Research Article

In vitro characterization of adipocyte plasma membrane-associated protein from poultry red mites, *Dermanyssus gallinae*, as a vaccine antigen for chickens

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[1] Abbreviations: PRM, poultry red mite; CDC, complement dependent cytotoxicity; APMAP, adipocyte plasma membrane-associated protein; PBS, phosphate-buffered saline; RNA-Seq, RNA-sequencing; ORF, open reading frame; LCM, laser-capture microdissection; RT-PCR, reverse transcription polymerase chain reaction; *Elf1a1*, elongation factor 1-α 1-like gene; qPCR, quantitative PCR; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; PBS-T, PBS containing 0.05% Tween 20; SDS-PAGE; SDS-polyacrylamide gel electrophoresis.

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Abstract

The poultry red mite (*Dermanyssus gallinae*; PRM) is a blood-sucking ectoparasite of chickens that is a threat to poultry farming worldwide and significantly reduces productivity in the egg-laying industry. Chemical acaricides that are widely used in poultry farms for the prevention of PRMs are frequently ineffective due to the emergence of acaricide-resistant PRMs. Therefore, alternative control methods are needed, and vaccination is a promising strategy for controlling PRMs. A novel adipocyte-plasma membrane-associated protein-like molecule (Dg-APMAP) is highly expressed in blood-fed PRMs according to a previous RNA sequencing analysis. Here, we attempted to identify the full sequence of Dg-APAMP, study its expression in different life stages of PRMs, and evaluate its potential as a vaccine antigen. *Dg-APMAP* mRNA was expressed in the midgut and ovaries, and in all life stages regardless of feeding states. Importantly, *in vitro* feeding of PRMs with plasma derived from chickens immunized with the recombinant protein of the extracellular region of Dg-APMAP significantly reduced their survival rate in nymphs and adults, which require blood meals. Our data suggest that the host immune responses induced by vaccination with Dg-APMAP could be an effective strategy to reduce the suffering caused by PRMs in the poultry industry.

Keywords: poultry red mite, adipocyte plasma membrane-associated protein, vaccine, Dg-

1. Introduction

Poultry red mites (*Dermanyssus gallinae*; PRM) are deleterious hematophagous ectoparasites of chickens. Mass infestation by PRMs causes various harmful effects in chickens, such as anemia, resulting in significant loss of productivity in poultry farming. Current prevention of PRMs with acaricides is usually insufficient because the mites hide in cracks and crevices after blood sucking, and due to the emergence of drug-resistant PRMs. Therefore, establishment of an alternative control method for PRMs is strongly needed. Recently, vaccines have emerged as a promising strategy for the prevention of ectoparasites including PRMs, and several studies have reported the antigen candidates and their efficacies [1–3]. However, vaccine efficacies have not been found to be sufficient in the practical application in the field [4]. Thus, vaccination strategies need to be improved and more effective vaccine antigens should be investigated to increase vaccine efficacies.

In ticks such as *Ixodes ricinus* and *Boophilus microplus*, two types of vaccine antigens can be considered based on their targets. The first target includes proteins secreted from salivary glands that facilitate blood sucking and attachment to the host skin by suppressing host immune responses [5,6]. The second includes antigens expressed in the midgut; since the sucked blood is accumulated in the midgut, midgut antigens can be efficiently exposed to antibodies contained in the blood sucked from immunized animals [7]. Whereas ticks infest hosts and continue blood

61 feeding for a longer period (3–10 days or more [8]), PRMs infest chickens for shorter periods (a
62 few minutes to one hour) and do not stay on the host body after blood sucking. In addition, PRMs
63 are intermittent feeders and suck blood repetitively during different life stages [9]. Therefore,
64 midgut proteins may be more suitable as vaccine antigens against PRMs.
65 The ideal vaccine antigens for an anti-PRM vaccine should have high expression levels and
66 should be constitutively expressed in all life-stages (or at least in the blood-fed stages of PRMs).
67 Additionally, antigens expressed on the plasma membrane are more desirable as vaccines
68 targeting plasma membrane proteins have shown tremendous efficacies in ticks; for instance,
69 although the physiological function of a transmembrane protein, BM86, is unknown, the
70 immunization of cattle with BM86 and its orthologs shows remarkable acaricidal effects on the
71 ticks *B. microplus* and *Hyalomma a. anatolicum*, respectively [10,11]. Collectively, plasma
72 membrane proteins expressed in the midgut with high expression levels would be suitable as
73 targets of vaccine antigens.
74 To investigate such molecules, we have previously conducted comparative transcriptome analyses
75 of blood-fed and starved PRMs [12] and identified a novel adipocyte plasma membrane-
76 associated protein-like transcript (*Dg-APMAP*) with high expression intensities in a blood-fed
77 state. In mammals, APMAP is expressed in a variety of tissues and organs including the liver and
78 pancreas and plays a role in adipocyte differentiation and carbohydrate metabolism, although the

79 physiological function of APMAP is yet to be fully understood [13,14]. This study aimed to
80 identify the full sequence of *Dg-APMAP,* analyze its expression profiles in each life-stage of
81 PRMs, and evaluate the potential of APMAP as a vaccine antigen.
82
83 **2. Materials and methods**
84 2.1. Ethics statement
85 Animal experiments were conducted according to relevant guidelines and regulations of Choka
86 Research Institute, Vaxxinova Japan K.K. (Tochigi, Japan), and the protocol was approved by the
87 Animal Care and Use Committee, Vaxxinova Japan K.K. (Approval number: AR007-C-EX-036).
88
89 2.2. PRM samples
90 Mixed developmental stages and sexes of PRMs were obtained from an egg-laying poultry farm
91 in Japan. PRMs were collected in a TubeSpin Bioreactor 600 bottle (TPP Techno Plastic Products
92 AG, Trasadingen, Switzerland). A few of the dark red, round PRMs were collected as “blood-fed
93 PRMs” in 1,200 μL extra-long filter tips (WATSON Bio Lab, Tokyo, Japan) within 2 days of
94 sample collection. The remaining PRMs were kept at 25 °C in 70% humidity for a 1-week period
95 and designated “starved PRMs.” Some blood-fed and starved PRMs were fixed with 70% ethanol,
96 and eggs, larvae, protonymphs, deutonymphs, and adults were segregated under microscopic

97 observation. The remaining PRMs were kept at 4 °C in 70% humidity or stored at -80 °C until
98 use for the *in vitro* feeding assays.
99
100 2.3. RNA isolation and cDNA synthesis
101 Segregated PRM samples were suspended in phosphate-buffered saline (PBS) and thoroughly
102 homogenized using a 1.5-mL homogenization pestle for a 1.5-mL microcentrifuge tube (Scientific
103 Specialties, Inc., Lodi, CA, USA) in 600 μL of buffer RLT Plus (RNeasy Plus Mini Kit, Qiagen,
104 Hilden, Germany). Total RNA was isolated using an RNeasy Plus Mini Kit according to the
105 manufacturer's instructions. cDNA was then synthesized from the isolated RNA with PrimeScript
106 reverse transcriptase (Takara Bio Inc., Shiga, Japan) using 200 pmol of oligo (dT)18 primer
107 (Hokkaido System Science, Hokkaido, Japan).
108
109 2.4. Identification of the cDNA sequence of *Dg-APMAP* in PRMs
110 The transcript of *Dg-APMAP* was identified using the contigs obtained from RNA sequencing
111 (RNA-seq) analysis (BioSample accession numbers: SAMD00228960, SAMD00229086) [12].
112 To determine the nucleotide sequence of the open reading frame (ORF) of *Dg-APMAP*, PCR was
113 performed with Ex-Taq polymerase (TaKaRa Bio Inc.) using the primer set DgAPMAP-ORF-F
114 and DgAPMAP-ORF-R (Table 1). The amplicon was then inserted into the pSMART2IF

115 linearized vector (TaKaRa Bio Inc.). The ORF sequence was confirmed by sequencing analysis
116 using the primer sets DgAPMAP-seq-F1-3 and DgAPMAP-seq-R1-3 (Table 1) and the 8-capillary
117 Beckman CEQ GeXP automated sequencer (Beckman Coulter, Brea, CA, USA) following the
118 manufacturers' instructions. The transmembrane site and the strictosidine synthase domain were
119 predicted with the software systems TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/)
120 and InterProScan program v5.32-71.0 (http://bioinf.wehi.edu.au/edgeR/), respectively. The
121 phylogenic tree was constructed with MEGA software version X [15], using the neighbor-joining
122 method with 1,000 bootstrap replicates.
123
124 2.5. Laser-capture microdissection
125 The cDNAs of the salivary glands, midguts, and ovaries of the PRMs were synthesized using
126 tissue samples extracted by laser-capture microdissection (LCM) as previously described [16].
127 Briefly, mixed life stages of starved PRMs were fixed with carnoy solution, embedded in paraffin,
128 and cut into 5 μm sections. Sections were then mounted on glass slides precoated with LCM films
129 (Meiwafosis, Tokyo, Japan) and stained with 1 % toluidine blue, and each tissue was dissected
130 with Ls-Pro300 (Meiwafosis). Total RNA was extracted from the dissected tissues using
131 RNAqueous®-Micro kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the
132 manufacturer's protocol. cDNA was synthesized with the SuperScript™ first-strand synthesis

133 system for RT-PCR (Invitrogen) using 300 pmol of random hexamer primers (Hokkaido System
134 Science).
135
136 2.6. Gene expression analysis of *Dg-APMAP*
137 Gene expression of *Dg-APMAP* in protonymphs, deutonymphs, and adults in blood-fed and
138 starved states, as well as in eggs and larvae, was examined by RT-PCR with Ex-Taq polymerase
139 (TaKaRa Bio Inc.) using the synthesized cDNA described above. In addition, to examine the
140 expression of *Dg-APMAP* in different tissues, RT-PCR was performed using the LCM samples.
141 The specific primers DgAPMAP-RT-F and DgAPMAP-RT-R (Table 1) were used. The *elongation*
142 *factor 1-α 1*-like gene (*Elf1a1*) was amplified as an internal control using the primers Elf1a1-F
143 and Elf1a1-R [17]. Since RNA samples extracted from salivary glands were of low quality or in
144 low quantity, target genes in salivary glands were detected by nested PCR using the same primer
145 set.
146
147 2.7. Quantitative PCR (qPCR)
148 The mRNA expression of *Dg-APMAP* in each life stage, was quantified by qPCR using the cDNA
149 synthesized from protonymphs, deutonymphs, and adults in blood-fed and starved states, as well
150 as that from larvae, with the LightCycler480® System II (Roche Diagnostics, Mannheim,

151 Germany) and the TB Green Premix DimerEraser (TaKaRa Bio Inc.) according to the
152 manufacturer's instructions. The *Dg-APMAP* gene was amplified using the primers DgAPMAP-
153 RT-F and DgAPMAP-RT-R, and the primers Elf1a1-F and Elf1a1-R were used to amplify the
154 *Elf1a1* gene as the internal control. The cycling conditions consisted of initial denaturation at
155 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. To
156 evaluate primer specificity, a final melting curve analysis was performed from 65 °C to 95 °C at
157 a rate of 0.1 °C/s. To construct standard curves for quantification, serial dilutions of T-vector
158 pMD20 (TaKaRa Bio Inc.)-inserted *Dg-APMAP* or *Elf1a1* were used. Each sample was tested in
159 triplicate, and *Dg-APMAP* mRNA expression was presented as the ratio obtained by dividing the
160 concentration of the *Dg-APMAP* mRNA by that of *Elf1a1* mRNA.
161
162 2.8. Preparation of recombinant Dg-APMAP
163 The recombinant protein of the extracellular region of Dg-APMAP was prepared as a fusion
164 protein with His-tag using the BIC system (TaKaRa Bio Inc.). To investigate the optimal size and
165 position of fragments to promote expression and secretion of recombinant proteins, we prepared
166 five expression plasmids containing different regions of the extracellular region of *Dg-APMAP*
167 (Dg-APMAP-N-his: N-terminal side of the extracellular region, Dg-APMAP-C-his: C-terminal
168 side of the extracellular region, Dg-APMAP-N2-his: N-terminal side of the extracellular region

without the strictosidine synthase domain, Dg-APMAP-D-his: strictosidine synthase domain, Dg-APMAP-C2-his: C-terminal side of the extracellular region without the strictosidine synthase domain) (Supplementary figure 2) and the entire extracellular region (Dg-APMAP-his), using the specific primer sets (Table 1). PCR was performed with KOD-Plus-Neo (TOYOBO Co., Ltd., Osaka, Japan) to amplify the target regions, and the fragments were introduced into *Brevibacillus* competent cells (Takara Bio Inc.). The gene fragments were inserted into the cloning site of pBIC4 vector (Takara Bio Inc.) by homologous recombination in the transformed bacteria. The transformed bacteria containing the expression plasmids were cultured in TM medium for 48 h at 32 ˚C following the manufacturer's instructions. The recombinant protein of Dg-APMAP-N-his was purified from the cultured supernatant using the TALON® metal affinity resin (Clontech Laboratory, Mountain View, CA, USA). The recombinant proteins were eluted with the elution buffer (0.1 M Tris, 276 mM NaCl, 5.4 mM KCl, 150 mM imidazole (pH 7.4)), and the eluted fractions were dialyzed with PBS using SnakeSkin™ dialysis tubing, 10 K MWCO (Thermo Fisher Scientific) overnight at 4 °C. To confirm the protein expression and purification, the obtained proteins were denatured by lysing in 2× SDS buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, and 20% glycerol) and boiled for 5 min, separated using 12% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The concentration of the proteins was determined with the Pierce™

187 BCA protein assay kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

188

189 2.10. Immunization of chickens with Dg-APMAP-N-his

190 To prepare the immune plasmas, specific pathogen-free chickens (VALO BioMedia) were
191 immunized with Dg-APMAP-N-his. The purified Dg-APMAP-N-his was mixed with light liquid
192 paraffin as the adjuvant (20 μg/mL). Briefly, 3,900 μL of light liquid paraffin HICALL M-52
193 (KANEDA Co., Ltd., Tokyo, Japan) was mixed with 300 μL of Rheodol AO-10V (Kao
194 Corporation, Tokyo, Japan) and 240 μL of 40% polysorbate 80 (FUJIFILM Wako Pure Chemical
195 Corporation) and agitated at 2,600 rpm for 2 min using Heidolph homogenizer DIAX 900 (Sigma-
196 Aldrich, St. Louis, MO, USA). After standing for 4 min, 1,560 μL of purified recombinant
197 APMAP prepared before (77 μg/mL) was added and agitated at 2,600 rpm for 2 min. After
198 standing for 4 min, the reagents were agitated again at 2,600 rpm for 2 min for emulsification.
199 The uniformity of droplets was confirmed under microscopic observation. Four chickens were
200 subcutaneously immunized with 20 μg of Dg-APMAP-N-his at 3 weeks of age (IM1, IM2, IM3,
201 and IM4). Four weeks later, the heparinized blood was collected, and the immune plasma was
202 isolated by centrifugation at 2,000 × G for 10 min. In addition, plasma was isolated from 4
203 unimmunized chickens of the same age (UN1, UN2, UN3, and UN4). All the procedures were
204 conducted at Vaxxinova Japan K.K., Tokyo, Japan, following the standard operating procedures.

205

206 2.11. Enzyme-linked immunosorbent assay (ELISA)

207 Antibody titers in the immune plasmas were determined by ELISA. Purified Dg-APMAM-N-his
208 was coated on the wells of 96-well plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan; 100
209 ng/well) for 16 h with carbon-bicarbonate buffer. After washing each well three times with PBS,
210 PBS containing 0.05% Tween 20 (PBS-T) with 1% bovine serum albumin was added and
211 incubated at 37 ˚C for 2 h. Wells were then washed five times with PBS-T, and immune plasmas
212 diluted 2,000×, 4,000× 8,000×, 16,000×, 32,000×, and 64,000× with PBS were added. After 1 h
213 incubation at room temperature, the wells were washed five times with PBS-T and incubated with
214 anti-chicken IgY peroxidase rabbit antibody (Sigma-Aldrich) diluted with PBS-T for 1 h at room
215 temperature. Finally, the wells were washed five times with PBS-T and reacted with the TMB one
216 component substrate (Bethyl Laboratories, Montgomery, TX, USA) for 20 min at room
217 temperature in the dark. The reaction was quenched with 0.18 M H_2SO_4 and the absorbance was
218 measured at 450 nm. The assay was performed in duplicate.

219

220 2.12. Western blotting

221 The production of specific antibodies against Dg-APMAP-N-his was examined by western
222 blotting. Purified Dg-APMAP-N-his was separated using 12 % SDS-polyacrylamide gel and then

223 transferred to polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA, USA).
224 The membrane was blocked overnight at 4 °C with PBS-T containing 1% skim milk. The
225 membranes were incubated at room temperature with the isolated immune plasmas, washed three
226 times with PBS-T, and incubated at room temperature with anti-chicken IgY peroxidase rabbit
227 antibody (Sigma-Aldrich). Finally, the membranes were incubated with Immobilon Western
228 Chemiluminescent HRP Substrate (Merck Millipore) to visualize the peroxidase signal.
229
230 2.13. *In vitro* feeding assay
231 Heparinized fresh chicken blood was collected from healthy chickens maintained at the Field
232 Science Center for Northern Biosphere, Hokkaido University, and incubated at 40 ˚C before use.
233 Plasmas from Dg-APMAP-N-his- immunized and unimmunized chickens were pooled separately.
234 After centrifugation of fresh chicken blood at 2,000 × G for 10 min, the plasma from fresh chicken
235 blood was discarded and replaced with equal volumes of pooled undiluted plasma from immune
236 or unimmunized chickens. Approximately 100 starved PRMs of mixed developmental stages were
237 collected in artificial feeding devices [17] and blood feeding was performed for 4 h at 40 ˚C in
238 dark, humid condition with modest shaking. Following this, only blood-fed PRMs were collected
239 in Pasteur pipettes (day 0) and kept at 25 °C in 70 % humidity during the observation period. The
240 numbers of dead PRMs were monitored for 1 week, and the anti-PRM property of Dg-APMAP-

241 N-his immunization was evaluated based on the survival rate of PRMs (calculated by dividing the
242 number of dead PRMs by the number of blood-fed PRMs). Adults and nymphs were collected
243 separately and the survival rate was analyzed independently. In addition, to evaluate the effects
244 of immune plasmas on PRM fecundity, the numbers of newborn larvae and protonymphs were
245 counted at day 7 and the reproductive capacity was calculated by dividing those by the number
246 of fed adults at day 0.
247
248 2.14. Statistical analysis
249 Differences were analyzed using Steel-Dwass test for gene expression analyses. To compare PRM
250 mortality between the immunized and unimmunized groups after *in vitro* feeding, we generated
251 Kaplan–Meier curves and performed a log-rank test. Additionally, we performed between-group
252 comparisons of the mortality and reproductive capacity of PRMs on each day using Fisher's exact test.
253 Moreover, the odds ratio and 95% confidence interval (CI) were estimated. p-values of < 0.05 and $<$
254 0.01 were considered statistically significant.
255
256 **Results**
257 3.1. Cloning and sequence analysis of *Dg-APMAP*
258 The transcript of an adipocyte plasma membrane-associated protein-like molecule was obtained

259 from the data of our RNA-seq analysis [12] and was designated as Dg-APMAP. The nucleotide
260 sequence of ORF was determined by PCR amplification and sequencing (Accession No.
261 LC647275). The expression intensity of *Dg-APMAP* was significantly higher in blood-fed PRMs
262 compared to that in starved PRMs (Supplementary table 1). The deduced amino acid sequence of
263 *Dg-APMAP* contained a putative transmembrane domain at positions 7–29 and a strictosidine
264 synthase domain site at positions 167–252 (Fig. 1A). Phylogenetic analysis using APMAP of the
265 chicken, mammals, ticks, and mites revealed that *Dg-APMAP* was close to that of the mite
266 *Galendromus occidentalis* (Fig. 1B).
267 .
268 3.2. Gene expression profile of *Dg-APMAP*
269 For the gene expression profiles of *Dg-APMAP*, we first examined the expression of *Dg-APMAP*
270 mRNA in the different life-stages and feeding states. *Dg-APMAP* mRNA was expressed in all the
271 life stages, regardless of blood-feeding state (Fig. 2A). qPCR analysis showed no significant
272 difference in the expression levels of *Dg-APMAP* mRNA in protonymphs, deutonymphs, and
273 adults in blood-fed and starved states, as well as in larvae (Fig. 2B). To examine the expression
274 of *Dg-APMAP* in different tissues, we performed LCM and RT-PCR/nested PCR using the tissue
275 samples from salivary glands, midguts, and ovaries. *Dg-APMAP* expression was observed in the
276 midguts and ovaries, but not in salivary glands (Fig. 2C). Collectively, *Dg-APMAP* appeared to

277 be constitutively expressed in all the life stages, and notably, it was expressed in the midguts and
278 ovaries.
279
280 3.3. Preparation of recombinant Dg-APMAP protein as a vaccine antigen
281 To evaluate the potential of Dg-APMAP as a vaccine antigen, we expressed the extracellular
282 region of Dg-APMAP (Dg-APMAP-his) using the BIC system. Although Dg-APMAP-his was
283 expressed in the cultured bacteria, secretion levels of the recombinant Dg-APMAP-his into the
284 supernatants were low (Supplementary figure 1). To promote the secretion of the recombinant
285 protein into the cultural supernatants, we partially expressed the extracellular region of Dg-
286 APMAP and assessed their secretory efficiencies into the supernatants. Among the 5 recombinant
287 proteins tested (Dg-APMAP-N-his, Dg-APMAP-c-his, Dg-APMAP-N2-his, Dg-APMAP-D-his,
288 Dg-APMAP-C2-his; Supplementary figure 2A), Dg-APMAP-N-his was efficiently secreted into
289 the culture supernatants (Supplementary figure 2B). Dg-APMAP-N-his was then purified from
290 the culture supernatants of transformed bacteria, and its purity was confirmed by SDS-PAGE and
291 by staining with Coomassie brilliant blue. Dg-APMAP-N-his was detected at the predicted
292 molecular weight (approximately 28.9 kDa; Fig. 3).
293
294 3.4. Immunization with Dg-APMAP-N-his

295 Chickens were subcutaneously immunized with the recombinant Dg-APMAP-N-his in an
296 emulsion with a light liquid paraffin. At 4 weeks post-immunization, the immune plasmas were
297 isolated. Increased antibody titers in the immune plasmas were determined by ELISA (Table 2),
298 and western blot analysis confirmed that the antibodies produced in IM1, IM2, IM3, and IM4
299 contained the specific antibodies against Dg-APMAP-N-his (Fig. 4).
300
301 3.5. Acaricidal potential of the plasma of chickens immunized with Dg-APMAP-N-his
302 Plasmas from immunized and unimmunized groups were used in an *in vitro* feeding assay to
303 evaluate anti-PRM effects. The plasmas of each group were pooled separately and fed to starved
304 PRMs using artificial feeding devices described previously [17]. At 4 hours after the *in vitro*
305 feeding, we collected blood-fed PRMs and monitored their survival rates for 1 week. The number
306 of blood-fed PRMs collected and their life-stages are summarized in Tables 3–5. The anti-PRM
307 effects were analyzed by log-rank test and Fisher's exact test at each time point. A comparison of
308 the Kaplan–Meier curves revealed that for PRMs fed on plasmas from chickens immunized with
309 Dg-APMAP-N-his, the survival rate was significantly lower than that of PRMs fed on plasma
310 from unimmunized chickens ($p < 0.001$; Fig. 5A). In addition, Fisher's exact test and odds ratio
311 analysis indicated that the survival rate of PRMs fed on immunized plasmas was decreased from
312 4 days post-feeding (Table 3).

313 To evaluate the anti-PRM effects in each life stage, we separated the data for PRMs of mixed
314 stages into those for adults and nymphs, and statistically analyzed the acaricidal activity of the
315 immunized plasma. Kaplan–Meier curve analysis and log-rank tests showed that survival rates
316 were lower in adults and nymphs fed plasma from immunized chickens, compared to those fed
317 plasma from unimmunized chickens ($p = 0.022$ and $p < 0.001$, respectively; Fig. 5B and C). This
318 decrease in survival rates was observed at 7 days post-feeding and 5 days post-feeding for adults
319 and nymphs, respectively, and the odds ratios significantly increased at the same time points
320 (Tables 4 and 5). In terms of reproductive capacity, however, no significant difference was
321 observed between each group (Supplementary figure 3). To clarify that the acaricidal effects
322 observed were mediated by antibodies against Dg-APMAP but not by the immune reactions to
323 adjuvant immunization, *in vitro* feeding was performed using plasmas from unimmunized
324 chickens and chickens immunized with emulsified PBS. There was no difference in survival rates
325 of PRMs, regardless of life stages (Supplementary tables 2–4; Supplementary figure 4). Taken
326 together, these results suggest that plasma from chickens immunized with Dg-APMAP-N
327 increased the mortality in both adults and nymphs, and therefore, Dg-APMAP could be one of the
328 candidates for vaccine antigens to effectively control PRMs.
329
330 **4. Discussion**

331 Current strategies for PRM prevention using chemical acaricides include various problems, such
332 as the selection of PRMs with acaricide-resistant properties for long-term use, and show
333 diminished efficacies. Therefore, the development of novel control methods for PRMs has been
334 enthusiastically studied and vaccination is seen as one of the most promising strategies. An anti-
335 tick vaccine targeting a transmembrane protein, BM86, has shown acaricidal effects on *B.*
336 *microplus* [10]. Moreover, a vaccine targeting BM86 also has an acaricidal property on PRMs
337 [18]. Thus, vaccines targeting transmembrane proteins in the midgut are expected to be effective.
338 Several studies have reported the efficacies of anti-PRM vaccines targeting midgut proteins;
339 however, most of the antigen candidates reported so far are secreted or intracellular proteins
340 [1,2,4,19–23]. In the present study, we characterized a newly identified protein, Dg-APMAP,
341 which was predicted to be associated with the plasma membrane and indicated the potential as a
342 vaccine antigen.
343 Based on the deduced amino acid sequence, Dg-APMAP was predicted as a single-pass
344 transmembrane protein with a strictosidine synthase domain. Strictosidine synthase is involved in
345 the biosynthetic pathway of the monoterpenoid indole alkaloid in plants, which produces various
346 alkaloids including pharmaceutical compounds applied to human disease treatment [24,25].
347 However, little is known about the functions and importance of APMAP in arthropods. In the
348 present study, PRMs fed on plasma from chickens immunized with Dg-APMAP showed higher

mortality. Therefore, Dg-APMAP may have important biological functions in PRMs. Further investigations such as gene silencing approaches are required to elucidate the physiological function of Dg-APMAP [26].

In this study, qPCR analysis showed no difference in the expression levels of *Dg-APMAP* mRNA between fed and starved PRMs in all the life stages. However, *Dg-APMAP* transcripts identified in RNA-seq data exhibited significantly higher expression intensity in fed PRMs. One possible cause of this difference is the incubation period for starvation. In the present study, we subjected PRMs to qPCR after a 1-week starvation period, whereas PRMs kept for a 2-week period were applied to RNA-seq analysis [12]. Additionally, RNA-seq was performed using PRMs of mixed life stages whereas qPCR was conducted independently for PRMs of each life stage, possibly biasing the results. Thus, Dg-APMAP expression levels may decrease depending on the starvation period. To precisely clarify the expression profile of *Dg-APMAP* mRNA, expression levels need to be quantified at several time points after blood feeding.

For the preparation of the recombinant Dg-APMAP as a vaccine antigen, we used the *Brevibacillus* expression system. This system can efficiently secrete the target protein into the cultural supernatant [27], reducing the risks induced by the endotoxin. However, the secretion efficiency was low for the full length of the extracellular domain of Dg-APMAP. Since Dg-APMAP is originally a plasma membrane protein, it may not be suitable for secretory expression.

If this expression system is applied for the production of the vaccine antigen, the suitable regions within the extracellular domain should be further optimized to enhance the secretion efficiency and efficacy as a vaccine antigen. In addition, the selection of an appropriate system for the production of Dg-APMAP may improve the expression efficiency.

To evaluate the potential of Dg-APMAP as a vaccine antigen, we assessed the acaricidal effects of plasmas derived from chickens immunized with Dg-APMAP-N-his *in vitro*. We observed drastic reduction in the survival rates of PRMs fed on immune plasmas. However, there was a difference in the acaricidal effect between nymphs and adults; statistically significant reduction in survival rate was observed from 5 days post-feeding in nymphs and 7 days post-feeding in adults. Although the underlying mechanism of the differences in acaricidal effect among life stages is unclear, it is possible that the expression pattern of Dg-APMAP is different after blood-feeding in each life-stage. Indeed, qPCR analysis indicated that the expression level of *Dg-APMAP* mRNA in blood-fed adults tended to be lower than those in fed nymphs, although the difference was not statistically significant. Therefore, the expression profiles of Dg-APMAP at each life-stage and in different tissues should be further investigated. Importantly, to the best of our knowledge, this is the first report showing that immunization with Dg-APMAP exhibited anti-PRM efficacies in both adults and nymphs. The previous studies did not assess the mortality in each life-stage independently.

Taken together, our data suggest that vaccination with Dg-APMAP could be an effective strategy to control PRM infestation in poultry farms. Vaccine efficacies have to be examined in PRM-infestation trials using immunized chickens to further evaluate its anti-PRM potential. Although previous studies have shown that several vaccine antigen candidates affect PRM fecundity [2,21,22], vaccination with Dg-APMAP did not affect the reproductive capacity of PRMs. Therefore, to enhance the vaccine efficacy of vaccination with Dg-APMAP, its potential for combined use with other antigens as a “cocktail vaccine” should be assessed.

Conflict of interest

TS, EO, and AT are employed by Vaxxinova Japan K.K, Tokyo, Japan. The other authors have no financial conflicts of interest.

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406

407 **Authors' contribution**

408 SF, SM, and MT conceived and designed the study. SF, SM, MT, JA, AM, MI, SYW, TA, TS, and
409 OI conducted the experiments. SF and SM analyzed the data. SM, EO, AT, NM, TO, SK, and KO
410 provided intellectual input, laboratory materials, reagents, and analytic tools. SF and SM wrote
411 the original draft. All authors reviewed and approved the final manuscript. All authors attest that
412 they meet the ICMJE criteria for authorship.

413

References

[1] Bartley K, Wright HW, Huntley JF, Manson ED, Inglis NF, McLean K, et al. Identification and evaluation of vaccine candidate antigens from the poultry red mite (Dermanyssus gallinae). Int J Parasitol 2015;45:819–30. https://doi.org/10.1016/j.ijpara.2015.07.004.

[2] Lima-Barbero JF, Contreras M, Mateos-Hernández L, Mata-Lorenzo FM, Triguero-Ocaña R, Sparagano O, et al. A vaccinology approach to the identification and characterization of Dermanyssus gallinae candidate protective antigens for the control of poultry red mite infestations. Vaccines 2019;7:190. https://doi.org/10.3390/vaccines7040190.

[3] Wright HW, Bartley K, Huntley JF, Nisbet AJ. Characterisation of tropomyosin and paramyosin as vaccine candidate molecules for the poultry red mite, Dermanyssus gallinae. Parasit Vectors 2016;9:544. https://doi.org/10.1186/s13071-016-1831-8.

[4] Bartley K, Turnbull F, Wright HW, Huntley JF, Palarea-Albaladejo J, Nath M, et al. Field evaluation of poultry red mite (Dermanyssus gallinae) native and recombinant prototype vaccines. Vet Parasitol 2017;244:25–34. https://doi.org/10.1016/j.vetpar.2017.06.020

[5] Willadsen P. Immunity to ticks. Adv Parasitol 1980;18:293–313. https://doi.org/10.1016/S0065-308X(08)60402-9.

[6] Wikel SK. Host immunity to ticks. Annu Rev Entomol 1996;41:1–22. https://doi.org/10.1146/annurev.en.41.010196.000245.

[7] Willadsen P, Riding GA, McKenna RV, Kemp DH, Tellam RL, Nielsen JN, et al. Immunologic control of a parasitic arthropod. Identification of a protective antigen from Boophilus microplus. J Immunol 1989;143:1346–51.

[8] Ribeiro JM, Francischetti IM. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. Annu Rev Entomol 2003;48:73–88. https://doi.org/10.1146/annurev.ento.48.060402.102812

[9] Pritchard J, Kuster T, Sparagano O, Tomley F. Understanding the biology and control of the poultry red mite Dermanyssus gallinae: a review. Avian Pathol 2015;44(3):143–53. https://doi.org/10.1080/03079457.2015.1030589.

[10] Rand KN, Moore T, Sriskantha A, Spring K, Tellam R, Willadsen P, et al. Cloning and expression of a protective antigen from the cattle tick Boophilus microplus. Proc Natl Acad Sci U S A 1989;86:9657–61. https://doi.org/10.1073/pnas.86.24.9657.

[11] Azhahianambi P, Ray DD, Chaudhuri P, Gupta R, Ghosh S. Vaccine efficacy of Bm86 ortholog of H. a. anatolicum, rHaa86 expressed in prokaryotic expression system. J Parasitol Res 2009;2009:165812. https://doi.org/10.1155/2009/165812.

[12] Fujisawa S, Murata S, Isezaki M, Oishi E, Taneno A, Maekawa N, et al. Transcriptome dynamics of blood-fed and starved poultry red mites, Dermanyssus gallinae. Parasitol Int 2020;78:102156. https://doi.org/10.1016/j.parint.2020.102156.

[13] Ilhan A, Gartner W, Nabokikh A, Daneva T, Majdic O, Cohen G, et al. Localization and characterization of the novel protein encoded by C20orf3. Biochem J 2008;414:485–95. https://doi.org/10.1042/BJ20080503.

[14] Ma Y, Gao J, Yin J, Gu L, Liu X, Chen S, et al. Identification of a novel function of adipocyte plasma membrane-associated protein (APMAP) in gestational diabetes mellitus by proteomic analysis of omental adipose tissue. J Proteome Res 2016;15:628–37. https://doi.org/10.1021/acs.jproteome.5b01030.

[15] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9.

[16] Ichii O, Kamikawa A, Otsuka S, Hashimoto Y, Sasaki N, Endoh D, et al. Overexpression of interferon-activated gene 202 (Ifi202) correlates with the progression of autoimmune glomerulonephritis associated with the MRL chromosome 1. Lupus 2010;19:897–905. https://doi.org/10.1177/0961203310362534.

[17] Ariizumi T, Murata S, Fujisawa S, Isezaki M, Maekawa N, Okagawa T, et al. Selection of reference genes for quantitative PCR analysis in poultry red mite (Dermanyssus gallinae). J Vet Med Sci 2021;83:558–65. https://doi.org/10.1292/jvms.20-0677.

[18] Harrington D, Canales M, de la Fuente J, de Luna C, Robinson K, Guy J, et al. Immunisation with recombinant proteins subolesin and Bm86 for the control of

468 Dermanyssus gallinae in poultry. Vaccine 2009;27:4056–63.
469 https://doi.org/10.1016/j.vaccine.2009.04.014.
470 [19] Wright HW, Bartley K, Nisbet AJ, McDevitt RM, Sparks NH, Brocklehurst S, et al. The
471 testing of antibodies raised against poultry red mite antigens in an in vitro feeding assay;
472 preliminary screen for vaccine candidates. Exp Appl Acarol 2009;48:81–91.
473 https://doi.org/10.1007/s10493-009-9243-5.
474 [20] Bartley K, Huntley JF, Wright HW, Nath M, Nisbet AJ. Assessment of cathepsin D and L-
475 like proteinases of poultry red mite, Dermanyssus gallinae (De Geer), as potential vaccine
476 antigens. Parasitology 2012;139:755–65. https://doi.org/10.1017/S0031182011002356.
477 [21] Lima-Barbero JF, Contreras M, Bartley K, Price DR, Nunn F, Sanchez-Sanchez M, et al.
478 Reduction in oviposition of poultry red mite (Dermanyssus gallinae) in hens vaccinated
479 with recombinant akirin. Vaccines 2019;7:121. https://doi.org/10.3390/vaccines7030121.
480 [22] Xu X, Wang C, Huang Y, Zhang S, Yu H, Meng J, et al. Evaluation of the vaccine efficacy
481 of three digestive protease antigens from Dermanyssus gallinae using an in vivo rearing
482 system. Vaccine 2020;38:7842–9. https://doi.org/10.1016/j.vaccine.2020.10.010.
483 [23] Murata S, Taniguchi A, Isezaki M, Fujisawa S, Sakai E, Taneno A, et al. Characterisation of
484 a cysteine protease from poultry red mites and its potential use as a vaccine for chickens.
485 Parasite 2021;28:9. https://doi.org/10.1051/parasite/2021005.

[24] Stöckigt J, Barleben L, Panjikar S, Loris EA. 3D-Structure and function of strictosidine synthase-the key enzyme of monoterpenoid indole alkaloid biosynthesis. Plant Physiol Biochem 2008;46:340–55. https://doi.org/10.1016/j.plaphy.2007.12.011.

[25] Fischereder E, Pressnitz D, Kroutil W, Lutz S. Engineering strictosidine synthase: rational design of a small, focused circular permutation library of the β-propeller fold enzyme. Bioorg Med Chem 2014;22:5633–7. https://doi.org/10.1016/j.bmc.2014.06.023.

[26] Chen W, Bartley K, Nunn F, Bowman AS, Sternberg JM, Burgess ST, et al. RNAi gene knockdown in the poultry red mite, Dermanyssus gallinae (De Geer 1778), a tool for functional genomics. Parasit Vectors 2021;14:57. https://doi.org/10.1186/s13071-020-04562-9.

[27] Mizukami M, Hanagata H, Miyauchi A. Brevibacillus expression system: hostvector system for efficient production of secretory proteins. Curr Pharm Biotechnol 2010;11:251–8. https://doi.org/10.2174/138920110791112031.

Figure captions

Fig. 1. Determination and phylogenetic tree analysis of a *Dermanyssus gallinae* adipocyte plasma membrane-associated protein-like molecule (Dg-APMAP).

(A) Deduced amino acid sequences of the open reading frame encoding Dg-APMAP. A putative transmembrane site is underlined, and the strictosidine synthase domain is boxed. (B) A phylogenic tree based on the deduced amino acid sequence of Dg-APMAP, built with neighbor-joining method using the MEGA X software [18]. Numbers indicate bootstrap percentage (1,000 replicates). The scale indicates the divergence time.

Fig. 2. Gene expression analysis of *Dg-APMAP.*

Dg-APMAP expression in poultry red mites (PRMs) as examined by RT-PCR/nested PCR for each life stage and blood-feeding state (A) and tissue type (C). *Elongation factor 1-alpha 1*-like gene (*Ef1a1*) is amplified as an internal control (B). *Dg-APMAP* expression is calculated by dividing the copy numbers of *Dg-APMAP* by those of *Elf1a1*. Each experiment was performed in triplicate and error bars indicate SEM. Statistical differences have been evaluated usingSteel-Dwass test. (C) *Dg-APMAP* expression in the midgut and ovaries (RT-PCR) and in the salivary glands (nested PCR).

518 **Fig. 3.** The purification of Dg-APMAP-N-his.

519 Dg-APMAP-N-his is purified from the culture supernatant by affinity purification, separated by

520 SDS-PAGE, and visualized by staining with Coomassie brilliant blue.

521

522 **Fig. 4.** Dg-APMAP-N-his-specific antibody production in immunized chickens.

523 The recombinant Dg-APMAP-N-his is reacted with the plasma from immunized chickens and

524 control chickens by western blotting. The predicted molecular size of Dg-APMAP-N-his is

525 approximately 28.9 kDa, and the specific signals are detected in plasma from the immunized

526 chickens.

527

528 **Fig. 5.** Anti-PRM effects of plasma from chickens immunized with Dg-APMAP-N-his.

529 The survival rate of PRMs fed on plasma from immunized chickens, assessed every day for a 1-

530 week period (A–C). Kaplan-Meier curves indicate survival in total PRMs (A), adults (B), and

531 nymphs (C). Statistical differences have been evaluated using the log-rank test; $p < 0.05$ is

532 considered statistically significant.

533 **Tables**

534 **Table 1.** Primers used for amplification of each gene

Primer	Primer sequences (5' - 3')
For gene cloning and sequencing	
DgAPMAP-ORF-F	GCAGAGCAGAGCAGAGCACGG
DgAPMAP-ORF-R	GTGGTGAGGCGGGCTCGAATG
DgAPMAP-seq-F1	ATTCCGCTCGACTTTGATCC
DgAPMAP-seq-F2	GGGGCTTCACCAAATCCTGT
DgAPMAP-seq-F3	CCATTCGTCACACTACACGC
DgAPMAP-seq-R1	ATCAGGATCTTTCCGTGCTC
DgAPMAP-seq-R2	CAACGGCAACTTCCTTGCGA
DgAPMAP-seq-R3	GGTCACGTACTTTGCGCTAA
For gene expression analysis	
DgAPMAP-RT-F	AGCTCCAGCTGGTTTTCGTA
DgAPMAP-RT-R	ACTGGGTTCCGGTGTAGATG

Elf1a1-F	GTCGGTGTCATCAAGTCCGT
Elf1a1-R	AGGGTCGAGAGTGTAGGGTC
For preparation of recombinant proteins	
pBIC-APMAP-F1	GATGACGATGACAAACTCGACTTTGATCCGGC
pBIC-APMAP-F2	GATGACGATGACAAAATCGATATCAAGACAGGC
pBIC-APMAP-F3	GATGACGATGACAAAACTAAGGGTAAGTTGACG
pBIC-APMAP-R1	CATCCTGTTAAGCTTTTACTTATGTTTCAAACG
pBIC-APMAP-R2	CATCCTGTTAAGCTTTTAGGCAAATAGCAGTTTCGAC
pBIC-APMAP-R3	CATCCTGTTAAGCTTTTAGCGAGGCGATCGGCGCAG

*Homologous recombination sites are underlined.

537 **Table 2.** Antibody titers in the plasmas of chickens immunized with Dg-APMAP-N-his

Experimental group		Antibody titers
Unimmunized	UN1	<800
	UN2	<800
	UN3	<800
	UN4	<800
Immunized	IM1	64,000
	IM2	64,000
	IM3	64,000
	IM4	64,000

538

539 **Table 3.** Summary of anti-PRM properties of Dg-APMAP-N-his immunization (Total)

	Days post-feeding						
	1	2	3	4	5	6	7
Immunized (*n* = 181)							
No. of dead PRMs	3	7	13	38	65	85	112
Survival rate (%)	98.34	96.13	92.82	79.01	64.09	53.04	38.12
Unimmunized (*n* = 210)							
No. of dead PRMs	4	12	19	27	42	59	85
Survival rate (%)	98.10	94.29	90.95	87.14	80.00	71.90	59.52
P value	1	0.483	0.581	0.041*	9.56E-04*	1.47E-04*	3.04E-05*
Odds ratio	0.112	0.664	0.778	1.798	2.172	2.261	2.382
95% CI (lower limit)	6.563	0.217	0.342	1.016	1.352	1.459	1.557
95% CI (upper limit)	0.859	1.879	1.719	3.22	3.516	3.524	3.663

540 The data were compared using Fisher's extract test between immunized and control groups.

541 *$p < 0.05$ was considered statistically significant.

542

543

544 **Table 4.** Summary of anti-PRM properties of Dg-APMAP-N-his immunization (Adults)

	Days post-feeding						
	1	2	3	4	5	6	7
Immunized (n = 65)							
No. of dead PRMs	0	2	5	11	18	29	40
Survival rate (%)	100.00	96.92	92.31	83.08	72.31	55.38	38.46
Unimmunized (n = 110)							
No. of dead PRMs	1	3	7	13	21	33	48
Survival rate (%)	99.09	97.27	93.64	88.18	80.91	70.00	56.36
P value	1	1	0.763	0.369	0.194	0.072	0.028*
Odds ratio	0	1.131	1.225	1.516	1.619	1.873	2.058
95% CI (lower limit)	0	0.092	0.293	0.572	0.735	0.945	1.057
95% CI (upper limit)	65.933	10.158	4.712	3.956	3.546	3.726	4.065

545 The data were compared by Fisher's extract test between immunized and control groups.

546 *$p < 0.05$ was considered statistically significant.

547

548 **Table 5.** Summary of anti-PRM properties of Dg-APMAP-N-his immunization (Nymphs)

	Days post-feeding						
	1	2	3	4	5	6	7
Immunized (*n* = 116)							
No. of dead PRMs	3	5	8	27	47	56	72
Survival rate (%)	97.41	95.69	93.10	76.72	59.48	51.72	37.93
Unimmunized (*n* = 100)							
No. of dead PRMs	3	9	12	14	21	26	37
Survival rate (%)	97.00	91.00	88.00	86.00	79.00	74.00	63.00
P value	1	0.178	0.242	0.117	0.005*	1.16E-03*	3.64E-04*
Odds ratio	0.859	0.457	0.545	1.858	2.405	2.644	2.772
95% CI (lower limit)	0.112	0.116	0.184	0.872	1.275	1.439	1.546
95% CI (upper limit)	6.563	1.582	1.523	4.109	4.639	4.946	5.031

549 The data were compared by Fisher's extract test between immunized and control groups.

550 *$p < 0.01$ was considered statistically significant.

Fig. 1.

(A)

```
1   M Y K L V K L Q L Q L V F V S L L A L L S L P F M P I P L D 30
31  F D P A A Y N V T L P A F E G V L A P N D A L D R A K M L F 60
61  Q Q R L R G P E S L A V K D G L I Y T G T Q F G D V F A I D 90
91  P V K E T L T K V A N T G S S C N G L H D E E K C G R V L G 120
121 L R F A P N G D L Y A A D A Y K G L L K I D I K T G K V E T 150
151 L V A S G S Y V G T S K L L F A D D L D I D N N G V I Y Y S 180
181 Q G S C R W G L H Q I L Y I I M E F D T T G R I L T Y D T K 210
211 T K K S G V L L E G L A F P N G V Q L T E D R S A L L F S E 240
241 L G K K R I M K Y Q L T G A T K G K L T V F A E N L P G G P 270
271 D N L R R S P R G T Y W V G I D I A R S N T T Q P L G D L I 300
301 A P Y P L V A K A T M R F C W L T G Q A L K Q I D S F V D H 330
331 P T L Q D L S A D F E H G K I L I N T V P N R G I I L E L G 360
361 P D G K I L R S L H S S H Y T L F S E V L E Y G G H L Y I G 390
391 S F I K P Y L L R L K H K
```

(B)

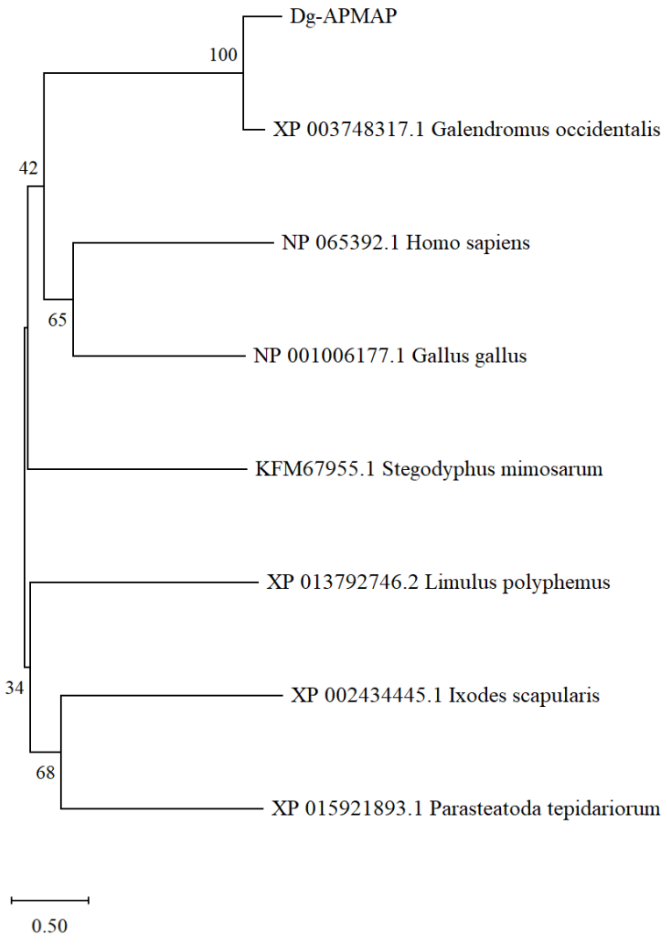


Fig. 2.

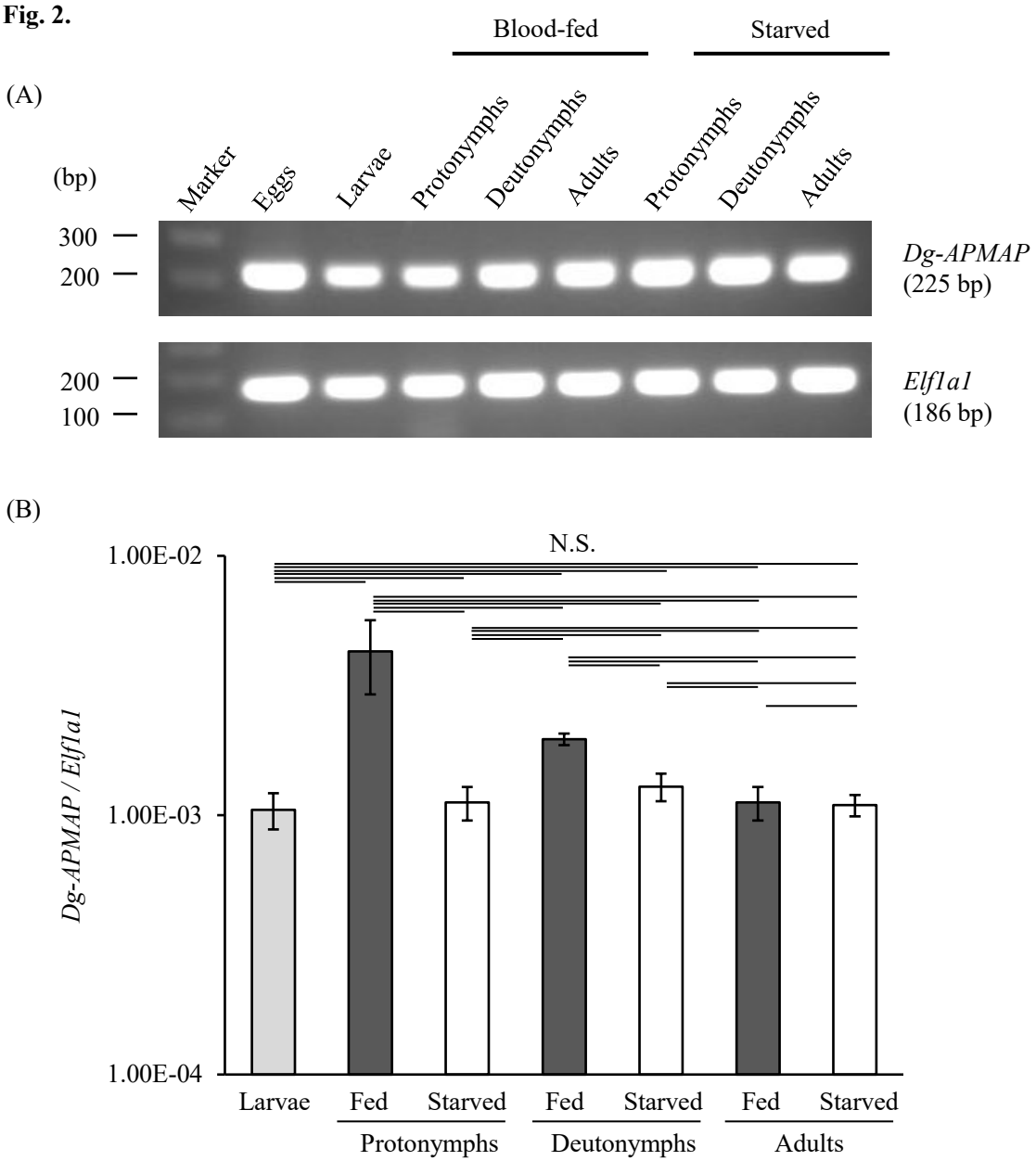

(A)
Blood-fed
Starved
(bp)
Marker
Eggs
Larvae
Protonymphs
Deutonymphs
Adults
Protonymphs
Deutonymphs
Adults
300
200
Dg-APMAP
(225 bp)
200
100
Elf1a1
(186 bp)
(B)
N.S.
1.00E-02
1.00E-03
1.00E-04
Dg-APMAP / Elf1a1
Larvae
Fed
Starved
Fed
Starved
Fed
Starved
Protonymphs
Deutonymphs
Adults
(C)
(bp)
Marker
Salivary glands
Midguts
Ovaries
300
200
Dg-APMAP
200
100
Elf1a1

Fig. 3.

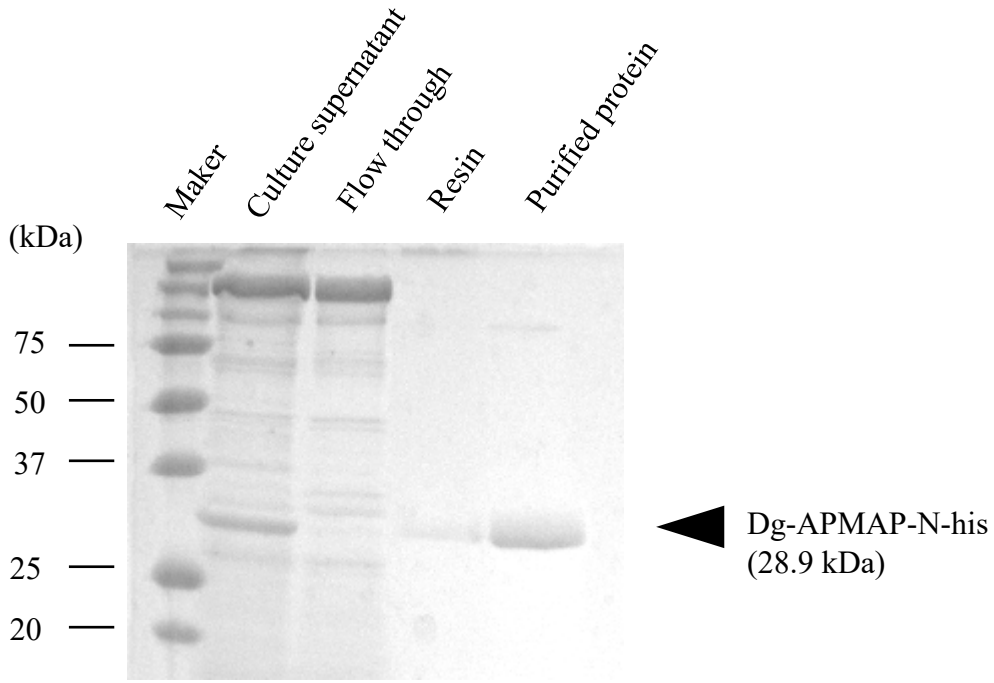

Maker
Culture supernatant
Flow through
Resin
Purified protein
(kDa)
75
50
37
25
20
Dg-APMAP-N-his
(28.9 kDa)

Fig. 4.

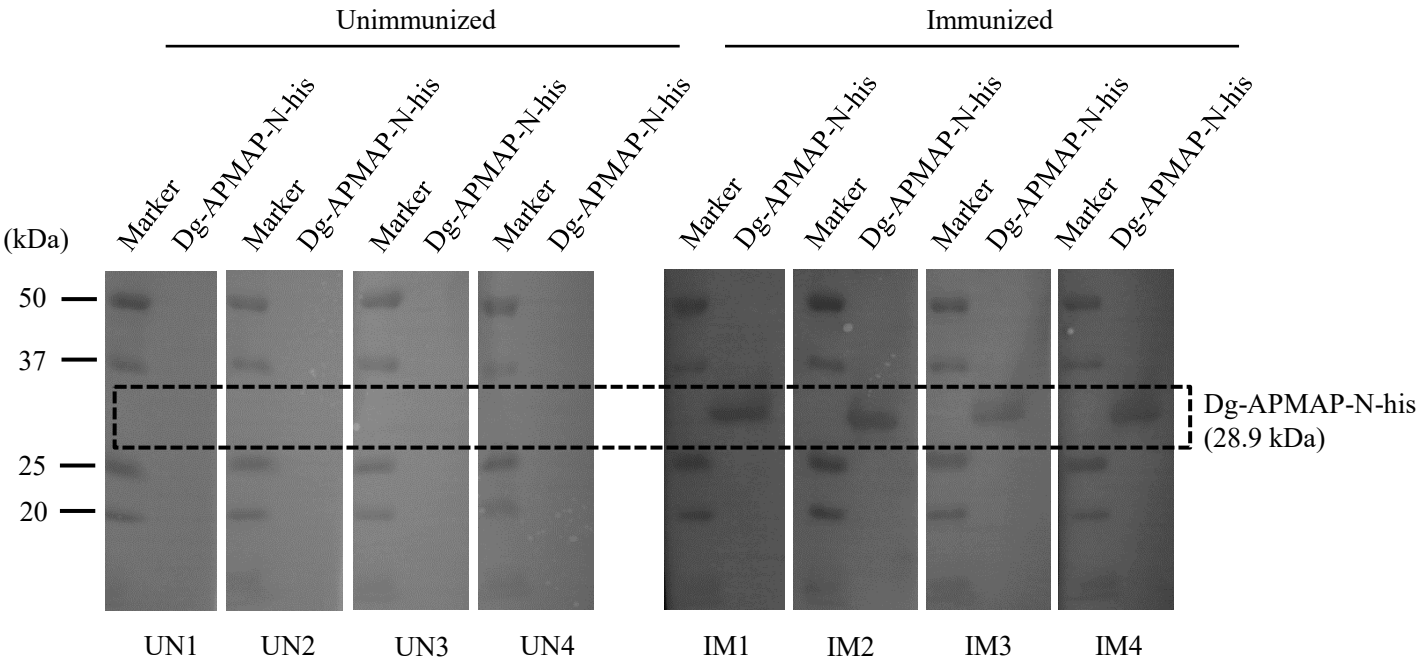
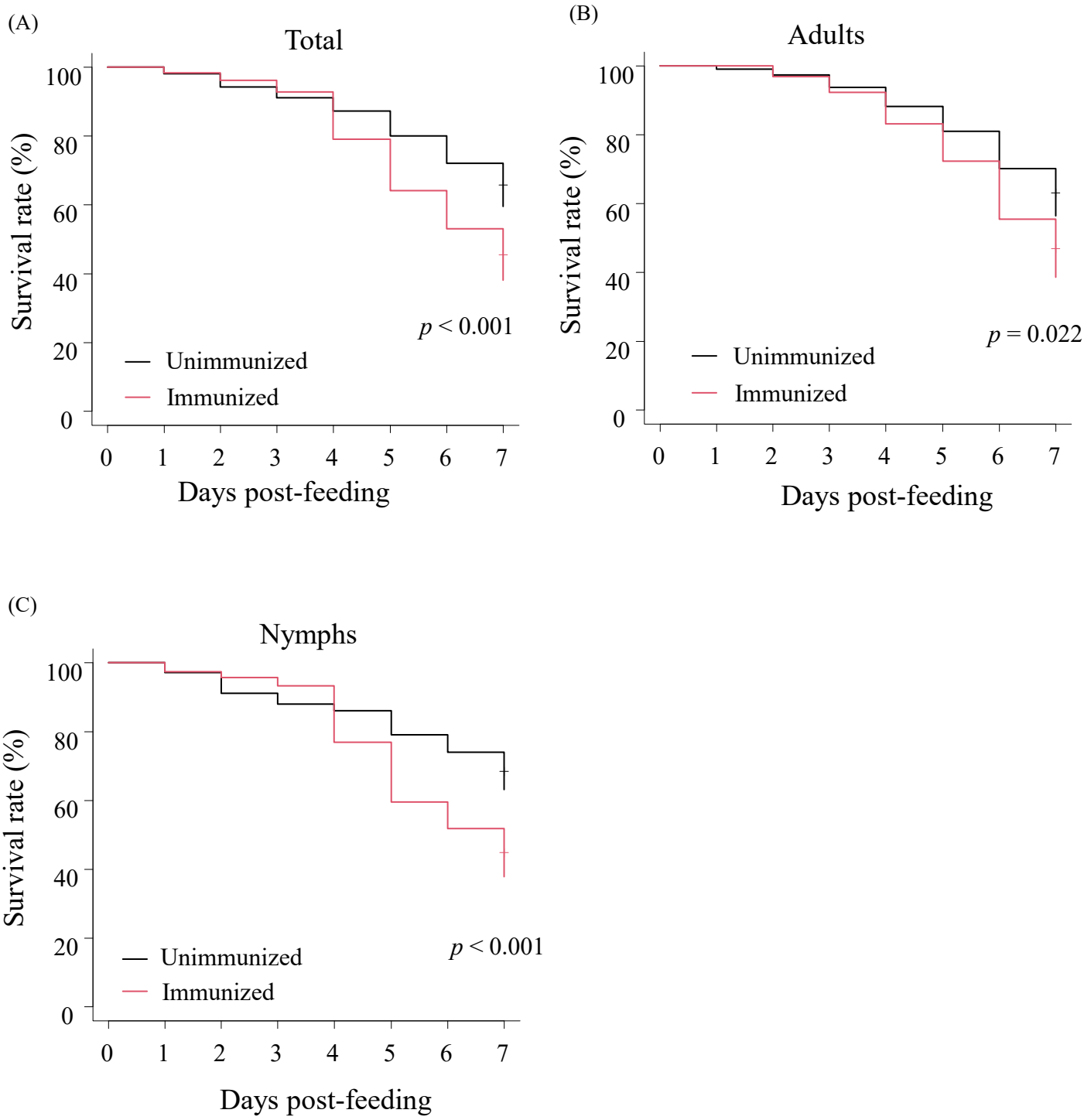

Unimmunized
Immunized
Marker
Dg-APMAP-N-his
(kDa)
50
37
25
20
Dg-APMAP-N-his
(28.9 kDa)
UN1
UN2
UN3
UN4
IM1
IM2
IM3
IM4

Fig. 5.


(A)
Total
Survival rate (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Days post-feeding
p < 0.001
Unimmunized
Immunized
(B)
Adults
Survival rate (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Days post-feeding
p = 0.022
Unimmunized
Immunized
(C)
Nymphs
Survival rate (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Days post-feeding
p < 0.001
Unimmunized
Immunized