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Author(s)	Horio, Fumiya; Seo, Hikari; Win, Shwe Yee et al.
Citation	Veterinary Immunology and Immunopathology, 283 https://doi.org/10.1016/j.vetimm.2025.110921
Issue Date	2025-05
Doc URL	https://hdl.handle.net/2115/97565
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Type	journal article
File Information	Horio_et_al.pdf

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Characterization of a lipocalin-like molecule from *Dermanyssus gallinae* as a potential vaccine antigen

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Abstract

Poultry red mites (PRMs, *Dermanyssus gallinae*) are hematophagous ectoparasites of chickens that pose a significant threat to the egg-laying industry. The emergence of acaricide-resistant PRMs raises the demand for alternative control approaches such as vaccination. However, several vaccine antigens have failed to suppress the growth of PRM populations in field trials due to difficulties in maintaining antibody levels. In ticks, the molecules exposed to the host, such as lipocalins, can facilitate antibody production, and are therefore considered advantageous as vaccine antigens. Therefore, we focused on a lipocalin-like molecule (Dg-Lipocalin) identified from an RNA-seq analysis reported by Fujisawa *et al*. (2020) and analyzed its exposure to the host and potential as a vaccine antigen. Western blotting using 500-fold diluted plasma of chickens from PRM-contaminated farms revealed the presence of antibodies against Dg-Lipocalin, suggesting its exposure to the host. To evaluate its potential as a vaccine antigen, PRMs were artificially fed immune plasma with 32,000- to 64,000-fold antibody titers or plasma from PBS-inoculated control chickens, and their mortality was observed for 7 days. In experiment 1, the immune plasma significantly increased PRM mortality compared to the control plasma. However, these effects were not observed in experiment 2, although the total mortality was significantly increased in immune plasma-fed PRMs. Thus, the efficacy of Dg-Lipocalin appears to be limited; however, its exposure to the

host may result in sustained antibody titers. Further investigation is required to evaluate its feasibility.

Keywords: Poultry red mite, *Dermanyssus gallinae*, lipocalin, Dg-Lipocalin, vaccine

Introduction

Poultry red mites (PRMs, *Dermanyssus gallinae*) are hematophagous ectoparasites that pose significant threats to egg-laying hens. The life cycle of PRMs comprises five developmental stages: eggs, larvae, protonymphs, deutonymphs, and adults, which are usually completed within 2 weeks [1]. PRMs live off the host for most of their lifetime, hiding in cracks and crevices of poultry houses, and contact the host only once every 2–4 days for a short duration of approximately an hour at night to feed on blood [1], making it challenging to detect PRMs. PRM infestation causes anemia, weight loss, abnormal behavior, and sometimes death in young chickens [1]. Additionally, PRMs serve as vectors for several avian pathogens such as *Escherichia coli*, *Salmonella enterica* serovars Enteriditis and Gallinarum, *Mycoplasma* spp. and avian influenza virus [2,3]. Thus, PRMs cause significant economic losses for the poultry industry [4].

Currently, the control of PRMs depends on the use of acaricides [4]. However, the characteristic of hiding away from the host after feeding makes it difficult for acaricides to reach PRMs. Moreover, the use of acaricides has led to the selection of acaricide-resistant PRMs [5]. Furthermore, the use of these chemical components in poultry farming in Europe is limited because of concerns regarding their negative effects on human health [1]. Therefore, novel strategies for controlling PRMs, including vaccination, have been investigated. The concept of the anti-PRM vaccine is to cause dysfunction of molecules crucial for the

61 physiology of mites with antibodies produced by vaccination in chickens. To date, several
62 candidates for vaccine antigens that show anti-PRM effects *in vitro* or *in vivo* have been
63 identified [6–16], most of which are expected to be expressed in the midgut. These antigens
64 include homogenized mite extracts [6]; histamine release factor (Dg-HRF-1) [7], which plays
65 for a role in histamine release; proteinases such as cathepsin D (Dg-CatD-1) [8]; cysteine
66 proteases 1 [9, 10] and 2 [11] (Deg-CPR-1 and 2); protease inhibitors, such as cystatin (Dg-
67 Cys) [12]; endopeptidases, such as legumain (Dg-Lgm) [13]; vitellogenin (Deg-VIT-1) [9,
68 14]; hemelipoglycoprotein (Deg-HGP-1) [9], which is involved in hemoglobin digestion by
69 PRMs; adipocyte plasma membrane-associated protein (Dg-APMAP) [15], which is involved
70 in adipocyte differentiation and carbohydrate metabolism; and copper transporter 1 (Deg-
71 Ctr1) [16], which is involved in copper uptake. However, in a field trial, immunization with
72 some antigens failed to exert sufficient effects on PRM control because antibody titers were
73 low when PRMs started to increase [14]. Thus, to apply anti-PRM vaccines in the field, high
74 antibody titers must be maintained for a prolonged period. In contrast, some exposed antigens,
75 which are components of saliva produced in the salivary glands and injected into the host
76 during feeding, have shown anti-PRM effects [6,8,10]. Immunization with soluble PRM
77 extracts containing saliva proteins successfully induced and maintained high IgY levels in
78 vaccinated chickens throughout the experimental period (A_{490nm} range 0.76–0.98 in the
79 vaccinated group vs 0.17–0.35 in the placebo group) and exhibited anti-PRM efficacy in a

field trial [14]. Thus, some molecules in the soluble extracts of PRMs may serve as anti-PRM vaccine antigens to maintain the antibody titers.

Hematophagous ectoparasites release several bioactive molecules in their saliva to engage the inflammatory responses of the host to maintain an undisturbed environment for blood feeding [17]. During the intrusion of the proboscis of arthropods, the host responds by priming the hemostatic machinery, including vasodilation, platelet aggregation, and blood clotting, at the biting site [18]. The host's acute inflammatory responses to infestation are accompanied by the activation of host immune cells including keratinocytes, endothelial cells, dendritic cells, and macrophages at the bite site, release of proinflammatory chemokines and cytokines such as interleukin-1 β and tumor necrosis factor-α, which are involved in neutrophil recruitment [17,18]. Subsequent infestation activates the adaptive immune responses involving T and B lymphocytes, which produce antibodies against the salivary molecules and the sensitization of complement systems, basophils, and mast cells to boost the inflammatory response [18]. On the contrary, several salivary molecules of ticks, such as serine protease inhibitors, enhance blood-feeding due to their anti-inflammatory effects in the host, such as blood anticoagulation, anti-hemostasis, and anti-complement effects [19], suggesting that antibodies against these antigens may interfere with tick attachment and blood feeding.

Lipocalin is a salivary protein produced in the salivary glands that is believed to bind to histamine and suppress inflammation at sites bitten by ticks, facilitating blood feeding [20,21]. Additionally, the immunization of rabbits with *Haemaphysalis longicornis* lipocalin reduced their engorgement weight, oviposition, and hatchability, indicating that lipocalin has potential as an anti-tick vaccine antigen in *H. longicornis* [22]. In our previous study, gene expression in blood-fed and -starved PRMs was comprehensively analyzed by RNA sequencing (RNA-seq) [23], and we identified lipocalin-like molecules with high expression intensities. This study aimed to focus on a lipocalin-like molecule (Dg-Lipocalin), and evaluate its exposure to the host and its potential as an anti-PRM vaccine antigen for future application in an *in vitro* vaccine cocktail.

Materials and methods

Ethics statement

The chickens used in this study were housed in an animal facility at the Faculty of Veterinary Medicine, Hokkaido University, with approval from the Institutional Animal Care and Use Committee of Hokkaido University (approval number: 20-0051). All experiments were performed in accordance with the relevant guidelines and regulations of the Faculty of Veterinary Medicine, Hokkaido University, which has been fully accredited by the Association of Laboratory Animal Care International (AAALAC).

Mite samples

PRMs of various developmental stages of both sexes were obtained from egg-laying farms in Japan. PRMs were collected in a TubeSpin bioreactor 600 bottle (TPP Techno Plastic Products AG, Trasadingen Switzerland) and were transferred to the laboratory at 4 ℃. The round and dark red PRMs were designated as “blood-fed PRMs” and were preserved at -80 °C. For further analysis, PRMs were stored at 25 ℃ and 70% humidity for a week without any blood meals to starve PRMs, and the starved mites were stored at 5 ℃.

RNA extraction and cDNA synthesis

Total RNA was extracted from PRMs using RNeasy® Plus Mini Kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer’s protocol. The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove unwanted DNA. cDNA was synthesized using PrimeScript Reverse Transcriptase (TaKaRa Bio Inc., Shiga, Japan) with 200 pmol of oligo (dT) 18 primers (Hokkaido System Science, Hokkaido, Japan).

Identification and genetic characterization of a lipocalin-like molecule in PRMs

The coding sequence of a lipocalin-like (*Dg-Lipocalin*) transcript was obtained from RNA-seq data (BioSample accessions: SAMD00228960, SAMD00229086) [23] and

136 determined using the Basic Local Alignment Search Tool of the National Center for
137 Biotechnology Information and InterPro 102.0 (*https://www.ebi.ac.uk/interpro/*) [24].
138 Phylogenetic analysis was conducted using MEGA 11 [25] using the maximum likelihood
139 method with 1,000 bootstrap replicates and the Hasegawa-Kishino-Yano model [26]. The
140 phylogenetic tree for *Dg-Lipocalin* was constructed using a discrete gamma distribution,
141 assuming that a certain fraction of the sites was evolutionarily invariable to improve the tree
142 topology.
143
144 *Preparation of the recombinant Dg-Lipocalin*
145 The coding regions of *Dg-Lipocalin* were amplified by PCR using specific primers
146 containing restriction enzyme sites (Table S1). The PCR amplicons of *Dg-Lipocalin* were
147 treated with BamHI (New England Biolabs, Ipswich, MA, USA) and NcoI (New England
148 Biolabs), cloned into pET-28a (+) (Merck KGaA, Darmstadt, Germany), and transformed into
149 *Escherichia coli* BL21 (DE3) (Merck KGaA). Recombinant Dg-Lipocalin (rDg-Lipocalin)
150 was expressed according to the manufacturer's instructions. Bacterial pellets were separated
151 into soluble and insoluble fractions using BugBuster solution (Merck KGaA), and the
152 recombinant protein was prepared from the insoluble fractions by solubilization in buffer
153 containing 0.3% N-lauroylsarcosine and 50 mM N-cyclohexyl-3-aminopropanesulfonic acid
154 (CAPS) (pH 11.0). The recombinant protein was purified with Ni Sepharose^{TM} 6 Fast Flow

resin (Cytiva Sweden AM, Uppsala, Sweden) and eluted with a buffer containing 0.3% N-lauroylsarcosine, 50 mM CAPS (pH 11.0), and 250 mM imidazole. The refolding was conducted by dialyzing in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 8.5) buffer containing 0.1 mM DL-Dithiothreitol for the first 24 h at 4 °C and in 10 mM Tris-HCl (pH 8.5) buffer for the next 24 h at 4 °C. The elution buffer was replaced with phosphate-buffered saline (PBS) via ultracentrifugation using an Amicon Ultra Centrifugal Filter (10 kDa MWCO; Merck KGaA). The protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). To confirm the expression and purification of rDg-Lipocalin, the protein was denatured in 2×Laemmli sample buffer (Bio-Rad Laboratories Inc., Hercules, CA, USA) containing 355 mM 2-mercaptoethanol, separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and stained with Quick CBB (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Immunization of chickens with rDg-Lipocalin

To immunize the chickens, fertilized eggs from commercial laying hens (White Leghorn) were purchased and day-old chicks were hatched in our laboratory. To prepare the immune plasmas against rDg-Lipocalin, four chickens were subcutaneously immunized with 20 μg/0.5 mL of rDg-Lipocalin mixed with the same volume of Freund's Incomplete

Adjuvant (FUJIFILM Wako Pure Chemical Corporation) at 3 and 7 weeks of age. As a control, four chickens were subcutaneously injected with PBS mixed with an equal volume of adjuvant. Three weeks after the second immunization, the chickens were euthanized by collecting heparinized whole blood from the hearts under deep general anesthesia by isoflurane inhalation (Zoetis Japan, Tokyo, Japan), and the plasma was separated by centrifugation. All housed animals were monitored for animal welfare concerns throughout the experimental period. No death, weight loss, or other clinical signs were observed during the experimental period.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to determine antibody titers in the immune plasma. Wells of the F96 MAXISORP NUNC-IMMUNO PLATE (Thermo Fisher Scientific Inc.) were coated with 1 μg/well of purified rDg-Lipocalin by incubating in carbonate-bicarbonate buffer (Merck KGaA) at 4 °C overnight. After washing five times with 200 μL of PBS containing 0.05% Tween20 (PBS-T), each well was blocked with PBS-T containing 1% bovine serum albumin (Merck KGaA) at 25 °C for 2 h. The wells were then washed five times with PBS-T, and 2,000-, 4,000-, 8,000-, 16,000-, 32,000-, and 64,000-fold PBS-T-diluted immune plasmas or 2,000-fold diluted control plasmas were added to each well and incubated at 25 °C for 1 h. Furthermore, the wells were washed five times with PBS-T and incubated with 10,000-fold

PBS-T diluted anti-chicken immunoglobulin Y (IgY) peroxidase rabbit antibody (Merck KGaA) at 25 °C for 1 h. After washing five times with PBS-T, the wells were reacted with 100 µL of TMB One Component HRP Microwell Substrate (Bethyl Laboratories, Montgomery, TX, USA) at 25 °C in the dark for 20 min. The reaction was terminated with 100 µL of 0.18 M sulphuric acid, and the absorbance was measured at 450 nm. The cutoff value was set at the maximum optical density value of the plasma obtained from the control group. All assays were conducted in duplicate.

Western blotting

rDg-Lipocalin was separated on a 15% sodium dodecyl sulfate polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Merck KGaA). Membranes were blocked with PBS-T containing 1% skim milk (skim milk PBS-T) at 25 °C for 1 h. To detect specific antibodies against rDg-Lipocalin in immunized chickens, the membranes were incubated with 2,000-fold diluted immune and control plasma in skim milk PBS-T at 25 °C for 1 h. To confirm the presence of antibodies against rDg-Lipocalin in the plasma of chickens infested with PRMs, the membranes were incubated with a 500-fold diluted plasma of chickens from PRM-contaminated farms or plasma samples from uncontaminated farms in skim milk PBS-T at 25 °C for 1 h. After washing with PBS-T, the membranes were incubated with a 5,000-fold diluted anti-chicken IgY peroxidase rabbit antibody (Merck KGaA) in skim

212 milk in PBS-T at 25 °C for 1 h. After washing with PBS-T again, the membranes were
213 incubated with Immobilon Western Chemiluminescent HRP Substrate (Merck KGaA) to
214 visualize peroxidase signals.
215
216 *In vitro feeding assay*
217 Fresh chicken blood was collected from healthy chickens at the Field Science Center
218 for Northern Biosphere, Hokkaido University, and the plasma was replaced with an equal
219 volume of immune or control plasma. Plasma samples from each chicken group were pooled
220 and used for assays. The blood containing the immune plasma or the control plasma was pre-
221 heated at 40 °C, and the starved PRMs, which were transferred from 4 °C to 25 °C a day
222 before the feeding assays, were artificially fed with fresh blood containing the immune
223 plasma or the control plasma using the *in vitro* feeding devices designed in the previous study
224 [27]. We used approximately 240–300 mites per group (40–50 mites/device × 6
225 devices/group) for *in vitro* feeding assays. Blood feeding was performed for 4 h at 40 °C
226 under humid conditions in the dark with shaking at 100 rpm. After feeding, approximately
227 20–50% of the mites were blood-fed and most of them were collected into Pasteur pipettes
228 and maintained at 25 °C under 60% humidity. The number of dead PRMs was counted daily
229 for 7 days, and the anti-PRM effects of the immune plasma were evaluated based on the
230 survival rate of blood-fed PRMs (number of dead PRMs / the number of blood-fed PRMs).

231

232 *Statistical analysis*

233 To compare PRM mortality between the immunized and control groups, Kaplan–

234 Meier curves were created, and log-rank tests were performed. Additionally, the mortality of

235 PRMs between the groups was compared daily using Fisher's exact test, and the odds ratio

236 and 95% confidence interval were estimated. Statistical significance was set at $p<0.05$. All

237 statistical analyses were conducted using EZR in R Commander Version 1.61 [28].

238

239 **Results**

240 *Identification and genetic characterization of Dg-Lipocalin*

241 The transcript of a lipocalin-like molecule with high expression intensity in both

242 blood-fed and starved PRMs was obtained from previous RNA-seq data (Table S2) [23] and

243 designated as *Dg-Lipocalin*. Phylogenetic analysis using *lipocalin* genes from chickens and

244 several mites, ticks, and other arthropod species revealed that *Dg-Lipocalin* is genetically

245 similar to *lipocalin* genes from two mite species, *Tropilaelaps mercedesae* and *Varroa*

246 *destructor* (Fig. 1).

247

248 *Production of the immune plasmas against rDg-Lipocalin*

To characterize Dg-Lipocalin, the recombinant protein rDg-Lipocalin was prepared using an *E. coli* expression system, and immune plasma against the recombinant protein was generated. rDg-Lipocalin was detected at a predicted molecular weight of 16 kDa (Fig. S1). Immune plasma was prepared from chickens immunized with rDg-Lipocalin. Western blotting revealed that the plasma obtained from immunized chickens contained antibodies specific to rDg-Lipocalin (Fig. 2A). Additionally, an increase in the antibody titer (32,000- to 64,000-fold) against rDg-Lipocalin was confirmed by ELISA 3 weeks after the second immunization (Table 1). The antibody titers were comparable to those of immunizations with other vaccine antigens in previous studies (8,000- to 64,000-fold) [12, 13, 15, 16]. Reactions with rDg-Lipocalin and an increase in the antibody titer were not observed in the plasma obtained from the control chickens (Fig. 2B, Table 1).

Exposure of Dg-Lipocalin to PRM-infested chickens

To determine whether chickens were exposed to Dg-Lipocalin, the presence of antibodies reacting with rDg-Lipocalin in the plasma of chickens on PRM-contaminated farms was investigated using western blotting. The plasma from chickens on PRM-contaminated farms contained rDg-Lipocalin (Fig. 3, lanes 1–4) with molecular weights similar to those detected in the immune plasma (Fig. 3, PC). In contrast, the presence of antibodies reacting with rDg-Lipocalin was not confirmed in the plasma of chickens that were

not infected with PRMs (Fig. 3, NC). These data suggest that chickens were exposed to Dg-Lipocalin during their blood feeding.

Acaricidal effect of the immune plasmas on PRMs

To assess the acaricidal potential of immune plasma, *in vitro* feeding assays were performed twice, as previously described [24]. In the first experiment, the Kaplan–Meier curve analysis and log-rank test indicated a significant reduction in the survival rate of PRMs fed with immune plasma against rDg-Lipocalin compared with that in the control group (Fig. 4A). Additionally, Fisher's exact test indicated that the mortality of PRMs fed with immune plasma significantly increased 5 days post-feeding compared with that in the control group (Table S3). However, in the second experiment, no significant differences in the survival rates were observed between the control and immunized groups (Fig. 4B, Table S4). When the data from the two experiments were combined, the survival rates of PRMs fed with the immune plasma against rDg-Lipocalin significantly decreased compared with that in the control group (Fig. 4C). Additionally, Fisher's exact test showed that the mortality rate of PRMs fed with immune plasma significantly increased on day 7 post-feeding in the immunized group compared with that in the control group (Table 2). These results suggested that immune plasmas against rDg-Lipocalin have acaricidal potential; however, their efficacy appears to be unstable and unlikely to be high.

Discussion

In this study, the potential of Dg-Lipocalin, showing high expression intensity in PRMs from previous RNA-seq data [20], as a vaccine antigen was analyzed. Western blot analysis revealed the presence of antibodies against Dg-Lipocalin in the plasma of chickens on PRM-contaminated farms, suggesting that chickens were exposed to Dg-Lipocalin. Immunization with rDg-Lipocalin-induced antibodies against rDg-Lipocalin and the immune plasma exhibited acaricidal effects on PRMs. However, the acaricidal effect was unstable in experiments 1 and 2. The number of PRMs per group used for the *in vitro* feeding assays was not significantly different from that used in previous studies [6–16], suggesting that the efficacy of Dg-Lipocalin as a vaccine antigen may be inferior to that previously reported.

Lipocalins are a superfamily of low molecular weight proteins that carry lipophilic binding molecules and participate in a variety of functions [29]. In ticks, lipocalin in saliva suppresses the inflammatory response at sites of blood feeding by binding to small molecules such as histamine and serotonin that are important mediators of the host immune response, acting on monocytes, dendritic cells, and lymphocytes [20,21]. In contrast, a subset of lipocalins was expressed in the midgut of *Ixodes ricinus* [30]. Additionally, in *Ornithorodos savignyi*, a subset of lipocalins is expressed in the midgut and hemocytes, and its expression increases in hemocytes after bacterial inoculation [31]. In *Amblyomma americanum*, lipocalin

306 expression has been detected in hemocytes, implying its role in the response against pathogen
307 infections [32]. In addition, lipocalin-like protein (PV5) of *Plasmodium falciparum* is
308 involved in oxidative damage control and detoxification of heme released by the catabolism
309 of erythrocytic hemoglobin to form hemozoin, which is important for the multiplication and
310 growth of the parasite [33,34]. Blood sucking arthropods including PRMs have adapted their
311 bloody diet via heme-detoxification mechanisms to avoid toxicity. Therefore, lipocalins may
312 be expressed in other cells, such as the midgut and hemocytes, in addition to the salivary
313 glands and may have multiple functions, including blood digestion in the midgut and
314 protection against pathogenic microorganisms. In this study, immune plasma containing
315 antibodies specific to Dg-Lipocalin exhibited partial acaricidal effects on PRMs upon single
316 feeding. Therefore, Dg-Lipocalin may have crucial roles in the homeostasis and heme
317 detoxification machinery of PRMs, but other lipocalins may potentially substitute for Dg-
318 Lipocalin because other subsets of lipocalins are present in PRMs [23]. To understand the
319 mechanisms underlying the acaricidal effects of immune plasma against rDg-Lipocalin,
320 further studies are required to clarify the function of Dg-Lipocalin. Additionally, it is
321 necessary to determine whether other lipocalins have acaricidal effects as vaccine antigens
322 and whether antibodies against Dg-Lipocalin can cross-react with other lipocalins and inhibit
323 their functions.

In the present study, immune plasma against rDg-Lipocalin showed acaricidal effects on PRMs. However, these effects were not stable in experiments 1 and 2, although the same experimental conditions with similar numbers of PRMs were set in both experiments. With reference to recent data on PRM mortality from *in vitro* feeding assays [35–37], we set the mortality at day 7 for the control group at 17% and for the immune group at 33%, the statistical power at 0.8, and the sample size at >88. In Experiment 1, the sample size of the control group was slightly lower than the set sample size but the statistical power was 0.83. Whereas, the statistical power of Experiment 2 was low, and the total of both experiments yielded a statistical power of 0.69, which was lower than expected. There are several possible reasons for this difference in the results between the experiments. In this study, we used mixed stages of PRMs, and the proportions of protonymphs included in the groups in each experiment were different. In experiment 1, the control and immunized groups included 30 and 32 protonymphs, respectively (35.3% and 32.0% of the total number of PRMs in each group). Whereas, experiment 2 included 24 protonymphs in the control group and 21 protonymphs in the immunized group (15.9% and 15.4% of the total number of PRMs in each group, respectively). Several previous studies showed a higher efficacy of immune plasma against vaccine antigens in protonymphs [11, 12, 16]; therefore, the difference in the number of protonymphs may have resulted in different efficacy outcomes between the experiments. On the contrary, some antigens are effective in increasing the mortality of both adults and

protonymphs, as well as reducing the reproductive efficiency of the adults [12, 15]. Compared to these antigens, the anti-PRM effects of immune plasma against rDg-Lipocalin on the mortality and fecundity may be unstable and unlikely to be high. Therefore, further investigation is required to clarify the fecundity and effect of immune plasma on each developmental stage to confirm the efficacy of Dg-Lipocalin as a vaccine antigen. Additionally, we used PRMs collected from poultry farms for artificial feedings in the present study. However, farm-sourced PRMs are assumed to be exposed to various external factors such as season, transportation conditions, and PRM control operations, which may have caused differences in the conditions of the PRMs in each experiment. Therefore, colonized PRMs maintained in the laboratory are preferable for research objectives [38]. However, long-term maintenance of colonies requires the exposure of chickens to PRMs for a long period; therefore, the establishment of a more convenient and stable method for maintaining PRM colonies is required. Moreover, PRMs feed on blood several times during their lifetime [1]; therefore, they have several opportunities to be affected by vaccination-induced antibodies, which may be more effective in long-term trials using immunized chickens. As the *in vitro* feedings in this study were conducted to determine the acaricidal effects on PRMs by a single feeding, PRM challenge trials on immunized chickens are required to determine the efficacy of the vaccine antigen in more detail, which may provide more stable results. For the development of vaccines using Dg-Lipocalin, the efficacy of Dg-Lipocalin must be verified

362 by conducting *in vivo* trials on immunized chickens under cage-rearing conditions [13], and
363 large-scale trials should be planned based on their results.

364 Because the exposed antigens are repeatedly injected into the host, antibody
365 production in immunized chickens may be enhanced by blood feeding in response to an
366 increase in the number of PRMs. Immunization with soluble mite extracts maintained high
367 antibody titers and prevented the growth of the PRM population in a field trial [14], and
368 feeding blood-containing immune plasma with a high antibody titer against a vaccine antigen
369 significantly increased the mortality of PRMs [14]. Thus, the maintenance of a high antibody
370 titer is required to exert sufficient acaricidal effects on PRMs by vaccination. In this study,
371 Dg-Lipocalin was identified as the exposed antigen; therefore, the application of Dg-
372 Lipocalin as a vaccine antigen in PRM-challenge trials on hens may lead to the maintenance
373 of antibody titers for a longer period and enhanced and prolonged efficacy. Further studies are
374 required to assess the efficacy of Dg-Lipocalin as a vaccine antigen.

375 In this study, we identified Dg-Lipocalin as the exposed antigen and determined its
376 acaricidal effects on PRMs; however, these effects were not stable in *in vitro* feeding assays of
377 a single feeding. Additionally, the duration of antibodies induced by immunization with Dg-
378 lipocalin must be clarified; thus, the efficacy of *Dg-Lipocalin* as a vaccine antigen must be
379 further assessed. Considering antigen modifications such as mutations, reconstruction as
380 chimeric antigens may improve vaccine-induced immunity. Additionally, the selection of an

effective adjuvant may increase the capability of the antigen to induce a stronger immune response. Moreover, a previous study showed that the combination of two immune plasmas significantly increased the mortality of PRMs compared with the single use of immune plasma [13]. Therefore, a cocktail vaccine containing several antigens that are essential for the physiology of PRMs, in addition to Dg-Lipocalin, may have improved efficacy. Evaluation of the efficacy of a combination of Dg-Lipocalin and vaccine candidates reported as anti-PRM vaccine antigens [6-16] would be required in the future.

In conclusion, Dg-Lipocalin was confirmed as an exposed antigen; therefore, its application as an antigen for cocktail vaccines may contribute to sustained antibody titers and better efficacy for a longer period. Thus, the search for other exposed antigens may lead to the development of more effective cocktail vaccines against PRMs.

Ethics approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Hokkaido University (Approval number: 20-0051). All experiments were performed in accordance with the relevant guidelines and regulations of the Faculty of Veterinary Medicine, Hokkaido University, which has been fully accredited by the Association of Laboratory Animal Care International (AAALAC).

Funding

This research was funded by Grants-in-Aid for Scientific Research, grant numbers JP23K27063 and JP23K27068 of the Japan Society for the Promotion of Science.

CRediT authorship contribution statement

Fumiya Horio: Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hikari Seo**: Resources, Methodology, Data curation. **Shwe Yee Win**: Investigation. **Jumpei Sato**: Investigation. **Yoshinosuke Motai**: Investigation. **Shunsuke Yamagami**: Investigation. **Takumi Sato**: Writing – review & editing, Resources. **Eiji Ohishi**: Writing – review & editing, Resources. **Naoya Maekawa**: Writing – review & editing, Validation, Resources, Formal analysis, Data curation. **Tomohiro Okagawa**: Writing – review & editing, Validation, Resources, Formal analysis, Data curation. **Satoru Konnai**: Writing – review & editing, Validation, Supervision, Resources, Formal analysis, Data curation, Conceptualization. **Kazuhiko Ohashi**: Writing – review & editing, Validation, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Shiro Murata**: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data Availability Statement

The data presented in this study are available in the article and supplementary materials.

Declaration of Competing Interest

Takumi Sato and Eiji Oishi are employed by Vaxxinova Japan K.K., Tokyo, Japan. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would also like to express sincere gratitude to Dr. Wataru Hashimoto, Japan Layer K.K., Gifu, Japan, and Dr. Akio Enya, and all famers for their kind assistance in the collection of the blood and the PRM samples throughout this study. We would also like to thank Editage (*www.editage.jp*) for English language editing.

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575

Figure captions

Fig. 1. Phylogenetic analysis of *Dg-Lipocalin.* The phylogenetic analysis of *Dg-Lipocalin* using *lipocalin* genes from mites, ticks, other arthropods, and chickens. Phylogenetic analysis was conducted using MEGA 11 [25] using the maximum likelihood method with 1,000 bootstrap replicates.

Fig. 2. Antibody production specific to recombinant *Dg-Lipocalin* (rDg-Lipocalin) in the immune plasmas. (A) Western blotting was conducted to detect specific antibodies against rDg-Lipocalin in the plasma from chickens immunized with rDg-Lipocalin mixed with the same volume of Freund's Incomplete Adjuvant. The arrowhead indicates the predicted molecular weight of rDg-Lipocalin (approximately 16 kDa). (B) As a control, chickens were injected with PBS mixed with an equal volume of adjuvant. No specific antibodies to rDg-Lipocalin were detected in the control plasma.

Fig. 3. Detection of antibodies specific to *Dg-Lipocalin* in the plasmas from chickens on farms contaminated with poultry red mites (PRMs). Production of antibodies specific to *Dg-Lipocalin* in the plasmas from chickens on the PRM-contaminated farms was examined by western blotting. Lanes 1–6, plasmas from chickens in PRM-contaminated farms; NC, negative control plasma from chickens that were not infested with PRMs; PC, positive control

595 plasma from chickens immunized with rDg-Lipocalin (immune plasma). The arrowhead
596 indicates the predicted molecular weight of rDg-Lipocalin (approximately 16 kDa), and the
597 white dashed square indicates reactive signals to rDg-lipocalin.
598
599 **Fig. 4. Acaricidal effects of the immune plasmas against rDg-Lipocalin on poultry red**
600 **mites (PRMs).** Starved PRMs of mixed developmental stages were artificially fed with fresh
601 chicken blood replacing its plasma with either immune plasma against rDg-Lipocalin or
602 control plasma from PBS-inoculated chickens. The acaricidal effects in the plasmas against
603 rDg-Lipocalin in experiment 1 (control, $n = 85$; rDg-Lipocalin, $n = 100$) (A), experiment 2
604 (control, $n = 151$; rDg-Lipocalin, $n = 136$) (B), and total (control, $n = 236$; rDg-Lipocalin, $n =$
605 236) (C) were examined by comparing the survival rates between the immunized and control
606 groups. The number of dead PRMs was monitored daily for 7 days and plotted to generate
607 Kaplan–Meier survival curves. Statistical analysis was conducted using the log-rank test.
608 Statistical significance was set at $p < 0.05$.
609
610

Table S1. Primers used for preparing recombinant proteins

Primers	Primer sequencing (5'-3')
pET-28a-Dg-Lipocalin F	CATGCCATGGCACTCCTAGGAACGTGGAAG
pET-28-a-Dg-Lipocalin R	CGGGATCCCACGTAGATTCGCGTGCAG

Restriction enzyme sites are underlined.

Table S2. Expression profiles of a *lipocalin*-like gene in poultry red mites

Antigen candidates	Expression level (FPKM*)		Domain search result (InterPro)
	unfed	fed	
Dg-Lipocalin	1396.13	622.06	Lipocalin/cytosolic fatty-acid binding domain

*FPKM, fragments per kilobase of exon per Million mapped reads

Table S3. Cumulative mortality of PRMs fed with plasma of chickens immunized with rDg-Lipocalin (Experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (n = 85)							
No. of dead PRMs post-feeding	1	2	3	7	7	8	12
Mortality (%)	1.2	2.4	3.5	8.2	8.2	9.4	14.1
Immunized group (rDg-Lipocalin, n = 100)							
No. of dead PRMs post-feeding	4	4	8	14	21	21	30
Mortality (%)	4.0	4.0	8.0	14.0	21.0	21.0	30.0
p value (Fisher's exact test, vs control)	0.377	0.689	0.230	0.251	0.022*	0.042*	0.013*
Odds ratio	3.45	1.72	2.37	1.81	2.95	2.55	2.59
95% confidence interval	0.34–174.00	0.24–19.50	0.55–14.30	0.64–5.58	1.13–8.69	1.01–7.07	1.18–6.03

$* p < 0.05$ was considered statistically significant.

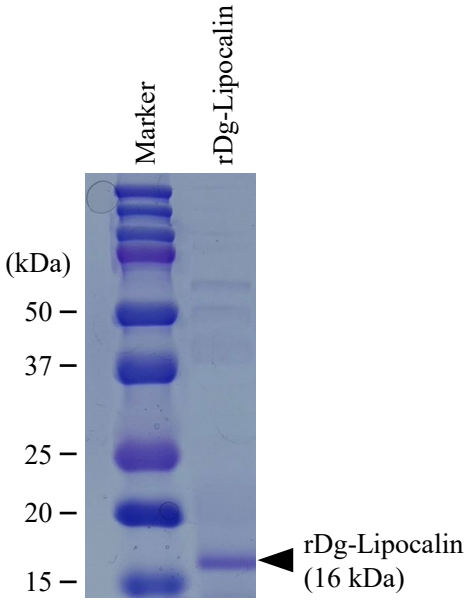
PRM, poultry red mite

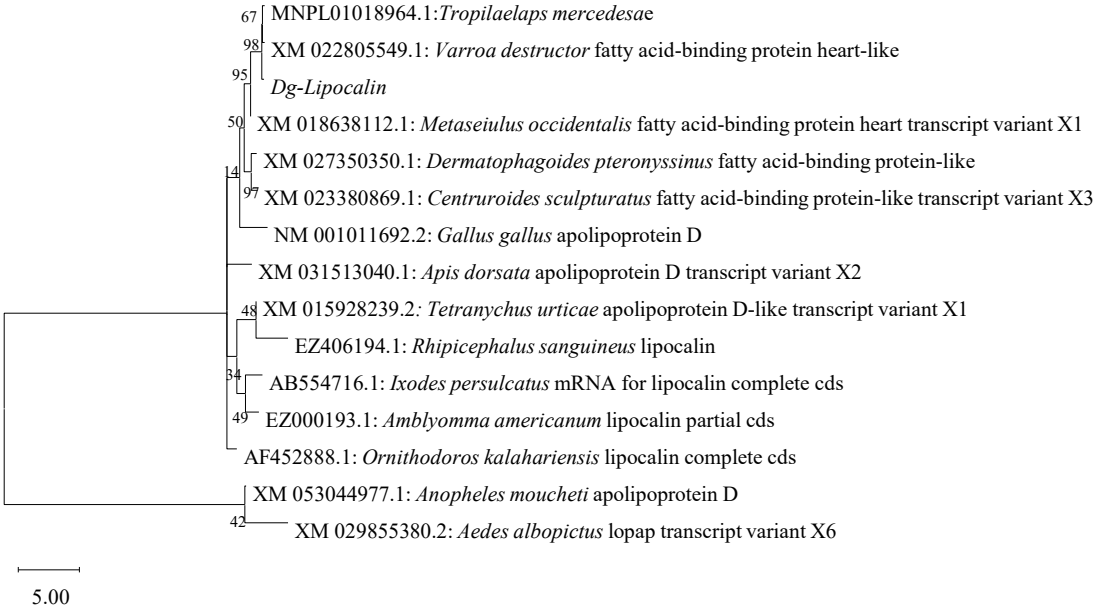
Table S4. Cumulative mortality of PRMs fed with plasma obtained from chickens immunized with rDg-Lipocalin (Experiment 2)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (n = 151)							
No. of dead PRMs post-feeding	9	12	12	15	16	18	23
Mortality (%)	6.0	8.0	8.0	9.9	10.6	11.9	15.2
Immunized group (rDg-Lipocalin, n = 136)							
No. of dead PRMs post-feeding	8	11	13	16	17	19	23
Mortality (%)	5.9	8.1	9.6	11.8	12.5	14.0	16.9
p value (Fisher's exact test, vs control)	1.000	1.000	0.679	0.704	0.712	0.725	0.749
Odds ratio	0.97	1.02	1.22	1.21	1.20	1.20	1.13
95% confidence interval	0.32–2.98	0.39–2.62	0.49–3.05	0.53–2.75	0.55–2.67	0.57–2.55	0.57–2.24

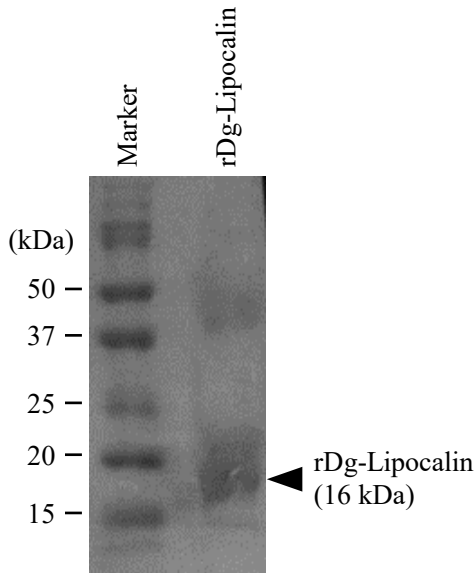
$* p < 0.05$ was considered statistically significant.

PRM, poultry red mite

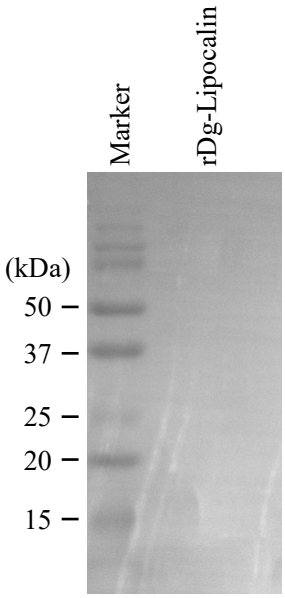

Marker
rDg-Lipocalin
(kDa)
50
37
25
20
15
rDg-Lipocalin
(16 kDa)

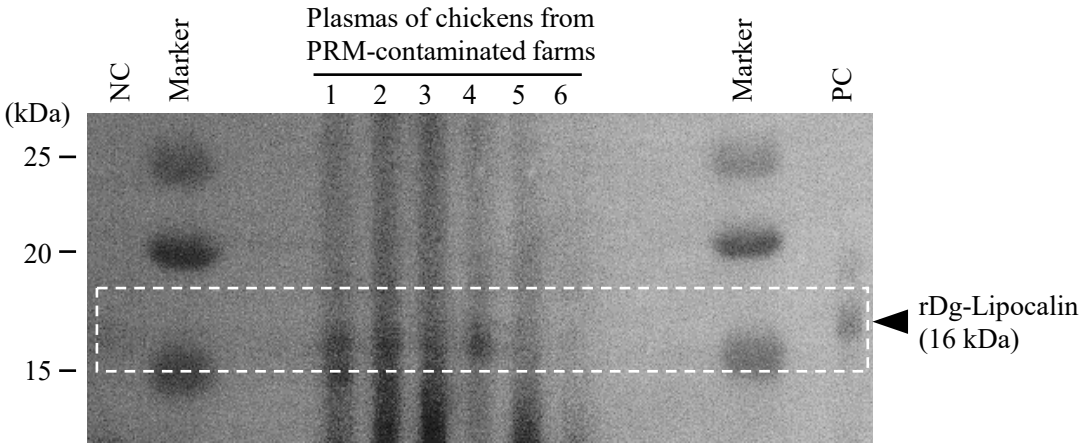

67
MNPL01018964.1:*Tropilaelaps mercedesa*e
98
XM 022805549.1: *Varroa destructor* fatty acid-binding protein heart-like
95
Dg-Lipocalin
50
XM 018638112.1: *Metaseiulus occidentalis* fatty acid-binding protein heart transcript variant X1
4
97
XM 027350350.1: *Dermatophagoides pteronyssinus* fatty acid-binding protein-like
XM 023380869.1: *Centruroides sculpturatus* fatty acid-binding protein-like transcript variant X3
NM 001011692.2: *Gallus gallus* apolipoprotein D
XM 031513040.1: *Apis dorsata* apolipoprotein D transcript variant X2
48
XM 015928239.2*: Tetranychus urticae* apolipoprotein D-like transcript variant X1
EZ406194.1: *Rhipicephalus sanguineus* lipocalin
34
AB554716.1: *Ixodes persulcatus* mRNA for lipocalin complete cds
49
EZ000193.1: *Amblyomma americanum* lipocalin partial cds
AF452888.1: *Ornithodoros kalahariensis* lipocalin complete cds
XM 053044977.1: *Anopheles moucheti* apolipoprotein D
42
XM 029855380.2: *Aedes albopictus* lopap transcript variant X6
5.00

(A) rDg-Lipocalin

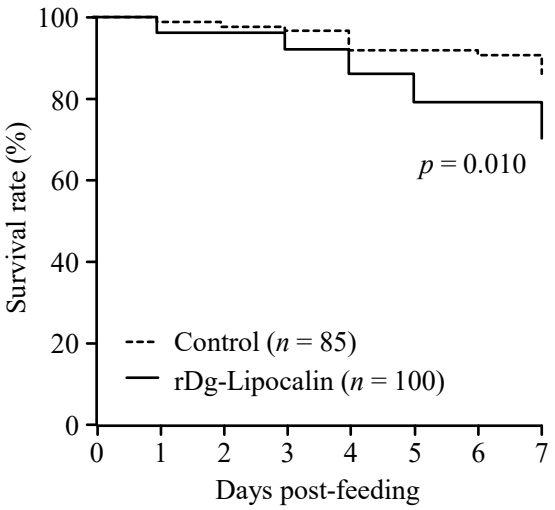


(B) Control

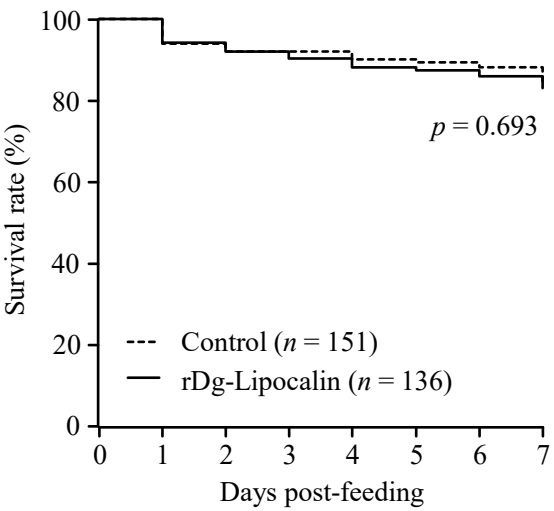



NC
Marker
Plasmas of chickens from
PRM-contaminated farms
1 2 3 4 5 6
Marker
PC
(kDa)
25
20
15
rDg-Lipocalin
(16 kDa)

(A) Experiment 1



(B) Experiment 2



(C) Total