Purification and characterization of a 45-kDa concealed antigen from the midgut membranes of *Ornithodoros erraticus* that induces lethal anti-tick immune responses in pigs

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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 the search for a vaccine against this parasite, a crude extract of tick midgut membranes (GME) was obtained that in pigs and mice induced a protective response able to kill up to 80% of the nymphs in the first 72 h post-feeding and to reduce the fecundity of females by more than 50%. To identify the protective antigens, the GME was subjected to successive biochemical fractionations and the resulting simpler protein fractions were inoculated in pigs. A 45-kDa antigen, the so-called Oe45, was detected, purified and demonstrated to be responsible for the protection induced by the GME. Oe45 seems to be a membrane protein that is presumably expressed on the luminal membrane of midgut epithelial cells. Oe45 consists of at least two differently charged bands (cationic and neutral), which show antigenic cross-reactivity. The possibility that these bands might be different isoforms of the same protein is discussed. Although Oe45 is constitutively expressed at low levels throughout the trophogonic cycle, its expression is up-regulated by the ingestion of blood, as suggested by the higher levels observed between 6 and 72 h post-feeding.

Keywords: *Ornithodoros erraticus*; Tick; Vaccine; Midgut antigen; Membrane protein; Oe45

1. Introduction.

In southern Europe, the argasid tick *Ornithodoros erraticus* lives in close association with swine on free-range pig farms, hidden in holes and fissures inside and around pig-pens (Oleaga-Pérez et al., 1990). The *O. erraticus* life cycle comprises the phases of egg, larva, up to five consecutive nymphal stages (nymph-1 to nymph-5) and adult. Except for the egg, all these developmental stages are haematophagous, requiring a blood meal to moult (immature forms) and reproduce (adults). *O. erraticus* is of veterinary importance because it is a well-established vector for the African swine fever virus (Sánchez Botija, 1963, 1982; Wilkinson, 1984), which causes a highly contagious disease in domestic swine with a broad range of clinical forms, varying from hyperacute to chronic or unapparent (Basto et al., 2006). In addition, *O. erraticus* is important from a medical point of view since it transmits several species of tick-borne relapsing fever borreliae, such as *Borrelia hispanica* and *B. crocidurae* (Piesman and Gage, 2004) to humans. Accordingly, control of *O. erraticus* would greatly improve the control of these diseases.

Current tick control is based on the use of acaricides, but these chemicals have serious drawbacks, including the development of resistance in ticks, toxicity, contamination of food products and environmental pollution (Graf et al., 2004). Moreover, in the case of *O. erraticus*, acaricide use has proved to be inefficient for the elimination of this tick from pig farms simply because it is not feasible to ensure that the acaricide will reach all the places where the parasite hides (Astigarraga et al., 1995).

This is why our team started to develop an anti-*O. erraticus* vaccine as an alternative method of control. In the search for suitable antigenic targets, which is the primary rate-limiting step in further vaccine development (Willadsen, 2004; Nuttall et al., 2006), we tested exposed - or salivary- antigens as well as several extracts of concealed antigens from the tick gut, haemolymph, synganglion and coxal glands (Astigarraga et al., 1995; Manzano-Román et al., 2006). Although some degree of protection -reflected as up to 50% inhibition of tick feeding- was achieved with salivary antigens (Astigarraga et al., loc. cit.), the highest degree of

protection was provided by an antigenic extract made of the midgut membranes of the tick, the so-called GME (Manzano-Román et al., loc. cit.).

Administration of the GME with Freund's adjuvants (FAs) to pigs and mice induced a protective response that killed up to 80% of the immature forms of the parasite in the first 72 hours post-feeding and reduced the fecundity of the females by more than 50%. The action of the vaccine was the result of damage to the midgut wall of the argasid, and, at least in mice, this damage is mediated by activation of the complement system. The protective antigens of the GME are expressed by all the developmental stages of *O. erraticus* and are probably membrane proteins of the luminal surface of midgut epithelial cells. These antigens are more abundant in recently fed parasites than in fasting ticks, suggesting that their expression is induced after blood ingestion (Manzano-Román et al., 2006).

The aim of the present work was to purify, characterize and identify the protective antigen(s) from the GME. This was accomplished following the pragmatic approach referred to by Willadsen (2004). That is, the GME was subjected to successive biochemical fractionations and the resulting simpler protein fractions were inoculated to the swine. In this way, a 45-kDa protective antigen was detected, purified and partly characterized.

2. Material and Methods.

2.1. Animals.

A total of 39 pigs were used as hosts in the vaccination experiments. All 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.

2.2. Parasites

In all experiments we used specimens of *O. erraticus* from a pathogen-free 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 midgut membrane extracts of O. erraticus

Extracts were routinely obtained from fed adult ticks taken at 72 hours post-feeding (p.f.). For one particular experiment, however, additional extracts were also prepared from fasting adults and from fed adults taken at 6 and 48 hours, and 15 and 23 days p.f. As a general rule, the specimens were processed in batches of 50 individuals per batch (25 males and 25 females).

A detailed description of the procedure for the preparation of the midgut membrane extracts can be found in Manzano-Román et al. (2006). Essentially, the procedure consisted in dissecting, emptying and washing the midgut, followed by osmotic lysis of the epithelial midgut cells in order to remove their cytoplasmic contents, and finally fragmentation of the cell membranes and basal membrane by sonication. The resulting suspension was the so-called midgut membrane extract (GME) and its protein concentration was estimated by the method of Markwell et al. (1978).

2.4. Fractionation of GME components.

GME components were first separated into three fractions according to their solubility. The GME was centrifuged for 1 h at 100,000 g and 4 ºC. The supernatant (containing water soluble proteins) was recovered, and the pellet was re-suspended in phosphate buffered saline (PBS) and centrifuged as before. The supernatant was then discarded and the new pellet was re-suspended in fresh PBS containing 1% Triton X-100 (TX-100) and allowed to incubate for 1 h

at 4 ºC with gentle shaking. After a new centrifugation step at 100,000 g, the resulting supernatant (enriched in TX-100-soluble membrane proteins from midgut epithelial cells) was recovered. The pellet was washed with PBS containing 1% TX-100, re-centrifuged and, finally, resuspended in fresh PBS. This final suspension contained the TX-100 insoluble proteins, which are most likely proteins of the basal membrane of the midgut wall (Balashov, 1972). The protein concentration of these three fractions was measured by the method of Markwell et al. (1978).

The TX-100-soluble membrane proteins were subsequently fractionated according to their electrical charge. This was carried out using the Sep-Pak disposable ion-exchange cartridges Accell™ Plus QMA -anion exchanger- and Accell™ Plus CM -cation exchanger- from Waters (Milford, Massachusetts) following the manufacturer's instructions. First, the sample containing the membrane proteins (nearly 3 mg in 4 ml) was concentrated to 0.3 ml through 3K Microsep filters (Filtron, Northborough, Massachusetts) and then diluted with 50 mM ammonium acetate buffer, pH 5.5, to a final volume of 3 ml. Then, both cartridges were connected in tandem and washed with 20 ml of ammonium acetate buffer, pH 5.5, at a flow rate of 2 ml/min. The protein sample was then applied on the upper cartridge at 0.5-1 ml/min and unbound (neutral) proteins were immediately eluted with 6 ml (0.5-1 ml/min) of ammonium acetate buffer, pH 5.5. Following this, the cartridges were disconnected and each of them was eluted with 5 ml (0.5-1 ml/min) of ammonium acetate buffer, pH 5.5, containing 1M NaCl to extract the anionic and the cationic proteins respectively. The three eluates were then concentrated through 3K Microsep filters and their final protein concentration was measured by the method of Markwell et al. (1978).

After each round of fractionation, each fraction was analysed by SDS-PAGE and its protective value was tested in pig vaccination trials.

2.5. Purification of the Oe45 antigen from the membrane protein fraction of the GME by gel electroelution.

As shown in the Results section, a potential protective antigen of 45 kDa was detected in the fraction of membrane proteins from the GME. This antigen, designated Oe45, was purified as follows. The membrane protein fraction of the GME was extracted with 1% TX-100 and resolved by SDS-PAGE. These gels were then stained with copper chloride (Harlow and Lane, 1988) to visualize the protein bands and cut those of 45 kDa. The gel pieces were destained and the protein electroeluted from the gel pieces in a model 4222 eluter from Bio-Rad (Hercules, CA) following the manufacturer's instructions. The eluted antigen was concentrated through 3K Microsep filters and its concentration assessed by the method of Markwell et al. (1978). Following this, its protective value was tested in a pig vaccination trial.

2.6. Vaccination procedure and collection of sera.

Unless otherwise indicated, the vaccination protocol was as follows. All pigs were vaccinated at 15-day intervals with three doses of the corresponding antigen (GME and its fractions and sub-fractions) administered subcutaneously at four points on their abdomens. Each dose contained between 150 and 5 µg of protein (Table 1) in 1 ml of PBS. The first dose was administered emulsified in an equivalent volume of Freund's complete adjuvant (FCA), the second with Freund's incomplete adjuvant (FIA) and the third dose with no adjuvant. The pigs were bled immediately before the administration of each dose and immediately after each infestation with parasites. Blood samples were allowed to clot and sera were removed and stored at -80ºC.

2.7. Vaccination trials and parasite infestations (Table 1).

Trial 1. The aim of this was to test which of the GME fractions obtained by differential solubilisation contained the protective antigens. Fifteen pigs were divided into five groups of 3 pigs each and treated as follows: Group 1, GME; Group 2, water-soluble proteins; Group 3, membrane proteins; Group 4, insoluble proteins; control group, adjuvant. Parasite infestations:

two infestations with 15 females, 25 males and 100 nymphs-2 per pig were carried out at 7 and 14 days after the third antigen dose.

Trial 2. The aim of this was to test which sub-fraction of the GME membrane proteins contained protective antigens. Fifteen pigs were divided into five groups of 3 pigs each and treated as follows: Group 1, GME membrane proteins; Group 2, cationic membrane proteins; Group 3, anionic membrane proteins; Group 4, neutral membrane proteins; control group, adjuvant. Parasite infestations: two infestations with 15 females, 25 males and 100 nymphs-2 per pig were carried out at 7 and 14 days after the third antigen dose.

Trial 3. The aim of this trial was to assess whether the eluted Oe45 antigen induces a protective response analogous to that induced by the whole fraction of membrane proteins of the GME. Nine pigs were divided into three groups of 3 pigs each: Group 1, GME membrane proteins; Group 2, Oe45 antigen; control group, adjuvant. Parasite infestations: two infestations with 15 females, 25 males, 100 nymphs-2 and 100 nymphs-1 per pig were carried out on days 7 and 14 after the third antigen dose.

2.8. Evaluation of the protection induced by the different antigenic extracts 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 (Manzano-Román et al., 2006). These parameters were feeding time, the amount of blood ingested, the number of eggs laid by the females, egg viability, the moult rate 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.

2.9. Humoral response.

219

220 The levels of IgG antibodies against the GME and its fractions and sub-fractions were
221 monitored by indirect ELISA according to the standard protocol described previously
222 (Manzano-Román et al., 2006). Briefly, polystyrene plates (Sigma) were coated with 1 µg of
223 antigen per well in 100 µl of carbonate buffer, pH 9.6, and post-coated with 1% bovine serum
224 albumin in PBS. The sera were analysed in duplicate at a dilution of 1/100 in TPBS (PBS
225 containing 0.05% Tween 20). Peroxidase-conjugated anti-pig IgG (Sigma) was used, diluted
226 1/6000 in TPBS. *Ortho*-phenylene-diamine was used as chromogen substrate for peroxidase and
227 the reactions were stopped with 3N sulphuric acid.

228

229 *2.10. SDS-PAGE and Western blots.*

230

231 SDS-PAGE and Western blotting were carried out essentially as described elsewhere
232 (Baranda et al., 2000). The GME and its successive fractions were subjected to SDS-PAGE in a
233 Laemmli (1970) discontinuous gel system using the Mini-Protean Cell system (Bio-Rad). The
234 stacking gel was 3% and the resolving gel was a 5-20% gradient. Typically, the gels were
235 loaded with 10 µg of protein per lane or each 5 mm of gel width. After running, the gels were
236 either electrotransferred onto nitrocellulose membranes or stained with silver nitrate and
237 Coomassie blue and dried. The electrotransfer step was carried out over 90 min at 400 mA in 2
238 mM Tris, 192 mM glycine buffer, pH 8. The sheets were cut into 5 mm strips, post-coated with
239 1.5% BSA in PBS and then incubated with different sera diluted 1/100. After washing, the strips
240 were incubated with 1/2000-diluted peroxidase-conjugated anti-pig-IgG. Finally, the reactive
241 bands were developed using 4-chloro-1-naphthol as chromogen. The dried gels and the
242 nitrocellulose strips were scanned directly and processed digitally to compose the corresponding
243 figures.

244

245 **3. Results**

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3.1. Vaccination of pigs with GME and GME fractions obtained by differential solubilisation: humoral response, antigens recognized by the vaccinated animals, and effect of the response in O. erraticus.

Fig. 1(A) shows that the three GME fractions induced medium to high anti-GME IgG serum levels in the pigs and that the whole GME induced an antibody response that was even higher than those induced by its fractions.

Almost 9% of the proteins of the GME were water-soluble; up to 60% were membrane proteins soluble in TX-100, while the remaining 31% were proteins insoluble in TX-100. Consequently, the whole GME and the membrane protein fraction showed quite similar band patterns in SDS-PAGE, whereas the fractions containing the water-soluble and TX-100-insoluble proteins showed band patterns that differed significantly (Fig. 1(B)). These similarities and differences were even more patent in the Western blots (Fig. 1(C)), where the pigs vaccinated with the whole GME and with the membrane proteins recognized almost the same antigens; namely, those of 100, 97, 73, 68, 45, 43 and 12-8 kDa, while the pigs vaccinated with the water-soluble and the TX-100-insoluble proteins showed completely different antigenic patterns, consisting in a few bands between 250 and 68 kDa.

The protective action of the response was assessed in 2 infestations with 25 males, 15 females and 100 nymphs-2 per pig and per infestation (Table 2). The GME fractions containing the water-soluble and the TX-100-insoluble proteins induced responses that were innocuous for the parasite since no differences were observed in any of the parameters measured between these two groups and the control group in any of the infestations. By contrast, the whole GME and the fraction containing its membrane proteins induced very similar protective responses which, although they did not affect the feeding time, the amount of blood ingested by the different developmental stages or the mortality of adults (data not shown) did elicit a nearly 50% reduction in the fecundity of females, together with the death of 58.1-71.9% of nymphs-2 in the first 72 h post-feeding (Table 2). The nymphs that did not die in the first 72 h survived with no apparent damage and moulted normally.

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276 *3.2. Vaccination of pigs with the fraction of membrane proteins and its sub-fractions containing*
277 *membrane proteins of different charge: humoral response, antigens recognized by the*
278 *vaccinated animals, and effect of the response in O. erraticus.*

279

280 Fig. 2(A) shows that all the antigenic extracts except the fraction of anionic proteins
281 induced similar, medium-to-high, IgG serum levels, which peaked after the third antigenic dose.
282 Anionic proteins, however, induced a noticeably weaker humoral response.

283 As can be observed in Fig. 2(B), among the membrane proteins the most numerous were
284 cationic, followed by neutral and anionic proteins. In terms of protein mass, the proportions of
285 cationic, neutral and anionic proteins were 80%, 18% and 2%, respectively.

286 Regarding the antigens recognized by the pigs, Fig. 2(C) shows that: (i), pigs vaccinated
287 with the whole fraction of membrane proteins (positive control group) recognized the same
288 antigens as the pigs vaccinated with the same extract in trial 1; namely, antigens of 100, 97, 73,
289 68, 45, 43 and 12-8 kDa; (ii), pigs inoculated with anionic proteins did not reveal any band; (iii),
290 all the pigs inoculated with cationic proteins revealed a unique band of 45 kDa, and (iv), two of
291 the three pigs vaccinated with the neutral proteins recognized five antigens of 100, 97, 73, 68
292 and 45 kDa, while the third pig recognized only the bands of 68 and 45 kDa, and did this so
293 weakly that those bands were hardly perceptible. In addition, Fig. 2(D) shows the results of a
294 crossed Western blot in which the pigs vaccinated with the cationic proteins recognised only the
295 antigen of 45 kDa on the neutral fraction, while pigs vaccinated with neutral proteins recognised
296 only the antigen of 45 kDa on the cationic fraction.

297 The action of these responses was assessed in two infestations with 25 males, 15
298 females, and 100 nymphs-2 per pig and per infestation (Table 3). As in the previous experiment,
299 the whole fraction of membrane proteins induced a protective response that reduced the
300 fecundity of females by more than 50%, and killed up to 68% of nymphs-2. Regarding the sub-
301 fractions, the anionic proteins did not induce any protection whereas the cationic and the neutral
302 proteins induced both the same protective effect and with almost the same intensity; that is, a

303 drop in the fecundity of the females between 30% and 62%, and the death of up to 25% of
304 immature forms (Table 3). The remaining parameters measured showed values similar to the
305 controls in all groups and infestations (data not shown).
306
307 *3.3. Vaccination of pigs with the Oe45 antigen purified by electroelution and with the whole*
308 *fraction of membrane proteins: humoral response, antigens recognized by the vaccinated*
309 *animals, and effect of the response in O. erraticus.*
310
311 As can be observed in Fig. 3(A), the Oe45 antigen administered alone induced a
312 humoral response almost as high as that induced by the whole membrane protein fraction, from
313 which it was purified and where it accounted for the 4% of the protein mass (Fig. 3(B)). Pigs
314 vaccinated with Oe45 recognized only this antigen on the whole GME (Fig. 3(C)) whereas pigs
315 vaccinated with the whole fraction of membrane proteins recognized a similar antigenic pattern
316 to that shown by pigs vaccinated with this same extract in trials 1 and 2.
317 The protective actions of the responses induced in this trial were assessed in two
318 infestations with 25 males, 15 females, 100 nymphs-2 and 100 nymphs-1 per pig and per
319 infestation (Table 4). As in trials 1 and 2, the response against the membrane protein fraction
320 reduced the fecundity of females by nearly 50% and killed between 65.1% and 78% of the
321 nymphs. The Oe45 antigen also induced the same protective effects but with less intensity, that
322 is, a 35%-42% reduction in the fecundity of females and the death of about 25% of the nymphs.
323
324 *3.4. Expression of the Oe45 antigen along the trophogonic cycle.*
325
326 The sera of the pigs vaccinated with Oe45 in trial 3 were used as a specific probe to
327 assess the presence of Oe45 in the GMEs obtained from fasted adults and from fed adults taken
328 at different times along the period of blood digestion. Fig. 4 shows that the Oe45 antigen was
329 present in the GME from fasted specimens as well as in the GME from specimens taken at any

time of their trophogonic cycle. However, Oe45 was clearly more abundant in the GME from specimens taken between 6 and 72 h p.f., and particularly between 48 and 72 h p.f.

4. Discussion

As indicated in the Introduction section, our aim in this work was to isolate, characterize and identify the protective antigen(s) from the crude extract of midgut membranes (GME) of *O. erraticus*. Since the GME obtained from adult ticks taken at 72 h p.f. (GME72h) was richer in protective antigens than the GME from fasted adults and even from adults taken at 6 h p.f. (Manzano-Román et al., 2006) we used GME72h as the starting material for the isolation and purification of the antigens. GME72h is an extract with a complex composition in which the sera from protected animals recognise many antigens, such that any of them could, in principle, be responsible for the protection. With a view to progressively pinpointing the range of potential protective antigens, the GME72h was subjected to successive biochemical fractionations and the resulting simpler protein fractions were inoculated in pigs.

In the first fractionation, carried out as a function of the solubility of the GME72h components, we observed that although GME72h is a membrane extract theoretically free of water-soluble remains, it still had 9% of water-soluble proteins (Fig. 2(B)). Although according to Astigarraga et al. (1995) these water-soluble proteins have no protective value, they were, however, administered to a batch of pigs before being fully discarded (trial 1). As expected, the response induced by this fraction was not protective at all (Table 2). Neither was protective the response induced by the fraction containing TX-100-insoluble components. This lack of protection was not surprising since this fraction would contain essentially insoluble proteins from the basal membrane lying beneath midgut epithelial cells (Balashov, 1972), such that these proteins are not directly exposed to the host's immune effectors (antibodies and complement) ingested with blood. Unlike the previous fractions, the one formed by the components solubilised with TX-100 induced a protective response similar to that of the complete GME (Table 2), indicating that it was indeed this fraction that contained the protective antigens. Since

this fraction is basically formed by TX-100-soluble membrane proteins (Van Renswoude and Kempf, 1984; Hjelmeland, 1990) it is reasonable to surmise that the protective antigens would be membrane proteins and that they would be expressed on the luminal membrane of midgut epithelial cells, where they can be readily targeted by the host antibodies and complement ingested with blood. Nevertheless, this presumed luminal expression of the protective antigens must be demonstrated directly (i.e., by immunolocalization) before any firm conclusions can be drawn.

Since the membrane fraction continued to be somewhat complex and still displayed many antigens recognised by the protected animals (Fig. 1(B), 1(C)), it was subjected to further fractionation, in this case as a function of the electrical charge of its components, after which we examined the protective value of the new fractions. The results of this experiment (trial 2) revealed, on one hand, that the anionic proteins induced weak responses, lacking protective value, and -on the other- that there were protective antigens in both the cationic and neutral protein fractions (Fig. 2 and Table 3).

At first sight, the weak response induced by the anionic fraction could be attributed to the lower amount of anionic protein administered per dose (10 µg) as compared to the doses of cationic (100 µg) and neutral fractions (50 µg). However we surmise that this was not the cause owing to the following reasons: first, because the anionic fraction contained only two bands (Fig. 2(B)), which suggests that we would have administered close to 5 µg of each anionic band per dose, and, second, because such a dose would be sufficiently high to induce a strong humoral response, as was actually the case of the purified 45 kDa band (see below). Instead, the weak and non-protective anti-anionic response could be attributed to a low immunogenicity of the anionic proteins. Evidently, demonstration of this would have required new immunizations and immunoassays with equivalent amounts of antigen fraction preparations. These assays were precluded by the very tedious, time-consuming and low-throughput procedure for the preparation of the fractions. Consequently we focused our attention on the two fractions that induced a protective immune response: namely, the cationic and neutral proteins.

Interestingly, trial 2 showed that although the fraction of cationic proteins was the most complex (Fig. 2(B)), only one of its components -that of 45 kDa- was recognized by the animals vaccinated -and protected- with this fraction (Fig. 2(C)). This result strongly suggested that this antigen of 45 kDa, then designated Oe45, could be responsible for the protection induced by this fraction.

In contrast, the pigs vaccinated with the neutral proteins recognised at least five antigens in the homologous fraction. Since one of these antigens had a molecular weight of 45 kDa, it was possible that that antigen could be an isoform of the cationic Oe45 or, at least, a cross-reactive protein of equal weight. To check this, we performed a crossed Western blot, which revealed that the only antigen recognised in both crossed combinations was the 45 kDa band, thus confirming that the two 45 kDa proteins shared epitopes (Fig. 2(D)). This finding supported the notion that both bands might be isoforms of the same protein (although confirmation of this point requires further studies) and, therefore, that the neutral Oe45 could be responsible for the protection induced by the fraction of neutral proteins. Were this indeed the case, it would also account for the lower degree of protection induced by the cationic and neutral protein fractions (around 25% of nymph mortality) as compared to the protection induced by the whole membrane protein fraction (up to 68% of nymph deaths) (Table 3), since with each fraction a smaller dose of protective molecules would have been administered than with the complete fraction of membrane proteins (see Table 1).

Definitive confirmation that Oe45 was indeed the protective antigen came from trial 3, after vaccinating a new batch of pigs with 5 µg/dose of Oe45 purified by electroelution and observing that it induced a protective response that was similar, although less potent, to that induced by 150 µg/dose of the complete fraction of membrane proteins (Table 4). Since Oe45 represents 4% of the protein mass of the whole membrane protein fraction, the pigs vaccinated with 150 µg/dose received the equivalent of about 6 µg of Oe45, very close to the dose of purified Oe45. Accordingly, we believe that the lower degree of protection obtained with the purified antigen was not due to a difference in the dose but to the probable loss of epitopes

412 caused by the denaturing of the proteins during their purification by electroelution from
413 polyacrylamide gels.

414 Once the protective antigen had been unveiled, we checked whether, according to the
415 hypothesis pointed out in the Introduction, its expression was induced by the ingestion of blood
416 by the parasites. In this sense, the results shown in Fig. 4 seem to confirm such a hypothesis
417 since although Oe45 was constitutively expressed at low levels throughout the trophogonic
418 cycle, its expression level increased notably between 6 and 72 h after blood ingestion. This
419 observation confirmed that, as anticipated by us, GME72h is richer in protective antigens than
420 the GME from fasting specimens or those taken at 6 h p.f. and, additionally, it would explain
421 why the parasites fed on the vaccinated swine died between 48 and 72 h p.f., since it was during
422 that period when they exposed a greater number of target molecules on their midgut, thereby
423 enhancing the damage derived from the binding of the host antibodies and complement to the
424 gut wall (see Manzano-Román et al., 2006). Since no data on Oe45 expression levels were
425 collected between 72 h p.f., when the highest Oe45 level was observed, and 15 days, when
426 Oe45 again showed basal expression levels, we simply do not know how Oe45 expression
427 levels change along this time period. However, having in mind that the nymphs that did not die
428 in the first 72 h p.f. survived with no apparent damage and moulted normally, it would be
429 reasonable to think that after 72 h p.f. the Oe45 expression levels would decrease rapidly.

430 Finally, although we attempted the molecular identification of the purified Oe45 by
431 MALDI-TOF mass spectrometry and Edman N-terminal sequencing we failed to obtain any
432 result. This outcome was most probably due to insufficient protein purity. Consequently,
433 forthcoming research will address the molecular identification and cloning of Oe45 in order to
434 obtain it as a recombinant protein and assess its protective value in combination with adjuvants
435 permitted for use in animal vaccines instead of Freund's adjuvants.

436

437 **5. Conclusions**

438

The antigen responsible for the protective response induced by GME from *O. erraticus* seems to be a membrane protein presumably expressed on the luminal membrane of midgut epithelial cells. This protein, designated Oe45, has a molecular weight of 45 kDa and splits into two differently charged bands -cationic and neutral- both of them able to induce protective responses. Although the Oe45 protein is constitutively expressed at low levels throughout the trophogonic cycle, its expression is up-regulated by the ingestion of blood, as suggested by the higher levels observed between 6 and 72 h post-feeding. The molecular identification and cloning of the Oe45 will be addressed in future work.

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498

499 Table 1.
500 Vaccination trials
501

Trial No.	Group	Antigen	Dose (µg)	Adjuvant[b] first-second-third	Infestation with
1	Control	None	-	FCA-FIA-PBS	25 males
	1	GME[a]	150	FCA-FIA-PBS	15 females
	2	Water-soluble proteins	50	FCA-FIA-PBS	100 nymph-2
	3	Membrane proteins	150	FCA-FIA-PBS	
	4	Insoluble proteins	150	FCA-FIA-PBS	
2	Control	None	-	FCA-FIA-PBS	25 males
	1	Membrane proteins	150	FCA-FIA-PBS	15 females
	2	Cationic membrane proteins	100	FCA-FIA-PBS	100 nymph-2
	3	Anionic membrane proteins	10	FCA-FIA-PBS	
	4	Neutral membrane proteins	50	FCA-FIA-PBS	
3	Control	None	-	FCA-FIA-PBS	25 males
	1	Membrane proteins	150	FCA-FIA-PBS	15 females
	2	Oe45	5	FCA-FIA-PBS	100 nymph-2 100 nymph-1

502
503 [a] GME, gut membrane extract.
504 [b] Adjuvants administered in the first, second and third antigen doses; FCA and IFA, complete and
505 incomplete Freund's adjuvant; PBS, phosphate buffered saline.
506

Table 2.

Trial 1. Mean ± SE of the parameters measured in *Ornithodoros erraticus* specimens fed on control pigs and on pigs vaccinated with GME and its fractions obtained by differential solubilisation along the two infestations.

Parameter	Developmental stage	Group/Antigenic extract	Infestation	
			First	Second
No. eggs laid	Females	Control	89.9 ± 7.3	76.6 ± 5.8
		GME	56.2 ± 6.7*	48.5 ± 4.5*
		Water-soluble proteins	73.9 ± 5.8	70.9 ± 8.2
		Membrane proteins	51.0 ± 6.9*	41.1 ± 10.4*
		Insoluble proteins	79.8 ± 9.1	69.9 ± 8.7
% Viability	Eggs	Control	99.3 ± 9.8	58.4 ± 6.2
		GME	53.8 ± 5.4*	15.1 ± 13.5*
		Water-soluble proteins	95.3 ± 12.3	49.4 ± 29.1
		Membrane proteins	35 ± 17.5*	19.3 ± 12.8*
		Insoluble proteins	58.3 ± 25.5*	23.9 ± 18.8
% Mortality (72 hours p.f.)	Nymphs-2	Control	7 ± 2.4	5.1 ± 1.1
		GME	71.8 ± 15.3*	74.3 ± 17.2*
		Water-soluble proteins	5.3 ± 1.4	3 ± 2.1
		Membrane proteins	66.6 ± 19*	60.1 ± 21.3*
		Insoluble proteins	8.6 ± 4.3	13.6 ± 8.4

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

513
514 Table 3.
515 Trial 2. Mean ± SE of the parameters measured in *Ornithodoros erraticus* specimens fed on control pigs
516 and on pigs vaccinated with the fraction of membrane proteins and the sub-fractions containing
517 membrane proteins of different charge along the two infestations.
518

Parameter	Developmental stage	Group/Antigenic extract	Infestation	
			First	Second
No. eggs laid	Females	Control	35 ± 5.5	27.3 ± 3.2
		Membrane proteins	14.1 ± 8.8*	12.9 ± 7.3*
		Cationic	13.3 ± 3.2*	17 ± 3.2*
		Anionic	26.5 ± 6.8	30.1 ± 2.3
		Neutral	19.1 ± 14.3	13.3 ± 12.8
% Viability	Eggs	Control	82.3 ± 6.1	36.5 ± 3
		Membrane proteins	83.6 ± 8.3	0 ± 0*
		Cationic	66.9 ± 12*	17.6 ± 4.6*
		Anionic	74.9 ± 7.8	19.4 ± 7.2
		Neutral	70.8 ± 21.2	22,9 ± 15.3
% Mortality (72 hours p.f.)	Nymphs-2	Control	0.7± 0.6	1 ± 1
		Membrane proteins	68 ± 8.9*	65.7 ± 6.4*
		Cationic	25.6 ± 22*	19.5 ± 2.8*
		Anionic	1.6 ± 0.6	1 ± 1
		Neutral	18.6 ± 13.4*	25.5 ± 14.3*

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

520
521 Table 4.
522 Trial 3. Mean ± SE of the parameters measured in *Ornithodoros erraticus* specimens fed on control pigs
523 and on pigs vaccinated with GME and the electroeluted Oe45 antigen along the two infestations
524

Parameter	Developmental stage	Group/Antigen	Infestation	
			First	Second
No. eggs laid	Females	Control	29.8 ± 11.2	21.1 ± 4
		Membrane proteins	17.7 ± 6.3	9.9 ± 9.1
		Oe45	18.3 ± 3.2	12 ± 3.7
% Viability	Eggs	Control	82.1 ± 6.1	28.9 ± 1.9
		Membrane proteins	81.9 ± 18.8	0 ± 0*
		Oe45	86.9 ± 12	18.5 ± 15.6
% Mortality (72 hours p.f.)	Nymphs-2	Control	0.9± 0.5	0 ± 0
		Membrane proteins	78 ± 10.9*	65.1 ± 7.8*
		Oe45	21.4 ± 23*	26.8 ± 2.8*
	Nymphs1	Control	1.6 ± 1.6	3.1 ± 3.1
		Membrane proteins	71 ± 5.9*	69.2 ± 12.5*
		Oe45	25.4 ± 0.9*	22.9 ± 1.4*

525 * $P < 0.05$ with respect to the control group
526

527

528 **Figure captions**

529

530 Figure 1. Vaccination of pigs with GME (gut membrane extract) and GME fractions obtained
531 by differential solubilisation: water-soluble proteins (Sol.); TX-100-soluble membrane proteins
532 (Memb.); insoluble proteins in 1% TX-100 (Insol.). (A) Anti-GME IgG serum levels (mean OD
533 ± SE) in control and vaccinated pigs. Asterisks indicate the weeks when the antigenic extract
534 was administered. The infestations with *O. erraticus* took place during weeks 5 and 6. (B) SDS-
535 PAGE: band patterns of the GME and the GME fractions. (C) Western blot: antigens recognised
536 on GME and GME fractions by the sera of pigs vaccinated with the homologous extract.

537

538 Figure 2. Vaccination of pigs with the fraction of membrane proteins (Memb.) and the sub-
539 fractions containing differently charged membrane proteins: cationic (+), anionic (-) and neutral
540 (Neu). (A) Anti-membrane protein IgG serum levels (mean OD ± SE) in control and vaccinated
541 pigs. Asterisks indicate the weeks when the antigenic extract was administered. Infestations
542 with *O. erraticus* took place during weeks 5 and 6. (B) SDS-PAGE: band patterns of the
543 membrane proteins and its sub-fractions. (C) Western blot: antigens recognised on the
544 homologous extract by sera of pigs vaccinated with the membrane proteins and its sub-fractions.
545 (D), Western blot: antigens recognised on the cationic proteins by pooled sera from pigs
546 vaccinated with neutral proteins (1), and antigens recognised on the neutral proteins by pooled
547 sera from pigs vaccinated with cationic proteins (2).

548

549 Figure 3. Vaccination of pigs with GME and with the antigen Oe45 purified by gel
550 electroelution. (A) Anti-GME IgG serum levels (mean OD ± SE) in the vaccinated pigs.
551 Asterisks indicate the weeks when the extract was administered. Infestations with *O. erraticus*
552 were carried out during weeks 5 and 6. (B) SDS-PAGE showing the purification of the
553 Oe45antigen: band patterns of the starting GME, its fraction of membrane proteins (Memb.) and

554 electroeluted antigen Oe45. (C) Western blot: antigens recognised on the GME by the sera from
555 pigs vaccinated with the membrane proteins and Oe45 alone.
556
557 Figure 4. (A) SDS-PAGE: band patterns of GMEs obtained from adult ticks before blood
558 ingestion (fasted) and from fed adults taken at different times along the period of blood
559 digestion. (B), Western blot: antigens recognized on the GMEs from fasted adults and from fed
560 adults taken at 6, 48 and 72 hours and 15 and 24 days post-feeding by a pool of sera from the
561 pigs vaccinated with the Oe45 antigen in trial 3.
562

Figure 1

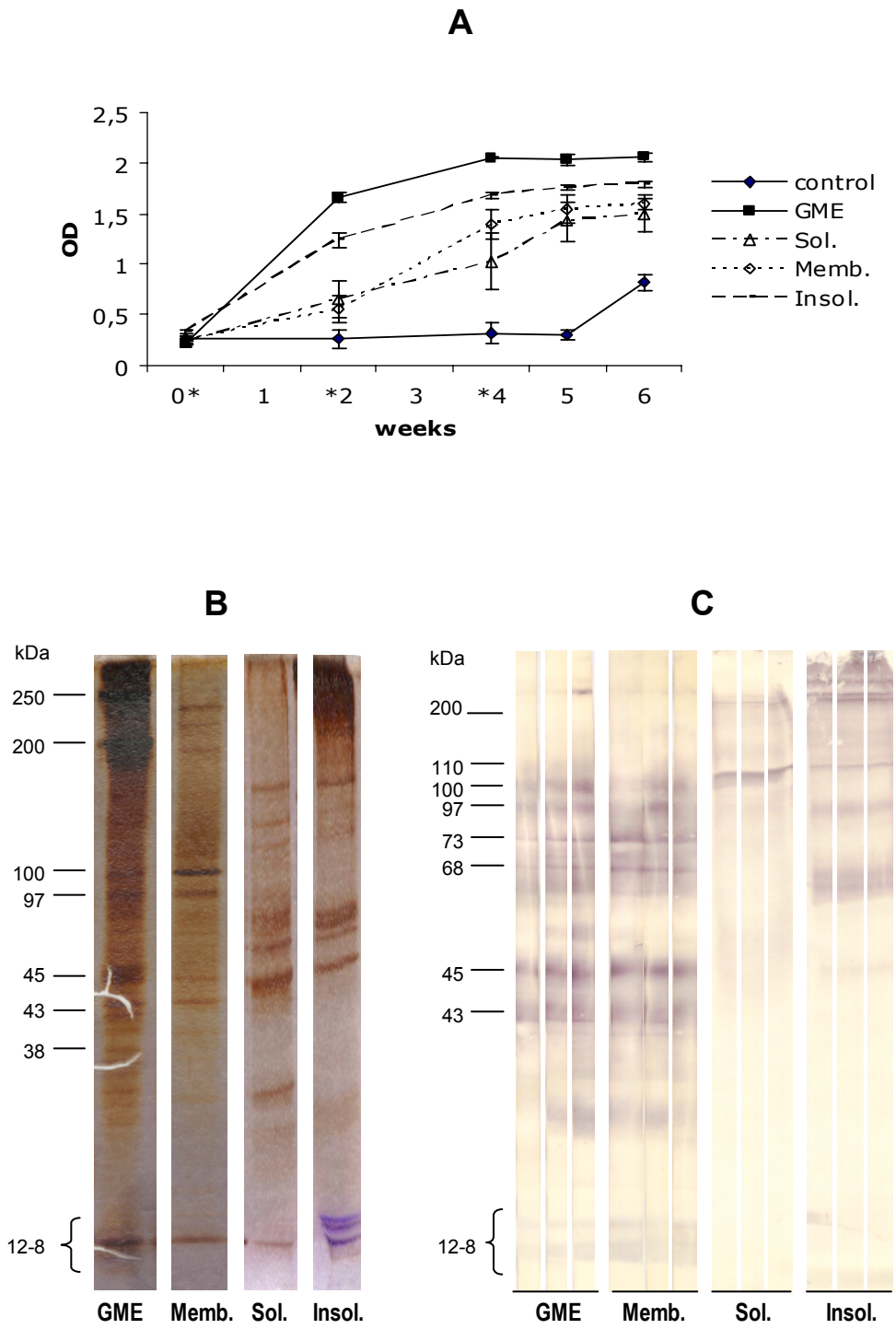
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Figure 2

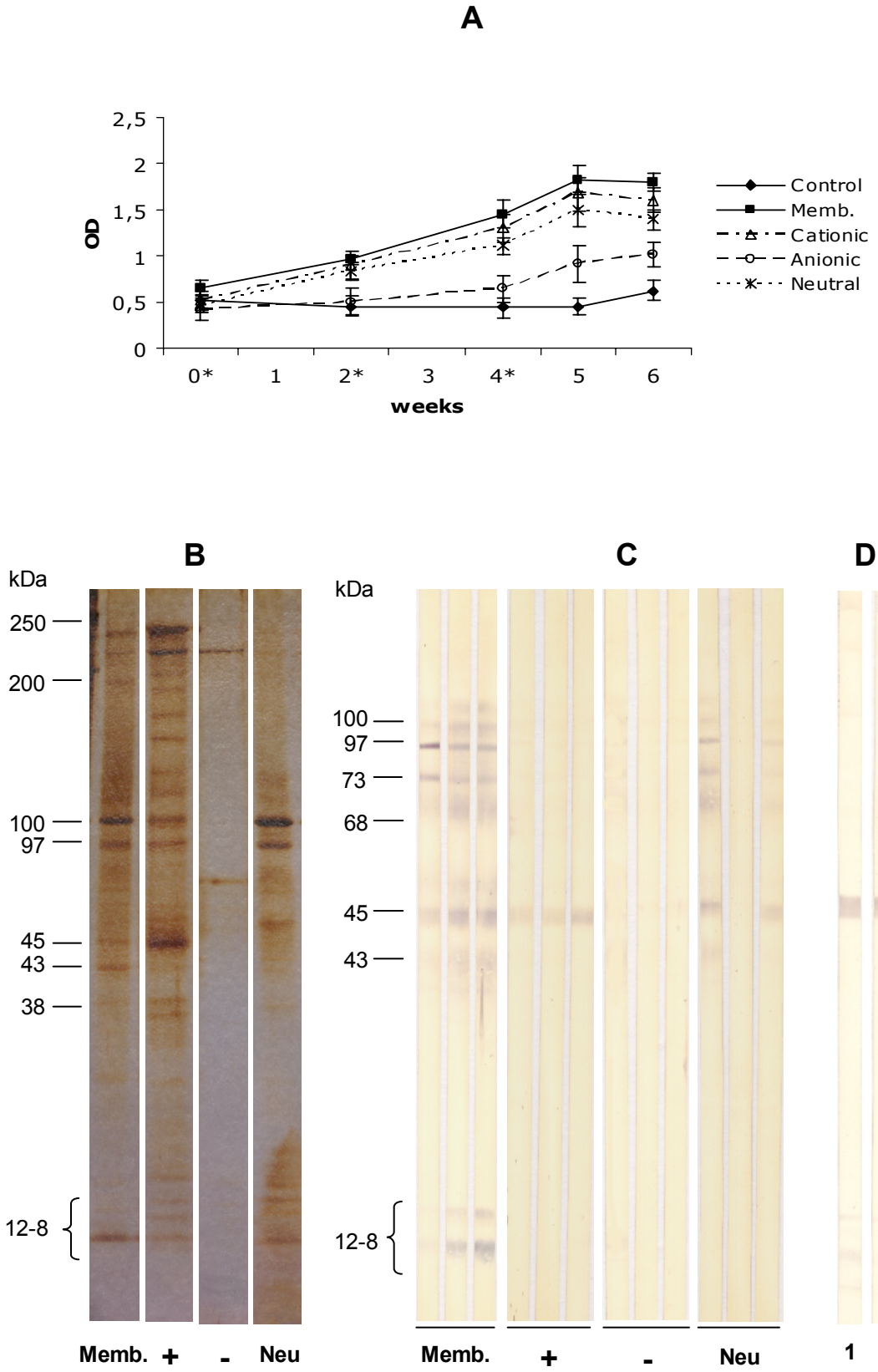
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Figure 3

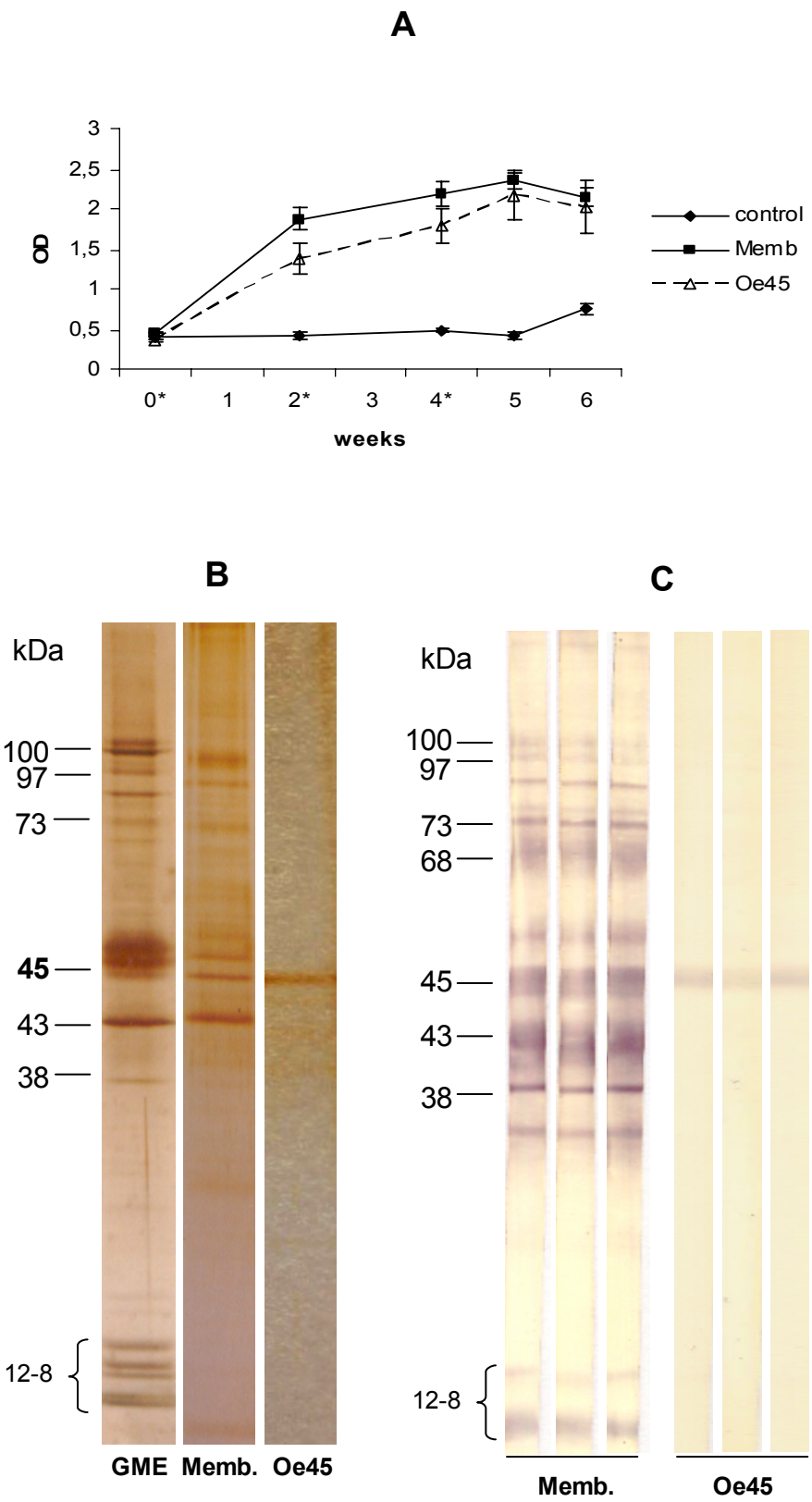
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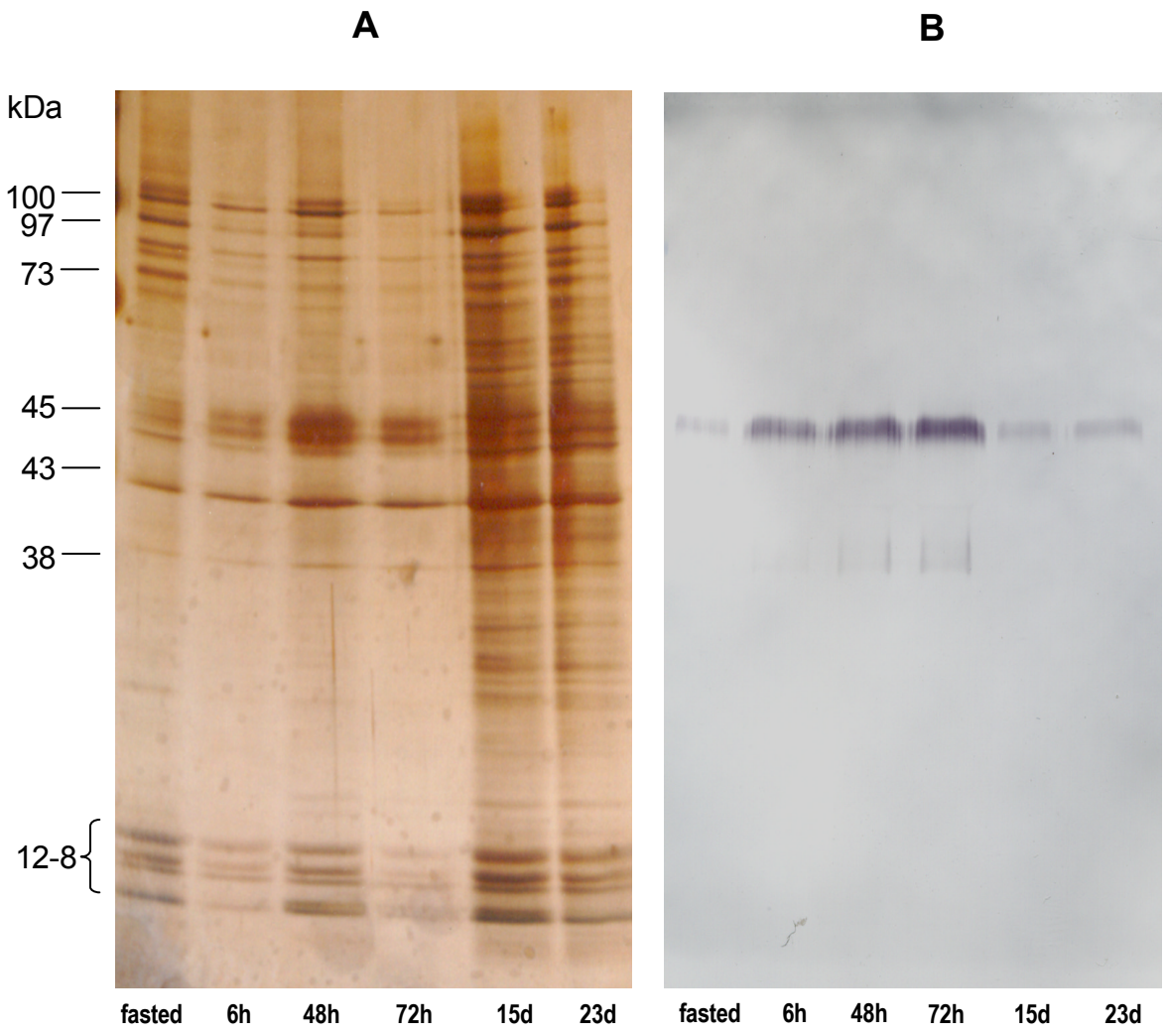
Figure 4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