would be insufficient to cause their death although it could hinder digestion of the blood ingested, which would be reflected in a reduced oviposition by the females, as was indeed observed.

The above necessarily implies that the adults and immature forms of *O. erraticus* share protective antigens and that vaccination with a GME6h from immature forms would presumably have the same effects as a GEM6h from adults. To check this possibility, we performed trial 2, in which we compared the protective capacity of GME6h from adults with that of GME6h obtained from nymphs-1. The results of this comparison revealed that the GME6h from both developmental stages had the same composition and that they induced almost identical protective responses (Table 3, Fig. 3). These observations suggest that the vaccination of pigs with GME6h from different developmental stages of *O. erraticus* induces protective responses that are always reflected in the same way, that is, a significant drop in female fecundity (up to 62.3%) and a high mortality rate in the immature forms (up to 83.4%), presumably as a consequence of the damage to the intestinal epithelium. It may also be stated that the adults are a better source of GME6h than nymphs owing to their better yield and ease of manipulation.

Since the most striking effect of the vaccine -the death of immature forms- occurred between 48 and 72 h p.f., we speculated that perhaps the protective antigens of the GME might be more abundant in an

extract taken at 72 h p.f. To check this, we vaccinated pigs with the aGME72h and the protection observed was compared with that induced by the aGME6h and the GME from fasting adults. The results of this experiment (trial 3) showed that all three types of GME contained protective antigens, but that these were more abundant in the fed than in the fasting specimens and even more so at 72 h p.f. than at 6 h p.f., as judged by the higher degree of protection, the level of antibodies and the intensity of antigen recognition (Table 4, Fig. 4). These findings suggest that the protective antigens would be constitutively expressed at low levels in the intestine and that the ingestion of blood would up-regulate their expression. The higher expression observed after the ingestion of blood is logical if the antigens somehow participate in the endocytosis or the digestion of blood, as occurs for example with the intestinal lysozyme of *Ornithodoros moubata,* whose expression in the intestine is maximum between 16 hours and 5 days post-feeding (Gluncová et al., 2003). Evidently, confirmation of this requires further studies.

The results of trial 3 also showed that the vaccine also causes the death of the immature forms not examined in the previous experiments (nymphs-2 and larvae) and that -in view of the results obtained on using FAs or alum as adjuvants as respective enhancers of one or the other type of response- Th1 type responses provide better protection than the Th2 type (Smith, 1992; Audibert and Lise, 1993).

Since the destruction of the gut observed in the dead specimens due to the vaccine could be a consequence of activation of the complement system on the surface of enterocytes (González et al., 2004), and since the isotypes/subclasses of antibodies derived from Th1 responses -for example, IgGa2 in mice- are active fixers of the complement system (Abbas et al., 1996, 2000), we considered it appropriate to attempt to confirm the participation of the complement system in the protection mechanism. This was checked in mice vaccinated with aGME6h + FAs. These mice developed an intense protective response of the Th1 type -as indicated by the high level of IgG2a (Fig. 5 (A)), and -as shown in table 5- decomplementation with CVF completely abolished protection, confirming the role of complement in the immune effector mechanism.

## 5. Conclusion

The vaccination of pigs and mice with an extract of gut membranes from *Ornithodoros erraticus* induces a protective response that reduces the fecundity of the females by more than 50% and kills up to 80% of immature forms. The action of the vaccine is a consequence of destruction of the intestinal wall of the argasid and is mediated by activation of the complement system. The protective antigens are present in all the developmental stages of the tick and are presumably membrane proteins of the luminal surface of enterocytes. These antigens are more abundant in recently fed parasites than in fasting ticks, such that their expression is probably induced after the ingestion of blood. The identification and characterisation of these protective antigens will be addressed in future work.

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Table 1.
Vaccination trials

| Trial No. (host) | *Group* | *Antigen*[a] | Dose | Adjuvant[b] 1st -- 2nd -- 3rd | Infestation with |
|---|---|---|---|---|---|
| 1 (swine) | Control | none | - | FCA-FIA-PBS | 25 males<br>15 females<br>100 nymph-3 |
| | 1 | aGME6h | 150 μg | FCA-FIA-PBS | |
| 2 (swine) | Control | none | - | FCA-FIA-PBS | 25 males<br>15 females<br>100 nymph-2<br>100 larvae |
| | Control | none | - | ALUM | |
| | 1 | aGMEfa | 150 μg | FCA-FIA-PBS | |
| | 2 | aGME6h | 150 μg | FCA-FIA-PBS | |
| | 3 | aGME72h | 150 μg | FCA-FIA-PBS | |
| | 4 | aGME6h | 150 μg | ALUM | |
| 3 (swine) | Control | none | - | FCA-FIA-PBS | 25 males<br>15 females<br>100 nymph-1 |
| | 1 | aGME6h | 150 μg | FCA-FIA-PBS | |
| | 2 | nGME6h | 150 μg | FCA-FIA-PBS | |
| 4 (mice) | Control | none | - | FCA-FIA-PBS | 50 nymph-2<br>50 larvae |
| | 1 | aGME6h | 50 μg | FCA-FIA-PBS | |

[a] aGMEfa, gut membrane extract from fasted adult specimens; aGME6h and aGME72h, gut membrane extracts from adult specimens taken at 6 and 72 hours post-feeding; nGME6h, gut membrane extracts from nymphs-1 taken at 6 hours post-feeding.

[b] Adjuvants administered in the first, second and third antigen doses; FCA and IFA, complete and incomplete Freund's adjuvant; PBS, phosphate buffered saline; ALUM, $Al(OH)_3$.

Table 2.

Trial 1. Mean ± SE of the parameters measured in *Ornithodoros erraticus* specimens fed on control pigs and on pigs vaccinated with aGME6h + FAs along the five infestations

| Parameter | Evolutive stage | Group | Infestation | | | | |
|---|---|---|---|---|---|---|---|
| | | | First | Second | Third | Forth | Fifth |
| Ingested blood (mg) | Males | Control | 3.8 ± 0.1 | 3.0 ± 0.1 | 3.2 ± 0.4 | 3.4 ± 0.2 | 3.0 ± 0.8 |
| | | aGME6h | 3.8 ± 0.4 | 3.2 ± 0.5 | 3.2 ± 0.1 | 3.5 ± 0.1 | 2.5 ± 0.4 |
| | Females | Control | 17.2 ± 2.0 | 11.7 ±0.6 | 4.2 ± 1.2 | 8.3 ± 1.8 | 4.1 ± 0.1 |
| | | aGME6h | 14.6 ± 2.0 | 10.8 ± 1.0 | 3.5 ± 1.5 | 13.6 ± 2.0 | 4.5 ± 1.2 |
| | Nymph-3 | Control | 2.40 ± 0.40 | 2.25 ± 0.15 | 1.32 ± 0.18 | 1.33 ± 0.20 | 1.04 ± 0.12 |
| | | aGME6h | 1.86 ± 0.29 | 2.64 ± 0.47 | 1.36 ± 0.24 | 1.26 ± 0.26 | 1.00 ± 0.10 |
| No. eggs laid | Females | Control | 95.9 ± 8.4 | 66.6 ± 5.8 | 9.9 ± 4.5 | 24.3 ± 7.1 | 10.4 ± 3.5 |
| | | aGME6h | 56.2 ± 6.7* | 48.5 ± 4.5* | 12.6 ± 5 | 22.7 ± 4.7 | 8.3 ± 4.1 |
| % Viability | Eggs | Control | 72.3 ± 5.8 | 78.4 ± 6.9 | 20.6 ± 8.8 | 38.5 ± 10.8 | 28.1 ± 14.5 |
| | | aGME6h | 55.8 ± 6.4* | 75.1 ± 6.5 | 22.8 ± 7.1 | 27.6 ± 8.1 | 23.8 ± 10.5 |
| % Mortality (72 hours p.f.) | Nymphs-3 | Control | 5.7 ± 5.1 | 0 ± 0 | 3.1±1.9 | 3 ± 0 | 1.9 ± 1.9 |
| | | aGME6h | 47.9 ± 25.7* | 17.9 ± 16.3* | 15.7 ± 3.7* | 12 ± 4* | 24.9 ± 11.2* |

* $P < 0.05$ with respect to the control group

Table 3.

Trial 2. Mean ± SE of the parameters measured in *Ornithodoros erraticus* specimens fed on control pigs and on pigs vaccinated with aGME6h and nGME6h along the two infestations

| | | | Infestation | |
|---|---|---|---|---|
| Parameter | Evolutive stage | Group | First | Second |
| No. eggs laid | Females | Control | 34.2 ± 11.4 | 37.9 ± 7.9 |
| | | adult GME6h | 12.9 ± 4.2* | 13.5 ± 3.7* |
| | | nymphal GME6h | 20.6 ± 6.5 | 18,6 ± 3.2* |
| % Viability | Eggs | Control | 92.3 ± 5.8 | 26.6 ± 0.3 |
| | | adult GME6h | 93.6 ± 7.3 | 0 ± 0* |
| | | nymphal GME6h | 0 ± 0* | 7.6 ± 7.6* |
| % Mortality (72 hours p.f.) | Nymphs-1 | Control | 1.6 ± 1.6 | 3.1 ± 2.1 |
| | | adult GME6h | 83.4 ± 7.1* | 7.9 ± 1.6* |
| | | nymphal GME6h | 65.5 ± 3.1* | 23.5 ± 14.2* |

* $P < 0.05$ with respect to the control group

Table 4.

Trial 3. Mean ± SE of the parameters measured in *Ornithodoros erraticus* specimens fed on control pigs and on pigs vaccinated with aGMEfa, aGME6h and aGME72h (+ FAs) and with aGME6h + alum along the two infestations.

| | | | Infestation | |
|---|---|---|---|---|
| Parameter | Evolutive stage | Group | First | Second |
| No. eggs laid | Females | Control[a] | 15 ± 4.3 | 17.3 ± 3.2 |
| | | aGMEfa | 9.3 ± 1.8 | 7.2 ± 4.3* |
| | | aGME6h | 6.6 ± 3.2* | 5 ± 3.2* |
| | | aGME72h | 6.5 ± 1.8* | 4.9 ± 2.3* |
| | | aGME6h + alum | 9.1 ± 4.3 | 7.3 ± 2.8* |
| % Viability | Eggs | Control[a] | 92.3 ± 5.8 | 26.6 ± 0.3 |
| | | aGMEfa | 93.6 ± 7.3 | 0 ± 0* |
| | | aGME6h | 0 ± 0* | 7.6 ± 7.6* |
| | | aGME72h | 90.6 ± 2.3 | 9.6 ±5.3* |
| | | aGME6h + alum | 95.6 ± 4.3 | 16 ± 12.2 |
| % Mortality (72 hours p.f.) | Nymphs-2 | Control[a] | 8 ± 2.4 | 4.9 ± 1.1 |
| | | aGMEfa | 5.4 ± 2.7 | 46.9 ± 18.6* |
| | | aGME6h | 53.8 ± 13.3* | 47.3 ± 17.2* |
| | | aGME72h | 81.1 ± 5.7* | 69.2 ± 27.5* |
| | | aGME6h + alum | 23.6 ± 11.6* | 5.2 ± 4.3 |
| | Larvae | Control[a] | 6.7 ± 3.6 | 0 ± 0 |
| | | aGMEfa | 28.8 ± 5.1* | 7.4 ± 1.2* |
| | | aGME6h | 19.1 ± 3.3* | 0 ± 0 |
| | | aGME72h | 21.1 ± 4.7* | 0 ± 0 |
| | | aGME6h + alum | 9.4 ± 4.9 | 0 ± 0 |

* $P < 0.05$ with respect to the control group

[a] Includes 6 pigs, three treated with FAs and the other three with alum

Table 5.
Mortality rate (%) (Mean ± SE) of the nymph-2 and larvae fed on control and aGME6h-vaccinated mice in the first infestation. Mortality rate (%) of the nymphs-2 fed on decomplemented (+CVF) and no decomplemented (-CVF) mice after a booster antigen dose (second infestation)

| | First infestation | | Second infestation (only Nymphs-2) | |
|---|---|---|---|---|
| Group | Nymphs-2 | Larvae | + CVF | - CVF |
| Control | 15.3 ± 7.7 | 6.9 ± 2.5 | 0 ± 0 | 1.3 ± 0.5 |
| Vaccinated | 67.4 ± 6.7* | 28.7 ± 14.1* | 0 ± 0 | 40.2 ± 10.1* |

* $P < 0.05$ with respect to the control group

512

513 **Figure captions**

514

515 Figure 1. Vaccination of pigs with aGME6h. (A) Anti-GME IgG serum levels (mean OD ± SE) in control
516 and vaccinated pigs. Asterisks indicate the weeks when the extract was administered. The infestations
517 with *O. erraticus* took place in weeks 5, 6, 13, 14 and 15. (B) IgG, IgG1 and IgG2 serum levels (Mean
518 OD ± SE) in control and vaccinated pigs seven days after the third dose of antigen (week 5). (C) SDS-
519 PAGE, protein composition of aGME6h. (D) Western blot, antigens recognised on aGME6h by the sera
520 of pigs vaccinated with the homologous extract (1-3), by the serum from a naturally infested pig (4) and
521 by the serum from a pig vaccinated with a salivary gland extract (5).

522

523 Figure 2. (A) Nymphs-3 of *O. erraticus* killed by the action of the vaccine or (B) by causes not
524 attributable to the vaccine. (C) Intestinal caeca of nymphs-3 killed by the action of the vaccine; except in
525 the case of the distal end (arrow), the intestinal wall has almost disappeared and only blood remains (D)
526 Intestinal caeca of surviving nymphs-3, unaffected by the action of the vaccine; after removing most of
527 the blood ingested and washing, the intestinal walls are seen to be intact..

528

529 Figure 3. Influence of the developmental stage of the specimens used as a source of GME in the immune
530 response. (A) Anti-GME IgG, IgG1 and IgG2 serum levels (mean OD ± SE) in control and vaccinated
531 pigs seven days after the third dose of antigen (week 5). (B) SDS-PAGE, protein composition of the
532 GME6h from adults and from nymphs-1. (C) Western blot, antigens recognised on the homologous
533 extract by sera from pigs vaccinated with aGME6h+FAs (1) and nGME6h+FAs (2).

534

535 Figure 4. Influence of adjuvant and of the physiological state of the specimens used as a source of GME
536 in the immune response. (A) Anti-GME IgG serum levels (mean OD ± SE) in the vaccinated pigs.
537 Asterisks indicate the weeks when the extract was administered. The infestations with *O. erraticus* were
538 done in weeks 5 and 6. (B) SDS-PAGE, protein composition of the GMEs from fasted adults and from
539 adults taken at 6 and 72 hours p.f. (C) Western blot, antigens recognised on the homologous extract by
540 sera from pigs vaccinated with aGMEfa+FAs (1), aGME6h+FAs (2), aGME72h+FAs (3) and
541 aGME6h+alum (4).

542

543 Figure 5. Vaccination of mice with aGME6h. (A) Anti-GME IgG, IgG1 and IgG2a serum levels (mean
544 OD ± SE) in control and vaccinated mice seven days after the third dose of antigen (week 5). (B) Western
545 blot, antigens recognised on the homologous extract by a pool of sera from mice vaccinated with
546 aGME6h.

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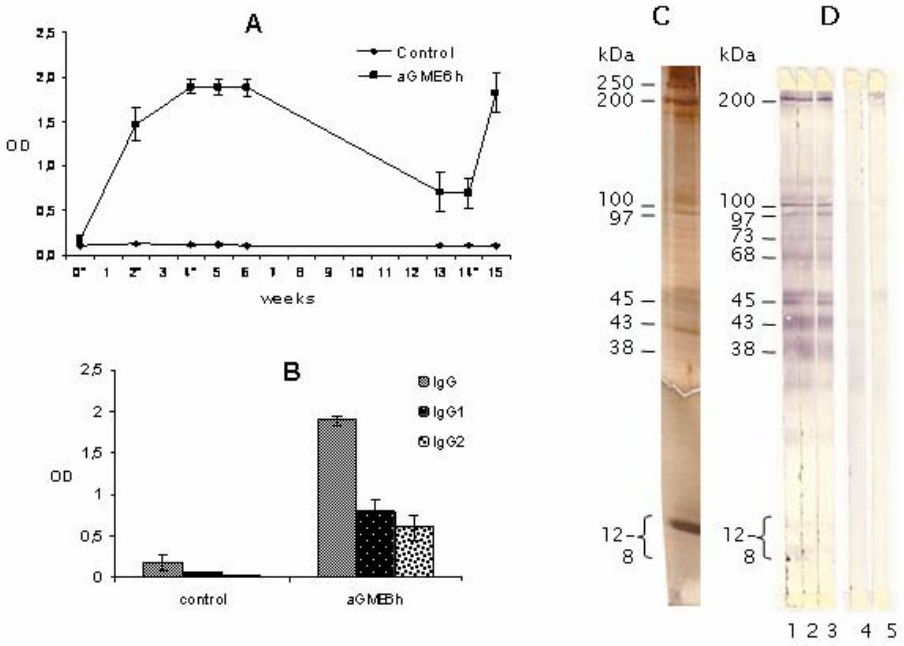
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Figure 1

550

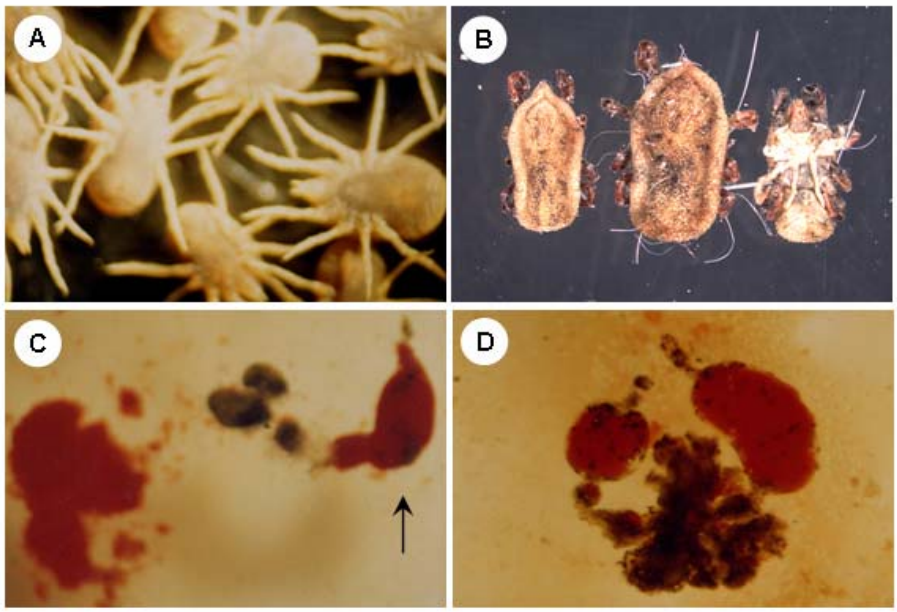


Figure 2

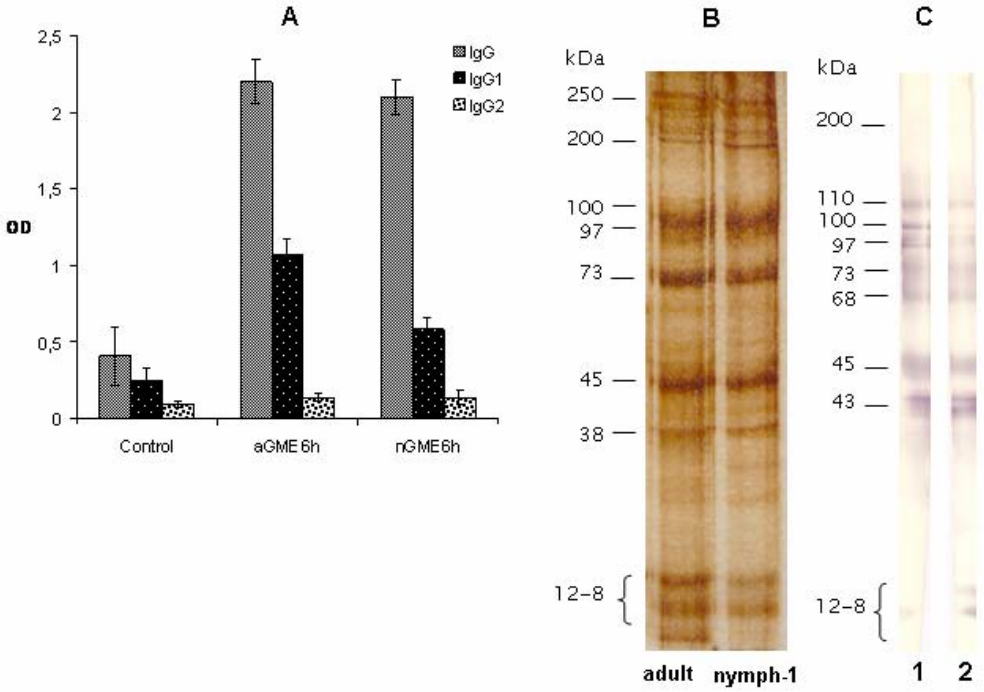
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Figure 3

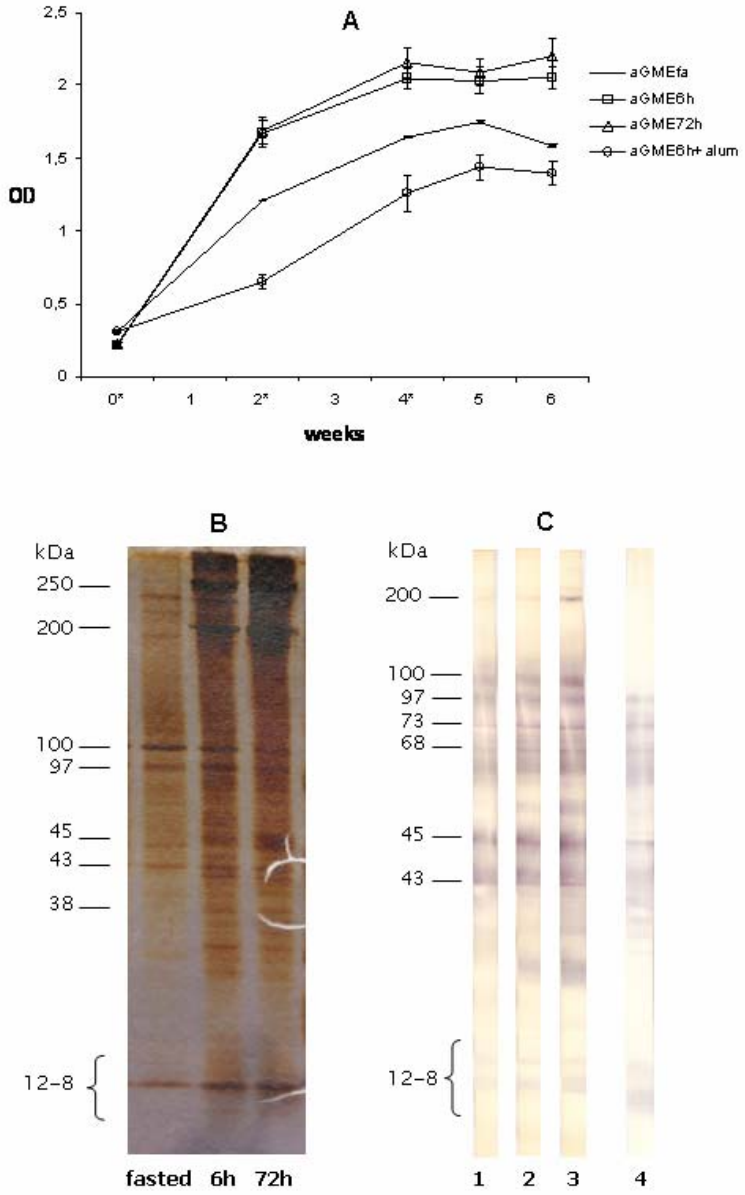
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Figure 4

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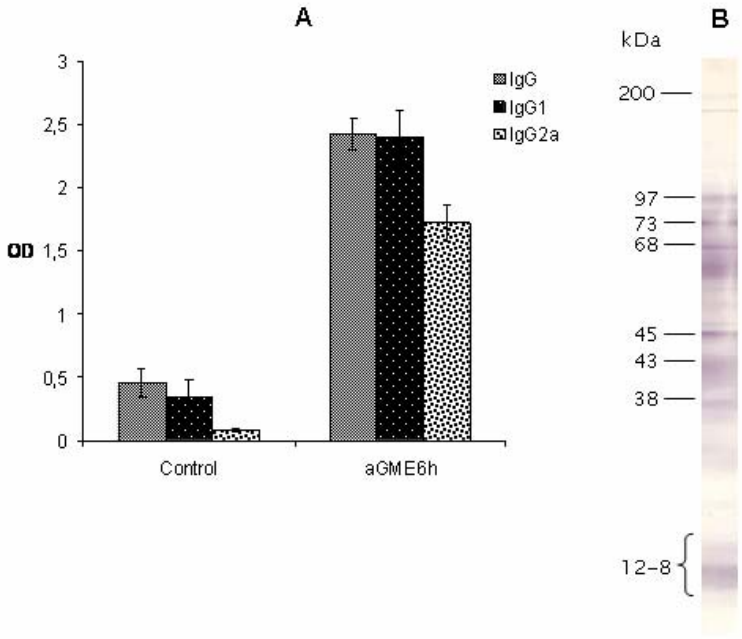
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Figure 5