Search for common antigens to develop universal vaccines to control avian mites, poultry red mites, tropical fowl mites, and northern fowl mites

ワクモ、トリサシダニ、及びミナミトリサシダニを防除するユニバーサルワクチン開発に向けた共通抗原の探索

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CONTENTS

ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
cDNA	complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
CI	confidence interval
CP	cysteine protease
CP-PD	peptidase domain of cysteine protease
DNA	deoxyribose nucleic acid
DTT	DL-Dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EV	extracellular vesicle
FER	ferritin
FER1	ferritin 1
FER2	ferritin 2
GSH	glutathione
GST	glutathione S-transferase
HRF	histamine release factor
HRP	horseradish peroxidase
Ig	immunoglobin
IL	interleukin
IPTG	isopropyl b-D-1-thiogalactopyranoside
NCBI	National Center for Biotechnology Information
NFM	northern fowl mite
OR	odds ratio
ORF	open reading frame
PBS	phosphate-buffered saline
PBS-T	PBS containing 0.05% Tween 20
PRM	poultry red mite
PVDF	polyvinylidene difluoride
RACE	rapid amplification of the cDNA ends
RNA	ribonucleic acid
RNAi	RNA interference
RPMC	rat peritoneal mast cell
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCTP	translationally controlled tumor protein
TFM	tropical fowl mite

NOTES

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PREFACE

According to the Food and Agriculture Organization (FAO) of the United Nations, the global poultry business is growing due to greater demand arising from the increased purchasing power of consumers. The production of chicken eggs and meats in 2022 increased by 69% and 111% in comparison to 2000, accounting for 87 (93% of the world's poultry egg production) and 120 million tons, respectively [FAO, 2022]. Along with the increased production, animal welfare has been a significant issue in the poultry industry; therefore, the poultry-rearing system, especially on egg-laying hen farms, has gradually shifted from intensive (cage) to extensive (cage-free) systems, such as organic and free-range poultry management systems [Majewski *et al*., 2024]. However, the shift to extensive systems increases the risks of exposure to several pathogens causing significant economic losses to the poultry industry, including parasites such as *Eimeria* spp., *Leucocytozoon* spp., helminths, and ectoparasites, bacteria such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp. and *Mycoplasma* spp., and viruses such as avian influenza virus, Newcastle disease virus, Marek's disease virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, and fowlpox virus [Bonnefous *et al*., 2022; Gentile *et al*., 2023]. Among these avian pathogens, ectoparasites, including avian mites belonging to Dermanyssidae and Macronyssidae families, fleas such as *Echidnophaga gallinacae*, lice such as *Menopon gallinea*, *Menacanthus stramineus*, *Goniodes gigas*, and *Goniocotes gallinae*, and ticks such as *Argus persicus* are common problems in the free-ranging poultry system due to the difficulty of sanitation measures. Notably, infestation with hematophagous avian mites, which negatively impact the health of poultry and cause the loss of poultry products, resulting in severe economic losses, has become a significant concern in the poultry industry worldwide.

Poultry red mites (*Dermanyssus gallinae*, PRMs), tropical fowl mites (*Ornithonyssus bursa*, TFMs), and northern fowl mites (*Ornithonyssus sylviarum*, NFMs), are known as obligatory pests of chickens including layers, broilers, and native chickens [Sparagano and Ho, 2020]. PRMs are major poultry pests worldwide and especially cause problems on laying hen farms [Chauve, 1998]. TFMs and NFMs are ectoparasites of wild birds [Knee and Proctor, 2007] and are widespread as serious poultry pests due to the migration of wild birds to poultry farms [Wood, 1920]. These avian mites have similar morphological and genetic characteristics [Takehara *et al*., 2019] and establish their life cycles by sucking blood from chickens. Generally, adult females of TFMs and NFMs (~0.6 mm) are smaller than those of PRMs (~1 mm), and they have distinguishable unique morphological characteristics [Di Palma *et al*., 2012; Murillo and Mullens, 2017]. PRMs have a square or keystone-shaped anal plate and whip-like chelicerae, while NFMs have a tear-drop-shaped anal plate and claw-like chelicerae. TFMs and NFMs resemble each other morphologically; however, they can

be distinguished based on the shape of the posterior dorsal plate (tear-drop-shaped) and the number of setae on the sternal plate. NFM has a sharp posterior dorsal plate and two pairs of setae on the sternal plate, while TFM has a more even posterior dorsal plate and three pairs of setae [Denmark and Cromroy, 2003]. The life cycles of avian mites include five stages, egg, larvae, protonymph, deutonymph, and adult, and complete in approximately 1–2 weeks. PRMs ingest the host's blood meal during the protonymph, deutonymph, and adult stages, while protonymphs and adults of TFMs/NFMs require blood meals [Hwang, 2023]. PRMs feed on host blood mostly at night and hide in dark places such as spider webs, cracks, and crevices in the daytime [Chauve, 1998]. This makes it challenging to detect PRMs on farms, thus favoring the rapid growth of mite populations. On the contrary, TFMs and NFMs are persistent in the vicinity of vent feathers, thus constantly sucking blood during the day [van Emous, 2005, Chen and Mullens, 2008, Murillo and Mullens, 2017]. In addition, the feathers of infested poultry seem to be dirty due to the accumulation of mites themselves, and cast skins, eggs, and feces of mites; therefore, TFMs and NFMs are more noticeable than PRMs. However, once these avian mites invade poultry farms, they may persist in farm facilities and/or poultry for long periods [McCulloch *et al*., 2020].

According to previous researches, mite numbers can be estimated at 50,000-500,000 for PRMs or 50,000-100,000 for NFMs per bird in severe cases, leading to 3% blood loss per night and 6% per day, respectively [DeLoach and DeVaney, 1981; Kilpinen *et al*., 2005; van Emous, 2005; Owen *et al*., 2009]. Chickens infested with these mites develop a variety of clinical signs, including anemia, irritation, dermatitis, pecking behavior and plucking feathers, skin wounds, secondary infections, and death in serious cases [Beugnet *et al*., 1997]. Therefore, several studies emphasize the issue of animal welfare because the infestation with avian mites on poultry farms leads to severe behavioral abnormalities and negative impacts on the health of chickens [Murillo *et al*., 2020; Temple *et al*., 2020; Jarrett *et al*., 2022]. Moreover, the infestation by avian mites causes lower feed conversion efficiency and reduces egg production and quality in laying hens [Sigognault Flochlay *et al*., 2017]. PRMs are distributed worldwide [George *et al*., 2015] and are detected in more than 46% of poultry farms in China and Japan [Sparagano and Ho, 2020], 90% in European countries [Sigognault Flochlay *et al*., 2017], including approximately 94% in Germany, Belgium, and the Netherlands [George *et al*., 2015]. The egg-laying industry in European nations annually costs €360 million due to the decreased productivity caused by PRMs and the countermeasures to control them [Hwang, 2023]. TFMs are distributed in warm and tropical regions [Sparagano and Ho, 2020; Waap *et al*., 2020]. In the Neotropical region, such as Brazil, TFMs are the most frequently occurring mites in farms and cause significant losses in poultry farming [Guimarães *et al*., 2001]. Although detailed economic losses related to TFM infestations are limited, their prevalence has been reported in several countries, including Asia, Africa, South America, and Australia

[Rahbari *et al*., 2009; Coimbra *et al*., 2012; Santillán *et al*., 2015; Arce *et al*., 2018; Silva *et al*., 2018; Takehara *et al*., 2019]. NFMs are considered a key pest for laying hens in North America, Brazil, China, and Australia [Murillo and Mullens, 2017]. The infestation with NFMs causes a reduction in egg production (2.1–4.0%), egg weight (0.5–2.2%) and feed conversion efficiency (5.7%), resulting in a profit loss of $0.07-0.10 per hen over a 10-week period [Mullens *et al*., 2009]. Moreover, mixed infestations of these avian mites have been detected in poultry farms in some areas such as Brazil, China, Myanmar, and India [Wang *et al*., 2010; Rezende *et al*., 2013, Sreenivasa Murthy and Panda, 2016; Takehara *et al*., 2019]. Collectively, these avian mites are ununiformly distributed and negatively impact on poultry sectors.

In addition to the direct harm caused by blood-sucking on the host, avian mites may act as vectors for pathogen transmission. In PRMs, pathogens such as *E. coli*, *Erysipleothrix rhusiopathiae*, *M. synoviae*, *M. gallisepticum*, *Plasmodium* spp. as well as viral pathogens such as fowlpox virus, Marek's disease virus, chicken anemia virus, fowl adenovirus have been detected [Chirico *et al*., 2003; Circella *et al*., 2011; Huong *et al*., 2014; George *et al*., 2015; Schiavone *et al*., 2020; Ciloglu *et al*., 2020; Oh *et al*., 2020; Kovács *et al*., 2024]. Moreover, a possible role of PRMs in the transmission of avian influenza A virus and *Salmonella enterica* subspecies *enterica* serovar Gallinarum, the causative agent of fowl typhoid, has been experimentally demonstrated [Sommer *et al*., 2016; Cocciolo *et al*., 2020]. These observations suggest their roles as vectors and/or reservoirs for avian pathogens in the flocks. In addition, several human cases such as urticaria, rash, and dermatitis, possibly caused by the bites of avian mites, have been reported [Cafiero *et al*., 2019; Waap *et al*., 2020; Sioutas *et al*., 2021]. Moreover, zoonotic pathogens such as *Coxiella burnetii* and *Borrelia burgdorferi* sensu lato, the causative agents of Q-fever and Lyme disease, respectively, have been detected in the PRMs collected from cases of itching dermatitis in urban areas [Raele *et al*., 2018]. In addition, several tick-borne pathogens, including *Ehrlichia* spp., *Bartonella* spp., and *Theileria* spp, were detected in PRMs collected from the dermatitis patients, suggesting the accidental infection by the co-feeding of other arthropods [Banović *et al*., 2024]. A recent study demonstrated that *Listeria monocytogenes* isolated in PRMs collected from a backyard chicken farm, showing their role as carriers for food-borne pathogens [Sioutas *et al*., 2023]. Collectively, the infestation of avian mites not only causes serious harm to poultry but also potentially poses a threat to human health.

The current approach for controlling avian mites is through commercial chemical compounds such as organophosphates, pyrethroid, carbamate, macrocyclic lactones, and dichlorodiphenyltrichloroethane [Silva *et al*., 2023]. As acaricides are commonly sprayed on farms, they have limited exposure to mites hiding in chicken feathers or crevices of poultry houses. In addition, the efficacy of acaricides used on farms has decreased because acaricide-resistant mites have been selected by its

prolonged and repetitive use or transmission of resistant genes with mechanisms that counteract the effects of acaricides [Meyer-Kühling *et al*., 2007; Abbas *et al*., 2014]. In addition, current commercial acaricides have short-term residual effects on mites and are substantially inactive on eggs; consequently, their eggs may resume the mite reproduction cycle on farms [Sigognault Flochlay *et al*., 2017]. Furthermore, restrictions are imposed on some chemicals authorized for application on poultry farms, because they are highly toxic and harmful to humans and animals and carry the risk to pollute environmental water and soil and to expose to customers via contaminated eggs and meats [Ansari *et al*., 2014]. In European countries, the use of organophosphates, pyrethroid, and carbamate has been banned to control PRMs [Guerrini *et al*., 2022]. Organophosphate phoxim had been licensed for the control of ectoparasites in livestock, including poultry. However, due to the emergence of resistance against phoxim [Meyer-Kühling *et al*., 2007], it is not available in all European countries [Sigognault Flochlay *et al*., 2017; Hwang, 2023]. Several acaricidal products such as pyrethroids, abamectin, and spinosad are available in some European countries for spraying poultry houses and equipment during empty periods between flocks. Currently, fluralaner, a novel miticide for administration in drinking water, is approved in Europe and widely used for PRM control [Sigognault Flochlay *et al*., 2017; Thomas *et al*., 2017]. Therefore, in the future, the use of chemical acaricides in food production systems may be further restricted to avoid contamination and reduce risks to human health. Furthermore, poultry farming systems have gradually shifted from battery cages to cage-free systems based on concerns for animal welfare [Hartcher and Jones, 2017; Leite *et al*., 2021], making it challenging to monitor and supervise the birds as they roam freely in the pastures. This would increase the risk of mite infestations and exacerbate the effects of chemical acaricides. Considering this scenario, innovative approaches for controlling avian mites are needed to be established as an alternative to chemical acaricides.

As alternative strategies, non-chemical approaches that utilized plant derivatives, silica-based products, entomopathogenic fungi, and semiochemicals, have been studied for PRM control [Rezende *et al*., 2013; Pereira *et al*., 2022; Silva *et al*., 2023]. Although plant-derivatives such as lavender oil, ajowan oil, and other essential oils derived from several plant products have been shown to have over 80% mortality rate of PRMs under laboratory conditions [Nechita *et al*., 2015; Lee *et al*., 2019; Baran *et al*., 2020], their effectiveness was limited only on trapped mites [Lundh *et al*., 2005]. In addition, entomopathogenic fungi such as *Beauveria bassiana*, *Metarhizium anisopliae*, *Trichoderma album*, and *Aspergillus oryzae* have been tested for anti-PRM efficacy *in vitro* [Kasburg, 2016; Miguel, 2019; Nascimento, 2019; Wang *et al*., 2019]. However, utilizing these fungi to combat avian mites in the field may pose several problems, including the potential for unwanted fungal growth that is hazardous to animals and humans [Kasburg, 2016; Miguel, 2019; Nascimento, 2019]. Silica-based

products such as silicon dioxide (SiO_2) have been shown to have acaricidal effects on PRMs by adhering to mites' bodies and immobilizing them, leading to their drying and death [Harrington *et al*., 2009; Schulz *et al*., 2014; Ulrichs *et al*., 2020]. However, even though these products showed sufficient acaricidal efficacy under laboratory conditions, the field application failed due to the influence of several environmental factors [Ulrichs *et al*., 2020]. Studies on the use of semiochemicals to repel PRMs showed that chickens fed with semiochemicals such as volatile organic compounds produced by uropygial glands of ducks (uninfested host) released the same chemicals, impeding PRM behaviors and thereby repelling them [Pageat, 2017; Hwang, 2023]. Nevertheless, the mechanisms involved in chemical-mediated repellency in PRMs are still poorly understood. Thus, these approaches have faced hardships and require further studies for their application in the field.

Rhipicephalus microplus, a well-known cattle tick, with acaricide resistance, has emerged widely in areas where the ticks are distributed [Rodriguez-Vivas *et al*., 2018; Hromníková *et al*., 2022]. *Rhipicephalus microplus* threatens the livestock industry and public health, because it transmits several tick-borne pathogens, including zoonotic agents [Jabbar *et al*., 2015; Perveen *et al*., 2021]. Additionally, ticks resistant to multiple acaricides have emerged in various parts of the world [Bishop *et al*., 2023], along with growing concerns about environmental pollution resulting from the widespread use of chemical acaricides. Anti-tick vaccines are safe and effective interventions to overcome the emergence of acaricide-resistant ticks and to improve tick control, thereby contributing to a reduction in the risks of tick-borne pathogen transmission [de la Fuente and Ghosh, 2024]. The protective mechanisms of the anti-tick vaccine are based on the concept that antibodies produced by vaccination cause dysfunction of target molecules through antibody-antigen interactions during blood feeding, resulting in physiological disorders such as impaired blood digestion, low engorgement weight, and reduced oviposition and hatching rate [de la Fuente and Kocan, 2014]. Therefore, the molecules critical for the physiological functions of ticks could be vaccine antigen candidates. Vaccine antigens against ticks are classified into exposed antigens (non-concealed), which are secreted in the saliva of ticks and are injected into the host during blood feeding, and concealed antigens, which are mostly expressed in the midgut of ticks [Willadsen, 2004; Githaka *et al*., 2020]. Exposed antigens, known as salivary proteins, are bioactive molecules that control the hemostatic and inflammatory responses of the host to create a suitable environment for blood feeding [Šimo *et al*., 2017]. On the other hand, concealed antigens are hidden proteins that are not naturally presented to the host immune machinery [Antunes and Domingos, 2023]. Immunization with exposed antigens can be constantly boosted by contact with ticks, whereas additional immunization with concealed antigens is necessary to maintain protective efficacy [Nuttall *et al*., 2006]. In recent years, several candidates for anti-tick vaccine antigens have been reported [de la Fuente and Ghosh,

2024], and a vaccine using Bm86/Bm95 antigens has been commercialized to control *R. microplus* in several parts of the world [de la Fuente *et al*., 2007; Rodríguez-Mallon, 2023]. Thus, research on the development of anti-tick vaccines has yielded significant achievements. In addition, the development of universal vaccines using protective antigens that are conserved across multi-tick species has become a growing concern, because the transmissions of tick-borne pathogens are a common problem in other tick species [Parizi *et al*., 2023; Nepveu-Traversy *et al*., 2024]. The application of universal vaccines against multiple ticks could greatly improve economic and public health issues.

Currently, vaccine-based strategies inspired by anti-tick vaccines are considered promising for the control of PRMs, and several antigen candidates with anti-PRM effects have been identified using *in vivo* and *in vitro* evaluation systems (Table 1). The principle of anti-PRM vaccine is based on the strategy of anti-tick vaccine; immunization with anti-PRM vaccines produces antigen-specific immunoglobulin (Ig)-Y in hens, which affects the survival and reproduction of PRMs through the interaction of antibodies with naïve antigens of PRMs (Figure 1). As part of recent vaccinomics, the identification of prospective vaccine antigens has focused on the integration of omics datasets [de la Fuente and Merino, 2013; Poland *et al*., 2013; Contreras *et al*., 2019]. Several transcriptome analyses of PRMs have provided a comprehensive understanding of molecules important for the physiology of these mites [Fujisawa *et al*., 2020; Huang *et al*., 2020; Bartley *et al*., 2021; Ribeiro *et al*., 2023]. Moreover, a proteomics analysis has identified the immunogenic antigens suitable for anti-PRM vaccine development [Bartley *et al*. 2015b]. Based on the framework of the anti-tick vaccines, immunization with exposed antigens can interfere with the creation of an environment for blood feeding. On the other hand, the immune response against concealed antigens exhibits lethal effects by inhibiting the protein functions in organs, especially in the midgut after blood feeding [Willadsen *et al*., 1989; Willadsen, 2004; Rodríguez-Mallon, 2023]. The blood-feeding period of PRMs seems to be short compared with ticks; therefore, concealed antigens may be more reliable vaccine antigens against PRMs. Thus, the molecules, which are involved in essential physiology such as blood digestion and reproduction and are expressed in the midgut, were considered promising vaccine antigens against PRMs. Although previous studies revealed the potential of several molecules as vaccine antigens [Harrington *et al*., 2009; Bartley *et al*., 2015b], their efficacies are required to be improved for practical use [Bartley *et al*., 2017]. Currently, there is no commercially available vaccine against PRMs, and for practical use, the efficacy of anti-PRM vaccines needs to be improved by searching for more potent antigens and/or combining multiple antigens as a cocktail vaccine. According to the strategy of anti-tick cocktail vaccines, the combination of multiple antigens could provide better protective efficacy than the use of a single antigen, since more physiological processes can be inhibited

[Lima-Barbero *et al*., 2019b]. In addition, a formulation of cocktail vaccines containing multiple antigens conserved among different tick species has the potential to broaden the protection range of the ticks and to enhance the efficacy against various developmental stages of ticks by disrupting biological functions more effectively [de la Fuente and Ghosh, 2024]. Regarding the combination of antigens, however, the concept of antigenic competition via inter- or intramolecular competitive processes is still debated [Lima-Barbero *et al*., 2019b]. TFMs and NFMs share biological and physical traits with PRMs, causing comparable problems on farms; nevertheless, no vaccines against TFMs and NFMs have been studied. Therefore, the search for anti-PRM vaccine antigens that are highly conserved among avian mites may offer broad-spectrum efficacies and contribute to minimizing economic losses on poultry farms in various regions, similar to the concepts of universal vaccines against ticks. Vaccines are widely accepted in the poultry industry and offer advantages over existing treatments, such as non-toxicity to the environment, inexpensive costs, and prolonged efficacy [Wright *et al*., 2016]. Thus, further evaluation of antigen candidates previously and newly identified in PRMs as universal vaccine antigens and their use as cocktail vaccines may lead to the development of a highly effective vaccine against multiple avian mites.

In this study, to develop the universal vaccine for controlling hematophagous avian mites, vaccine antigens, which were previously evaluated in PRMs, were identified from TFMs and NFMs, and their potentials as universal vaccine antigens were assessed. Considering the future application of multiple antigens, in the present study, the feasibility of four molecules as universal vaccine antigens was investigated. In Chapter I, histamine release factor (HRF) also known as translationally controlled tumor protein (TCTP) [Bartley *et al*., 2009], a multifunctional protein involved in biological activities and allergic responses; in Chapter II, glutathione S-transferase (GST) [Bartley *et al*., 2015a], a multifunctional enzyme vital for the detoxification of drugs; in Chapter III, cysteine protease (CP) [Bartley *et al*., 2012; Murata *et al*., 2021], a digestive enzyme important for hemoglobin digestion; and in Chapter IV, ferritin 2 (FER2) [Xu *et al*., 2021], an iron-binding protein involved in iron transport and metabolism, were evaluated as potential antigens.

Table 1. Antigen candidates used for vaccines against poultry red mites

Antigen	Predicted function	Method	Efficacy*	Reference
Dg-HRF-1	Histamine release factor	*in vitro*	Increased mortality	Bartley *et al*., 2009
Dg-CatD-1, Dg-CatL-1	Aspartic protease, cysteine protease	*in vitro*	Increased mortality	Bartley *et al*., 2012
Deg-SRP-1, Deg-VIT-1, Deg-HGP-1	Serpin, Vitellogenin, Hemelipoglycoprotein	*in vitro*	Increased mortality	Bartley *et al*., 2015b
PRM extracts	Mixed stages of PRMs	*in vitro*	Increased mortality	Makert *et al*., 2016
Der g 10, Der g 11	Tropomyosin, Paramyosin	*in vitro*	Increased mortality	Wright *et al*., 2016
SME	Soluble mite extract	*in vivo*	Increased mortality	Bartley *et al*., 2017
Deg-SRP-1+Deg-VIT-1+Deg-HGP-1	Serpin, Vitellogenin, Hemelipoglycoprotein	*in vivo*	No significant difference (field trial)	Bartley *et al*., 2017
Dg-CatD-1	Aspartic protease	*in vitro*	Decreased reproductive capacity	Price *et al*., 2019
Deg-AKR	Akirin	*in vitro*	Decreased reproductive capacity	Lima-Barbero *et al*., 2019a
Deg-CALU, Rhm-SUB	Calumenin, *Rhipicephalus microplus* Subolesin	*in vitro*	Decreased reproductive capacity	Lima-Barbero *et al*., 2019b
Dg-CatD-1, Dg-CatL-1, Dg-Lgm	Aspartic protease, cysteine protease, legumain	*in vivo*	Increased mortality, decreased reproductive capacity	Xu *et al*., 2020
Deg-CPR-1	Cysteine protease	*in vitro*	Increased mortality	Murata *et al*., 2021
Dg-Cys	Cysteine protease inhibitor	*in vitro*	Increased mortality (nymphs), decreased reproductive capacity	Fujisawa *et al*., 2021a
Dg-APMAP	Adipocyte plasma membrane-associated protein	*in vitro*	Increased mortality	Fujisawa *et al*., 2021b
Dg-FER1, Dg-FER2	Ferritin 2, Ferritin 1	*in vivo*	Increased mortality, decreased reproductive capacity	Xu *et al*., 2021
Deg-CPR2	Cysteine protease	*in vitro*	Increased mortality (nymphs)	Ariizumi *et al*., 2022
Dg-Ctr1	Copper transporter 1	*in vitro*	Increased mortality (nymphs)	Fujisawa *et al*., 2022

* Mortality included the efficacies against mixed stages of PRMs, adults, or nymphs. Reproductive capacity was evaluated based on oviposition, hatching rates, and abilities to produce the progenies per adult.

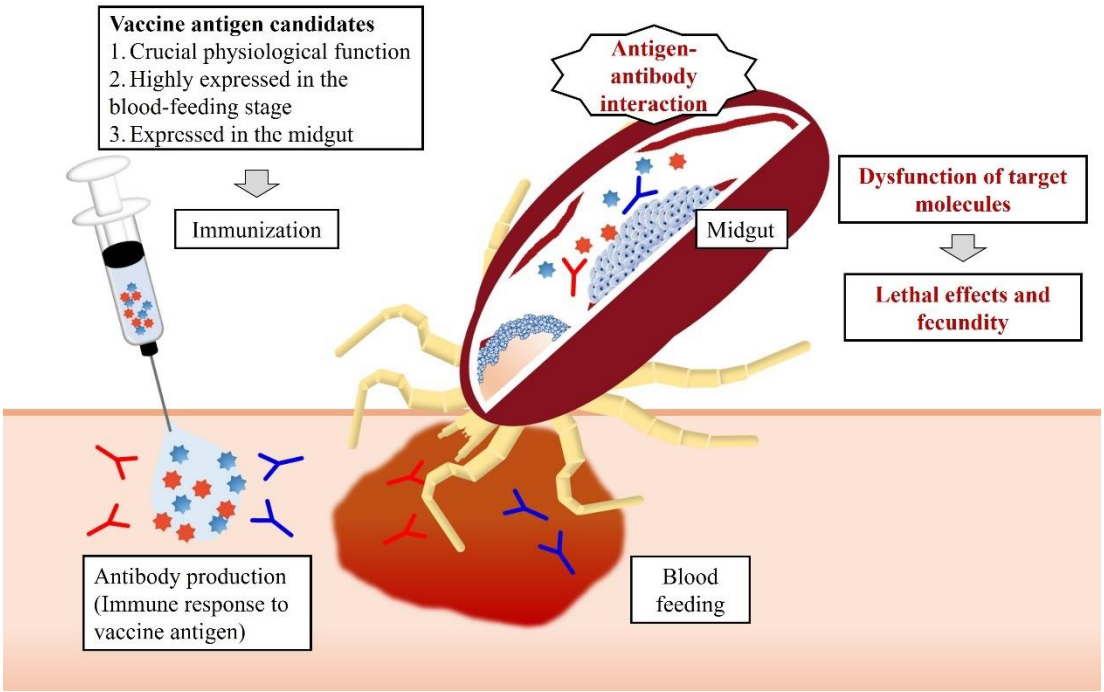

Figure 1. Principle of anti-PRM vaccine.

Chickens are immunized with vaccine antigens which are essential for the physiological activity of PRMs. Antigen-specific antibodies produced in immunized animals are ingested by PRMs during blood feeding and induce dysfunction of target molecules through the interaction of antibodies with naïve molecules of PRMs, thereby exerting lethal effects and decreased fecundity on PRMs.

CHAPTER I

In vitro evaluation of histamine release factor as a universal vaccine antigen against poultry red mites, tropical fowl mites, and northern fowl mites

INTRODUCTION

Currently, chemical acaricides authorized for application in poultry farms to control avian mites have been restricted due to their negative impact on the environment and human health [Abbas *et al*., 2014; Ansari *et al*., 2014]. In addition, prolonged use of the same chemicals or exposure to sublethal doses of acaricides resulted in the selection of acaricide-resistant mites, thereby diminishing their efficacy [Nordenfors *et al*., 2001; Marangi *et al*., 2012]. A vaccine-based control strategy is an alternative approach and several antigen candidates with anti-PRM effects have been identified by an *in vitro* evaluation system [Fujisawa *et al*., 2021a, b; Murata *et al*., 2021; Ariizumi *et al*., 2022; Fujisawa *et al*., 2022]. However, the efficacy of anti-PRM vaccines requires further improvement for the field application [Bartley *et al*., 2017]. The combined use of multiple antigens as a cocktail vaccine could be a predictive approach to improve the protective efficacy. In addition, the application of highly conserved molecules among avian mites as vaccine antigens may provide broad-spectrum acaricidal effects against multiple species of avian mites and lead to the establishment of methods for their control in various areas where different mites are distributed. Moreover, developing a universal vaccine is a desirable approach to improve animal welfare and production losses in poultry farms worldwide. For these reasons, identification and evaluation of multiple antigens suitable for utilization as universal vaccines, as well as for future application as cocktail vaccines, are required.

Histamine release factor (HRF) is a ubiquitous protein that is widely expressed in eukaryotic organisms [Taylor *et al*., 2015] and has intercellular and extracellular functions [Macdonald, 2012]. HRF/TCTP has multiple functions in cellular biological processes, such as cell growth, regulation of protein synthesis and degradation, microtubule stabilization, calcium-binding activities, and apoptosis [Bommer and Kawakami, 2021]. Extracellular HRF/TCTP activates various human immune cells, such as basophils, eosinophils, T cells, and B cells, and facilitates the release of various mediators, such as histamine and cytokines involved in allergic responses [Macdonald, 2012; Lee *et al*., 2020]. Moreover, HRF/TCTP has been identified in some parasites and has been targeted for therapeutics or vaccines. Immunization with recombinant HRF/TCTP of *Plasmodium falciparum*, *P. berghei*, or *P. yoelli* in mice showed reduced levels of parasitemia when they were challenged with the YM strain of *P. yoelli*. These observations suggest that HRF/TCTP plays a significant role in the establishment of parasitemia in animals infected with *Plasmodium* parasite [Taylor *et al*., 2015]. The knockdown of the *HRF*/*TCTP* genes in *Schistosoma japonicum* by RNA interference negatively affected their development, survival, embryo viability, and fecundity [Zhong *et al*., 2022]. Furthermore, immunization with recombinant HRF/TCTP from *S. japonicum* (r*Sj*TCTP) induced higher levels of serum immunoglobin G (IgG), interleukin

(IL)-2, and IL-10 in mice and reduced the number of parasites, egg burden in the liver, and hatching rate [Zhong *et al*., 2022]. The recombinant *Sarcoptes scabiei* HRF/TCTP (rSsHRF/TCTP) induced histamine release in mice *in vivo* and promoted host inflammatory response. Moreover, rSsHRF/TCTP exhibited degranulation in KU812, a human basophilic leukemia cell line, and increased the production of IL4, IL6, and IL13, in addition to histamine release [Xu *et al*., 2022]. In addition, the elevated *HRF*/*TCTP* mRNA expression level of *Ixodes scapularis* in *B. burgdorferi*-infected ticks was observed during the rapid feeding phase. Furthermore, gene silencing of *HRF/TCTP* impaired tick blood feeding and decreased *B. burgdorferi* load in ticks, suggesting an important role in tick engorgement and efficient *B*. *burgdorferi* transmission [Dai *et al*., 2010]. Collectively, HRF/TCTP is a candidate vaccine antigen for controlling parasites, including arthropods. Among avian mites, HRF was identified in PRMs. The *HRF* gene was ubiquitously expressed in various tissues of PRMs, and the recombinant protein of HRF (rHRF) induced degranulation of rat peritoneal mast cells (RPMCs). Furthermore, the *in vitro* feeding with yolk-derived anti-PRM-HRF IgY antibodies showed acaricidal effects on PRMs [Bartley *et al*., 2009]. However, the HRFs of TFMs and NFMs have not yet been identified, and their similarities to those of PRMs remain to be determined.

In Chapter I, the *HRF* genes from TFMs and NFMs were genetically characterized, and their potential as common antigens for application as a universal vaccine across avian mite species was assessed. The *HRF* genes from TFMs and NFMs were identified and compared with those of PRMs. The rHRFs of PRMs, TFMs, and NFMs were generated, and chickens were immunized with each rHRF to produce immune plasma. To evaluate the potential of HRFs as universal vaccine antigens, the cross-reactivity of immune plasmas with rHRFs from different avian mites was analyzed. In addition, *in vitro* feeding assays using PRMs were conducted to evaluate the acaricidal effects of immune plasma against the rHRFs of TFMs or NFMs on PRMs.

MATERIALS AND METHODS

Ethical statement

1. Genetic recombination experiment

All genetic recombination experiments were performed in accordance with the Manual for Safety Management on Genetic Recombination Experiment in Hokkaido University (Approval number: 2019-024).

2. Animal experiments

The animal experiments were performed in accordance with the regulations fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The immunizations of chickens with rHRF of PRMs, TFMs, and NFMs were approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval number 22-0051), and chickens were housed in the animal facility at the Faculty of Veterinary Medicine, Hokkaido University.

Mite samples

PRM samples were collected in TubeSpin Bioreactor 600 bottles (TPP Techno Plastic Products AG, Trasadingen, Switzerland) from PRM-contaminated egg-laying farms in Japan and transferred to the laboratory at 4 °C. PRMs were kept at 25 °C and 70% humidity without blood feeding for a week, and these starved PRMs were maintained at 5 °C and 70% humidity for further use. Mixed stages of blood-fed PRMs (20–30 mites) and samples of TFMs and NFMs that were previously collected in the Republic of the Union of Myanmar (Burma) and were genetically and morphologically identified [Takehara *et al*., 2019] were used for RNA extraction.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was isolated from the homogenized mite samples using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove unwanted DNA. cDNA was synthesized from 1μg of RNA using PrimeScript Reverse Transcriptase (Takara Bio Inc., Shiga, Japan) and 200 pmol of oligo (dT)18 primer (Hokkaido System Science, Hokkaido, Japan).

Identification of *HRF* genes from TFMs and NFMs

The partial *HRF* genes of TFMs and NFMs were amplified using primers listed in Table I-1. The primers were designed based on the conserved regions of *HRF* nucleotide sequences from *D. gallinae* (FM179713), *Tropilaelaps mercedesae* (OQR67526) and *Varroa destructor* (XM022810042). The amplified products were

cloned into the pMD20 vector (Takara Bio Inc) and transformed into competent DH5α *E coli* cells (Takara Bio Inc.). Nucleotide sequences were obtained using an automated CEQ GeXP sequencer (Beckman Coulter Inc., Brea, CA, USA). To obtain the open reading frame (ORF) sequences of the *HRF* genes from TFMs and NFMs, 3′ and 5′ regions were determined using the rapid amplification of the cDNA ends (RACE) system (Invitrogen) according to the manufacturer's instructions. The primers used for the RACE analysis were designed based on the partial nucleotide sequences of *HRF* genes (Table I-1). The amplified DNA products were cloned into pGEMT easy vector (Promega, Madison, WI, USA) and transformed into competent DH5α *E. coli* cells. The ORF sequences of *HRF* genes of TFMs (LC813285) and NFMs (LC813286) were determined. The sequence homology of the *HRF* genes of TFMs and NFMs with those of PRMs and other vertebrates was analyzed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). A maximum likelihood phylogenetic tree analysis was performed using the nucleotide sequences of the *HRF* genes of mites, ticks, fruit flies, mosquitoes, and chickens (Table I-2). MEGA software (version X) was used to construct a phylogenetic tree with 1,000 bootstrap replicates and a discrete gamma distribution (+G), assuming that a certain fraction of sites was evolutionarily invariable (+I) to improve tree topology [Kumar *et al*., 2018].

Generation of rHRF proteins

The nucleotide sequence of the *HRF* gene from PRMs (accession no. FM179713) was used to generate the recombinant protein. The coding regions of the *HRF* genes from PRMs, TFMs, and NFMs were amplified using primers, including the NdeI and XhoI restriction sites (Table I-1). The products were cloned into the NdeI and XhoI restriction sites of the pET19b vector (Merck & Co., Inc., Rahway, NJ, USA) and transformed into *E. coli* (DE3) (Merck & Co., Inc.). The recombinant protein expression was induced with 1 mM Isopropyl b-D-1-thiogalactopyranoside (IPTG), and the bacteria were grown at 37 °C for 4 h until the OD value reached 0.6. Recombinant proteins were extracted from the inclusion body fractions using the BugBuster solution (Merck & Co., Inc.) and solubilized in a buffer containing 0.3% N-lauroylsarcosine and 50 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS; Merck & Co., Inc.) (pH 11.0). The rHRFs were purified with Ni Sepharose™ 6 Fast Flow resin (GE Healthcare, Chicago, IL, USA) and eluted with 0.3% N-lauroylsarcosine and 50 mM N-cyclohexyl-3-aminopropanesulfonic-acid (CAPS) (Merck & Co., Inc.) (pH 11.0) buffer containing 250 mM imidazole (Nacalai Tesque, Tokyo, Japan). The refolding dialysis was performed against 10 mM Tris-HCL (pH 8.5) buffer containing 0.1 mM DL-Dithiothreitol (DTT) (Merck & Co., Inc.) at 4 ℃ overnight. The purity of rHRFs was confirmed by 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie Brilliant Blue (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The concentration of

rHRF was determined using a Pierce™ Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Production of immune plasma against rHRF proteins

To produce the immune plasma against rHRFs of PRMs, TFMs and NFMs, four chickens per group were immunized with 20 μg per 0.5 mL of each rHRF emulsified with Freund's incomplete adjuvant (FUJIFILM Wako Pure Chemical Corporation). The control chickens were injected with phosphate-buffered saline (PBS, pH 7.2) with the same adjuvant. The chickens were subcutaneously immunized twice at 3 and 7 weeks of age. Three weeks after the second immunization, chickens were euthanized by collecting heparinized whole blood from the hearts under deep general anesthesia with isoflurane inhalation (Zoetis Japan, Tokyo, Japan). Approximately 25–30 mL of heparinized blood was collected from each chicken. The immune plasma against each rHRF was isolated for further analysis by centrifugation and stored at -80 °C until use. During the experimental trial, the health status of chickens was recorded, and there were no instances of mortality, reduction in body weight, or any other noticeable symptoms in all immunized groups.

Enzyme-linked immunosorbent assay (ELISA)

The antibody titer in the plasmas obtained from each immunized chicken was confirmed by ELISA. The rHRFs of PRMs, TFMs and NFMs (100 ng/well) was coated onto a 96-well plate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) using carbon-bicarbonate buffer (pH 9.8) at 4 °C overnight. The plate was washed three times with PBS containing 0.05 % Tween 20 (PBS-T). The plate was blocked with 1% bovine serum albumin (BSA) at 37 °C for 1 h. Then, the plate was washed five times with PBS-T. The antigen-coated plate was reacted with 100 μL of diluted immune plasma (×2,000, ×4,000, ×8,000, ×16,000, ×32,000, and ×64,000) obtained from each chicken as the primary antibody. After the plate was washed with PBS-T five times, the reaction complex was labeled with goat anti-chicken IgY[IgG](H+L)-horseradish peroxidase (HRP) (Bethyl Laboratories, Inc., Montgomery, TX, USA) for 1 h at 37 °C. Thereafter, the plate was washed five times with PBS-T and the 3, 3', 5, 5'-tetramethylbenzidine (TMB) One Component HRP Microwell Substrate (Bethyl Laboratories, Inc.) was added to each well and incubated for 20 min at room temperature (RT) in the dark. Finally, the reaction was stopped by adding 100 μL of 0.18 M H_2SO_4 to each well. The absorbance was measured at 450 nm using an ELISA plate reader MTP 900 (Corona Electric Co., Ltd, Ibaraki, Japan). The cutoff value was set as the value of the control plasma at a 2,000-fold dilution. Each dilution of plasma from immunized chickens was considered positive if the absorbance values were above the cutoff value.

Western blotting

The production of specific antibodies against each rHRF and the cross-reactivity of each immune plasma with rHRFs from different mites were confirmed by western blotting. Each rHRF was separated by 13% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Merck & Co., Inc.). The membranes were blocked with 3% skim milk in PBS-T overnight at 4 ℃. The membranes were washed with PBS-T three times and reacted with each immune plasma (1:1,000) at 25 ℃ for 1 h. Membranes were washed with PBS-T three times and reacted with anti-chicken IgY peroxidase rabbit antibody (1:10,000) (Sigma-Aldrich, St. Louis, MO, USA) at 25 ℃ for 1 h. To visualize the signals, the Immobilon Western Chemiluminescent Horseradish Peroxidase (HRP) Substrate (Merck & Co., Inc.) was added to the membranes and incubated at 25 ℃ for 5 min. To confirm His-tagged rHRFs purification, each rHRF was incubated with HRP-conjugated anti-6-his, mouse monoclonal antibody (1:6,000; Merck & Co., Inc.) and labeled with goat anti-mouse IgG antibody (1:10,000; Merck & Co., Inc.).

Assessment of acaricidal effects of immune plasmas on PRMs

In vitro feeding assays were conducted to assess the acaricidal potential of the immune plasma against each rHRF [Ariizumi *et al*., 2021]. In this study, due to the limited availability of TFMs and NFMs in Japan, only PRMs were used for analysis. Fresh blood was collected from healthy chickens housed at the Field Science Center for the Northern Biosphere, Hokkaido University, and plasma was replaced with pooled immune plasma from each immunized or control group. To construct the devices for the artificial feedings, 10 mL serological disposable pipets (Corning Inc., Corning, NY, USA) were cut and assembled across stretched Parafilm (Bemis Co., Inc., Neenah, WI, USA). Mixed stages of starved PRMs stored at 5 °C were transferred at 25 °C one day before the assays and collected using 300 μL Universal Barrier Tips (Neptune Scientific, San Diego, CA, USA). Tips containing starved PRMs were inserted into the devices, and subsequently, the open ends of the pipettes were closed with rubber caps (Cytiva, Uppsala, Sweden). For air ventilation, 27-G needles were stabbed into the rubber caps. After placing the starved PRMs in the devices, the tops of the devices were filled with 300 μL of blood containing immune plasma, and blood feeding was performed in a dark and humid environment with gentle shaking for 4 h at 40 ℃ [Figure I-1]. Blood-fed PRMs were collected using a pasture pipette and maintained at 25 ℃, and the mortality rate was monitored every day for seven days. The number of dead adult and nymph PRMs was recorded separately. The *in vitro* feeding assays were conducted three times. To reduce the bias caused by differences in the condition of PRMs, the same lot of PRMs, which were stored in the same single tubes from collection on farms to maintenance in the laboratory, were used for each experiment.

Statistical analysis

For statistical analysis, EZR statistical software was used [Kanda, 2013]. To compare the daily mortality rate of PRMs between the immunized and control groups after *in vitro* feeding, Fisher's exact test and the calculation of odds ratios (ORs) with 95% confidence intervals (CI) were performed. To compare the mortality rate of PRMs for seven days after *in vitro* feeding, a Kaplan–Meier survival curve was generated, and a log-rank test was performed. A significant difference from the control group was set at $p<0.05$.

Table I-1. Primers used for amplification of *histamine release factor* gene in this study

Primer	Mite species	Intended use	Sequences (5′-3′)
HRF-F	NFM & TFM	Partial gene amplification	GAYGAGATGTTCACCGATACG
HRF-R1			GGGTTCAT GACTCGCCKAGG
HRF-R2			GGCTGTCYTTGTCVATCTTGC
GSP1	NFM & TFM	3′RACE	CCGGCTACAAGCTCTACCTG
GSP2			GACTCACCGAGACGACCTTC
GSP3			CCGGCTACAAGCTCTACCTG
GSP1	NFM & TFM	5′RACE	CAAGAACAACATTACG
GSP2			ACTGGCTGTCCTCGTCAAGC
GSP3			GCGCCTTCGTGTACGTCTTC
HRF ORF-F	PRM, TFM & NFM	ORF confirmation	ATGTTCACCGATACGKYKAAG
HRF ORF-R			YTAGMMYTTCTCYTCCKCRAC
rHRF PRM-F	PRM	Construction for expression plasmids	aagcatatgATGCTCATCTACAGGGACATCATCTCGG
rHRF PRM-R			atcctcgagCTAGACCTTCTCTTCCGCAACGCC
rHRF TFM-F	NFM & TFM		aagcatatgATGTTCACCGATACGTCTAAGGTGACC
rHRF TFM-R			atcctcgagTTAGCATTTCTCCTCCTCGACGCC

*The NdeI and XhoI sites are underlined.
PRM, poultry red mite; NFM, northern fowl mite; TFM, tropical fowl mite

Table I-2. The *histamine release factor* genes used for phylogenetic analysis in Figure I-1-b

Accession no.	Registered name in GenBank	Organism
FM179713	mRNA for histamine release factor (HRF-1 gene)	*Dermanyssus gallinae*
OQR67526	Histamine release factor-like	*Tropilaelaps mercedesae*
XM022810042	Translationally-controlled tumor protein homolog (LOC111252342) mRNA	*Varroa destructor*
XM003748309	Translationally-controlled tumor protein homolog (LOC100906672) mRNA	*Metaseiulus occidentalis*
XM037655552	Translationally-controlled tumor protein homolog (LOC119387963) mRNA	*Rhipicephalus sanguineus*
KP184513	Histamine release factor mRNA complete cds	*Hyalomma anatolicum*
XM037707824	Translationally-controlled tumor protein homolog (LOC119442797) mRNA	*Dermacentor silvarum*
AF467699	IgE-dependent histamine release factor mRNA complete cds	*Dermacentor variabilis*
Q009479	Histamine release factor mRNA complete cds	*Boophilus microplus*
XM016051387	Translationally-controlled tumor protein homolog (LOC107438960) mRNA	*Parasteatoda tepidariorum*
XM027342537	Translationally-controlled tumor protein homolog (LOC113792627) mRNA	*Dermatophagoides pteronyssinus*
XM017639074	Translationally-controlled tumor protein homolog (LOC108382703) mRNA	*Rhagoletis zephyria*
XM001845223	Translationally-controlled tumor protein homolog (LOC6035030), mRNA	*Culex quinquefasciatus*
XM001664232	Translationally-controlled tumor protein homolog (LOC5579186), mRNA	*Aedes aegypti*
AY826164	Translationally controlled tumor protein mRNA, complete cds	*Aedes albopictus*
AY383617	Growth-related translationally-controlled tumor protein gene, intron and partial cds	*Gallus gallus*

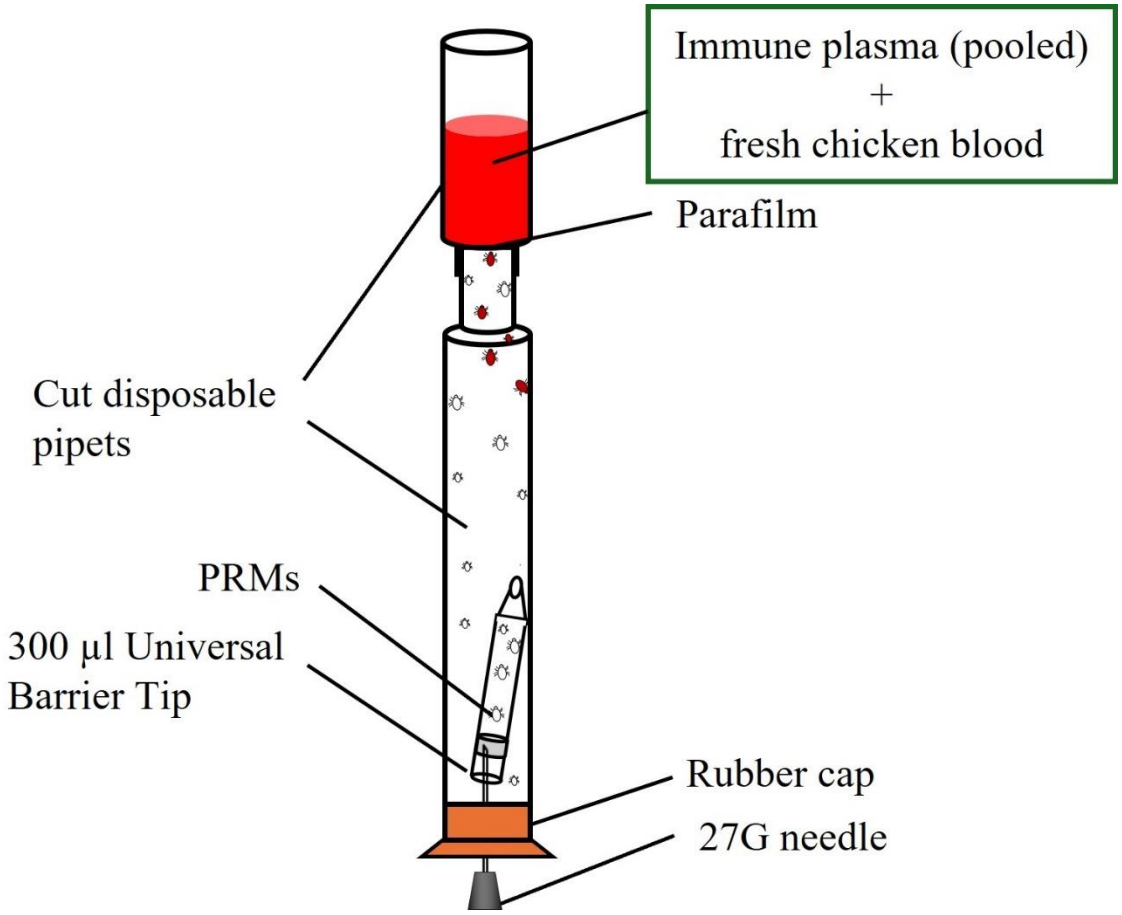

Figure I-1. The device used for *in vitro* feeding assay.
Disposable plastic pipettes (10 mL) were cut and assembled across stretched parafilm. Mixed stages of starved PRMs were collected using a 300 µL universal barrier tip and enclosed in the devices. The cut ends of the devices were closed with rubber caps, which were penetrated with 27-G needles for ventilation. Fresh chicken blood containing plasma obtained from immunized chickens was added to the devices. Blood feeding was performed at 40 °C for 4 h in the dark (Ariizumi *et al*., 2021).

RESULTS

Genetic characterization of *HRF* genes of avian mites

The nucleotide sequences of the *HRF* ORFs of TFMs (LC813285) and NFMs (LC813286) were identical, with a length of 492 bp, encoding 163 amino acids. The nucleotide and deduced amino acid sequences of the HRF ORFs of TFMs and NFMs showed 81.5% and 75.5% homology, respectively, with those of PRMs (Table I-3). In the multiple alignments of the deduced amino acid sequences of HRFs from PRMs (FM179713), TFMs and NFMs, *T. mercedesae* (OQR67526), *V. destructor* (XM022810042) and *M. occidentalis* (XM003748309), the HRFs were highly conserved among avian mites. However, the HRFs of TFMs and NFMs lacked 12 amino acids at the N-terminal region compared to those of PRMs and other mites (Figure I-2-a). Similar to other mites, signal peptides were absent in the HRF sequences of avian mites. Phylogenetic tree analysis using the nucleotide sequences of the *HRF* genes of arthropods and chickens showed that the *HRF* genes of avian mites were classified into a cluster to which the *HRF* genes of PRMs belonged, and this cluster consisted of the *HRF* genes of mite species (Cluster 1) (Figure I-2-b). *HRF* genes of other arthropods were distinctly classified into cluster 2. The *HRF* gene of chicken was distantly related to those of arthropods and showed 49.0% homology to that of PRMs and 48.0% homology to those of TFMs and NFMs, respectively.

Production of immune plasmas against each rHRF

The HRFs from PRMs, TFMs, and NFMs were expressed in the *E.coli* system and purified by metal affinity chromatography. All rHRFs were successfully expressed, and their molecular weights were approximately 23–25 kDa. The rHRFs were subsequently designated as rHRF PRM, rHRF TFM, or rHRF NFM (Figure I-3-a). rHRF PRM was expressed at a slightly lower molecular weight than the rHRFs of TFMs and NFMs. Successful purification of rHRFs was confirmed by western blotting using an anti-His-tag antibody (Figure I-3-b). To produce specific antibodies containing immune plasma, the chickens were immunized with each rHRF. After the second immunization, plasma samples were isolated from each immunized chicken, and the increase in antibody titers in all immunized chickens was confirmed by ELISA (Table I-4).

Cross-reactivities of immune plasma with rHRFs from different mites

The production of antibodies specific to each rHRF and the cross-reactivity of immune plasma with rHRFs from different mites were analyzed by western blotting. The immune plasma against rHRF PRM cross-reacted with rHRF TFM, and rHRF NFM (Figure I-4). In addition, the immune plasma against rHRF TFM and rHRF NFM showed

cross-reactivity with rHRF PRM (Figure I-4), suggesting the utility of HRFs as antigens common to avian mites.

Acaricidal effects of immune plasmas on PRMs

To assess the acaricidal activity of the immune plasma against each rHRF, *in vitro* feeding assays using PRMs were conducted three times (Experiments 1, 2, and 3). In this study, pooled plasma from each immunized group was used. Because the acaricidal effects could depend on the antibody titer [Murata *et al*., 2021], the acaricidal effects of immune plasma were assessed by comparing the mortality rates between PRMs fed with control plasma and each immune plasma. In addition, to assess the acaricidal effects on different developmental stages, the mortality of nymphs and adults in Experiments 1 and 2 were separately analyzed. In Experiment 1, a significantly lower survival rate of mixed-stage PRMs was observed in all immunized groups than that of the control group (Figure I-5-a), and their mortality rates significantly increased at 6 and 7, 2–7, and 7 days post-feeding in the immunized groups of rHRF PRM, rHRF TFM, and rHRF NFM, respectively, compared to those of the control group (Table I-5). The Kaplan–Meier survival curves revealed that no significant difference in the survival rates of adults in all immunized groups was observed compared to that of the control group (Figure I-5-b). In contrast, the survival rate of nymphs in all immunized groups significantly decreased (Figure I-5-c). In addition, the mortality rates of nymphs significantly increased at 5–7, 4–7, and 7 days post-feeding in the groups of rHRF PRM, rHRF TFM, and rHRF NFM, respectively (Table I-6), whereas no differences in mortality of adults were observed between the groups (Table I-7). A similar result was observed in Experiment 2. The Kaplan–Meier survival analysis showed that mixed stages of PRMs fed with immune plasma against rHRF TFM showed a significantly increased mortality rate. An increased tendency of mortality in the rHRF PRM immunized group (p=0.09) was recorded; however, no significant difference was observed in rHRF NFM immunized group (Figure I-6-a). The survival rates of nymphs in all immunized groups were significantly lower than those in the control group, whereas no difference in the survival rates of adults was observed (Figure I-6-b,c). Significantly increased mortality in the mixed stage of PRMs was observed at 3, 6, and 3 days post-feeding in the rHRF PRM, rHRF TFM, and rHRF NFM immunized groups, respectively, and the mortality of nymphs significantly increased at 3 and 6 days post-feeding in the rHRF PRM and rHRF TFM immunized groups, respectively (Tables I-8–10). To further confirm the effects of each type of immune plasma on the nymphs, an *in vitro* feeding assay using nymph PRMs was conducted (Experiment 3). The survival rate of nymphs in the rHRF PRM, rHRF TFM, and rHRF NFM immunized groups was significantly lower than that of the control group (Figure I-6-d). The mortality of nymphs in each immunized group was higher than that in the control group at 1–5 (PRM), 5 (TFM), and 3 (NFM) days post-feeding (Table I-11).

Therefore, the immune plasma against each rHRF exhibited acaricidal effects on nymph PRMs, suggesting that immunization with rHRFs is effective in reducing the number of nymphs and that HRF can be a candidate for universal vaccine antigens across avian mite species.

Table I-3. Sequences homology of histamine release factors from poultry red mites (PRMs), tropical fowl mites (TFMs) and northern fowl mites (NFMs)

		Ammino acid sequence (%)		
		PRM	TFM	NFM
Nucleotide sequence (%)	PRM	-	75.5	75.5
	TFM	81.5	-	100
	NFM	81.5	100	-

(a)

```
PRM_HRF          MLIYRDIISGDEMFTDTVKITVVENSVYEVTCKHVVRKEGEVVLAGSNP-AEG-EADEGT  [ 58]
TFM_HRF          ------------.....S.V.....A....V..........I.......-...ADDEG..  [ 47]
NFM_HRF          ------------.....S.V.....A....V..........I.......-...ADDEG..  [ 47]
T. mercedesae    ..V...SV...........VN............Y.T.R...I.......-...-......  [ 58]
V. destructor    ....K..V..........RV.L...C..............I.......S...EG.....  [ 60]
M. occidentalis  ....K....N.......T.V.L........L......R...I.......S..--.....A  [ 58]

PRM_HRF          DEHTEAGLDVVLNQRLQETSFDKGSYKTYLKTYTKALQDKWKELEYSADKITELKNQCMA  [118]
TFM_HRF          ...V............T..T...AG..L..............M.F.DE..A...S...T  [107]
NFM_HRF          ...V............T..T...AG..L..............M.F.DE..A...S...T  [107]
T. mercedesae    E..V.S.....................AG..L.....................P.E..A...S...T  [118]
V. destructor    ........................NG..M.............NK....EA..A...T..L.  [120]
M. occidentalis  .DSCQ..................AG.............E........DE..A...SS...  [118]

PRM_HRF          GVKYMVGKIDKDSQYFLGESMNPDGCVAILNYRDGADGNEEAIMMFLKAGVAEEKV [174]
TFM_HRF          ....IL..L.E.................V......P......V.L......E...C [163]
NFM_HRF          ....IL..L.E.................V......P......V.L......E...C [163]
T. mercedesae    ....IL.........Y.............V.T....................LD...C [174]
V. destructor    .A..IIS.....C..............V.T....................E...C [176]
M. occidentalis  ...F.LS.M................M..V......E.............LE...C [174]
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(b)

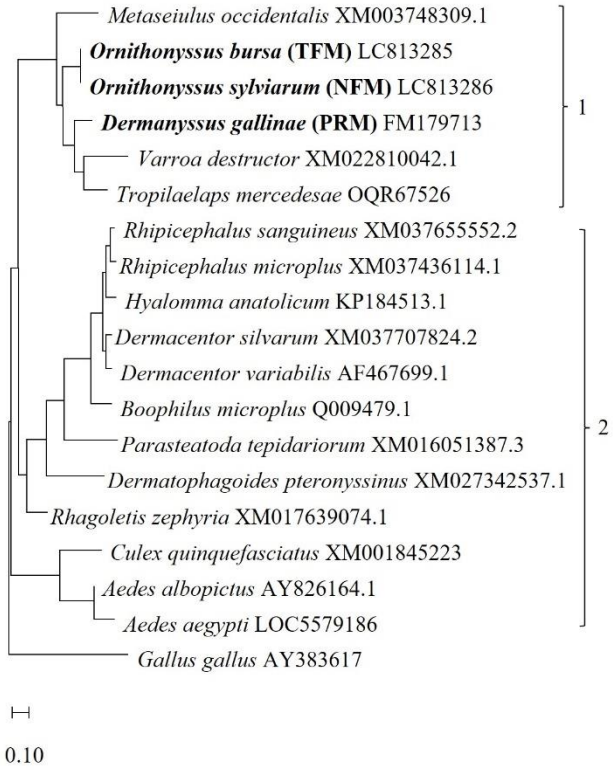

Figure I-2. Genetic characterization of the *HRF* genes of avian mites.

(a) The multiple alignments of the deduced amino acid sequences of HRFs of PRMs, TFMs, NFMs, *T. mercedesae*, *V. destructor,* and *M. occidentalis*. (b) Phylogenetic analysis of *HRF* genes of avian mites, other arthropods, and chickens. The tree was constructed using the maximum-likelihood method. Cluster 1 includes the *HRF* genes of mites, including PRMs, TFMs (bold), and NFMs (bold). The HRF/TCTP genes of ticks, mosquitos, and other insects are classified into cluster 2.

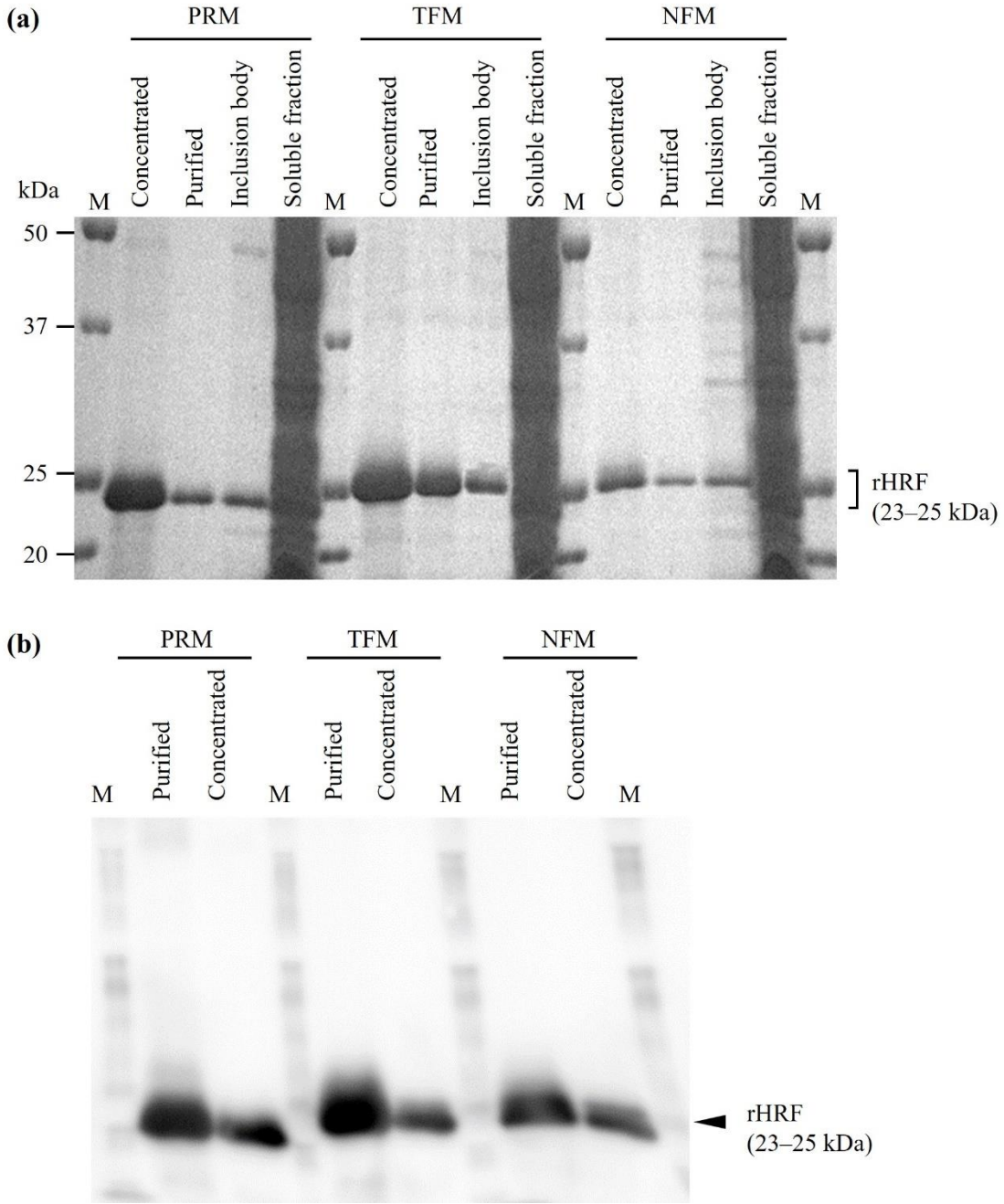

Figure I-3. Expression and purification of rHRF proteins of avian mites.
The rHRFs from PRMs, TFMs, and NFMs were generated and named rHRF PRM, TFM, and NFM, respectively. Each rHRF was fused with histidine-tag and expressed in *E. coli* system. The purity of rHRFs was confirmed by SDS-PAGE (a) and western blotting using anti-his tag antibody (b). M, Marker (Precision Plus Protein™ All Blue Prestained Protein Standards, Bio-Rad, CA, USA).

Table I-4. Antibody titers in plasma obtained from chickens immunized with recombinant histamine release factors

Group	Chicken	Antibody titer
Control	C1	1:2,000
	C2	1:2,000
	C3	1:2,000
	C4	1:2,000
Immunized- rHRF PRM	PH1	1:16,000
	PH2	1:64,000
	PH3	1:64,000
	PH4	1:64,000
Immunized- rHRF TFM	TH1	1:64,000
	TH2	1:64,000
	TH3	1:64,000
	TH4	1:64,000
Immunized- rHRF NFM	NH1	1:64,000
	NH2	1:64,000
	NH3	1:64,000
	NH4	1:64,000

PRM, poultry red mite; NFM, northern fowl mite; TFM, tropical fowl mite; rHRF, recombinant histamine release factor

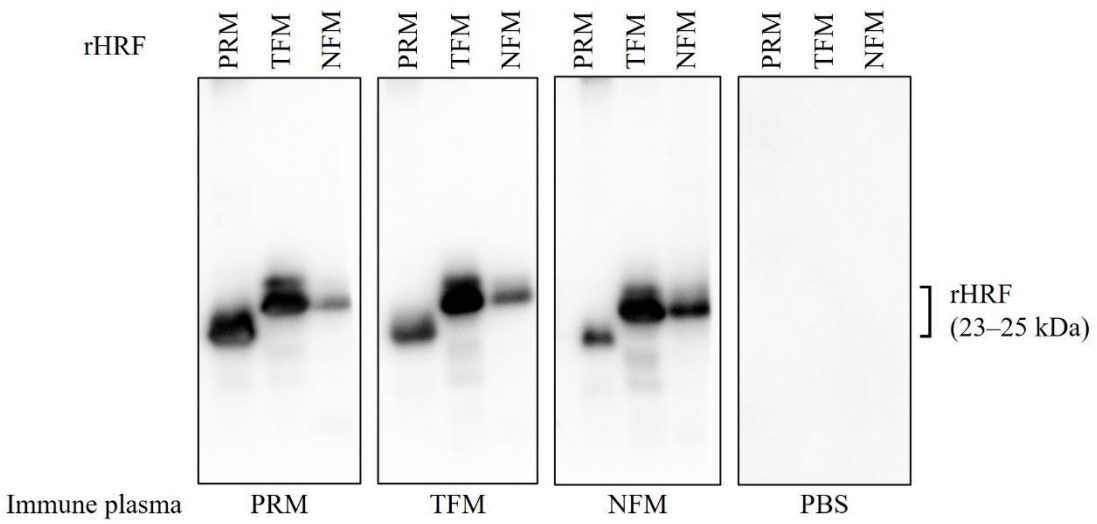

Figure I-4. Cross-reactivities between plasmas obtained from chickens immunized with rHRFs from avian mites.

The plasma was isolated from chickens immunized with each rHRF. The production of antibodies specific to each rHRF in the plasma and cross-reactivities of immune plasmas with rHRFs were analyzed by western blotting. The predicted molecular weights of rHRFs were approximately 23–25 kDa.

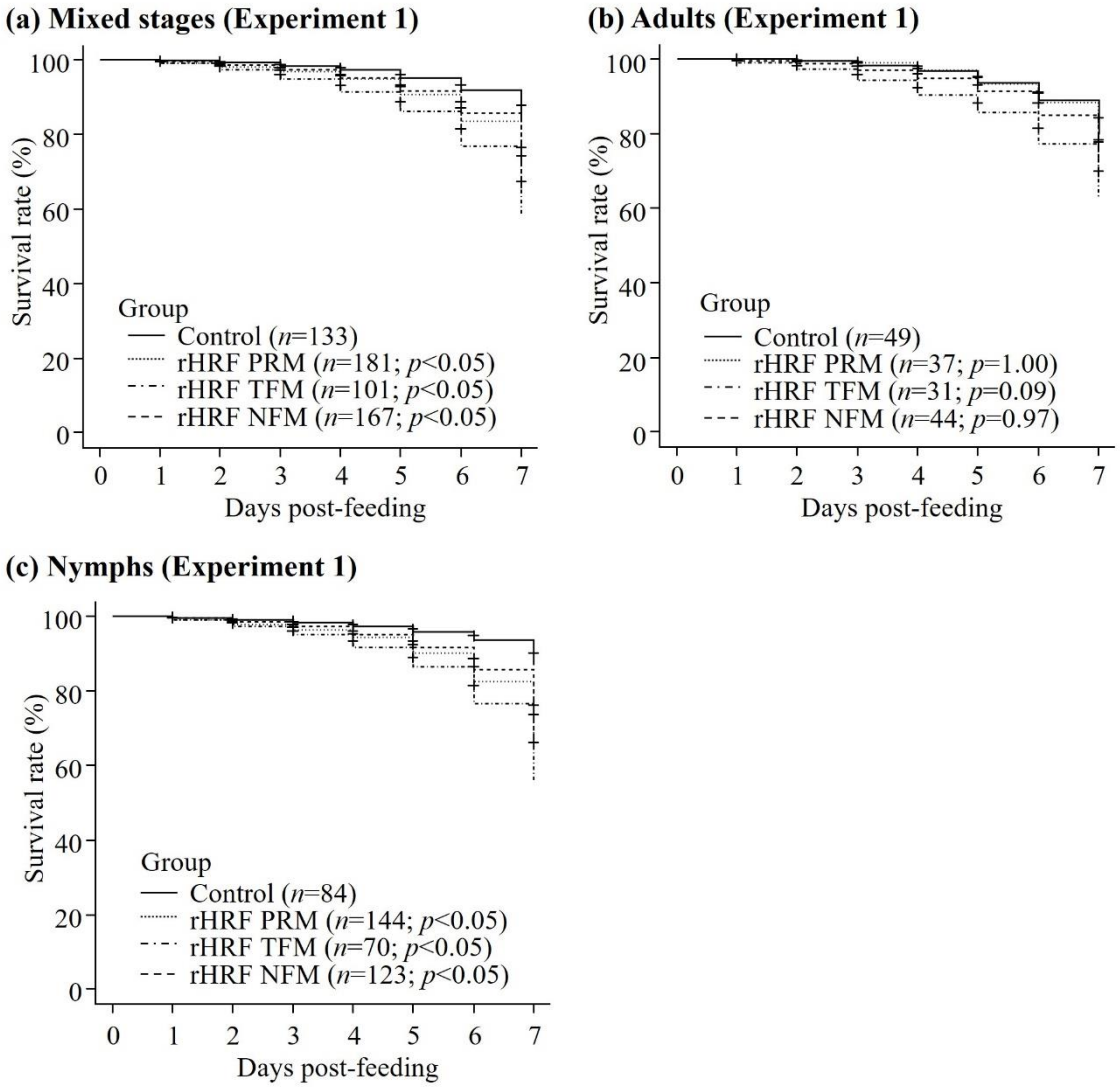

Figure I-5. Assessment of the acaricidal effects of plasmas obtained from chickens immunized with rHRFs (Experiment 1).

The mixed stage of PRMs was artificially fed with immune plasmas containing antibodies against each rHRF. The survival rate of PRMs from each group was compared with that of the control group. The survival rates of mixed stages of PRMs (a), adults (b), and nymphs (c) were assessed. The total numbers of mixed stages of PRMs, adults, and nymphs used in this experiment are as follows; (a) Mixed stage: Control (n=33), rHRF PRM (n=181), rHRF TFM (n=101), rHRF NFM (n=167); (b) Adults: Control (n=49), rHRF PRM (n=37), rHRF TFM (n=31), rHRF NFM (n=44); (c) Nymphs: Control (n=84), rHRF PRM (n=144), rHRF TFM (n=70), rHRF NFM (n=123). Statistical test was conducted using a log-rank test. Results with p<0.05 were considered statistically significant.

Table I-5. Mortality of mixed-stage PRMs fed plasma from chickens immunized with recombinant histamine release factors from different species of mites (Experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=133)							
No. of dead PRMs post-feeding	2	4	6	6	9	9	11
Mortality (%)	1.50	3.01	4.51	4.51	6.77	6.77	8.27
Immunized group (rHRF PRM, *n*=181)							
No. of dead PRMs post-feeding	8	12	12	15	23	29	40
Mortality (%)	4.42	6.63	6.63	8.29	12.71	16.02	22.10
p value (Fisher's exact)	0.199	0.197	0.472	0.254	0.093	0.014*	0.001*
Odds ratio	3.02	2.28	1.50	1.91	2.00	2.62	3.14
95% confidence interval	0.58–29.65	0.67–9.94	0.51–5.01	0.67–6.17	0.85–5.09	1.15–6.53	1.49–7.08
Immunized group (rHRF TFM, *n*=101)							
No. of dead PRMs post-feeding	6	11	13	15	17	22	25
Mortality (%)	5.94	10.89	12.87	14.85	16.83	21.78	24.75
p value (Fisher's exact)	0.079	0.028*	0.028*	0.010*	0.020*	0.001*	0.001*
Odds ratio	4.11	3.92	3.11	3.67	2.78	3.81	3.63
95% confidence interval	0.71–42.48	1.11–17.4	1.05–10.38	1.28–12.02	1.11–7.42	1.59–9.91	1.61–8.66
Immunized group (rHRF NFM, *n*=167)							
No. of dead PRMs post-feeding	5	9	12	15	18	22	35
Mortality (%)	2.99	5.39	7.19	8.98	10.78	13.17	20.96
p value (Fisher's exact)	0.469	0.399	0.464	0.172	0.310	0.086	0.003*
Odds ratio	2.02	1.83	1.64	2.08	1.66	2.09	2.93
95% confidence interval	0.32–21.51	0.49–8.33	0.54–5.46	0.73–6.75	0.68–4.35	0.88–5.34	1.38–6.69

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table I-6. Mortality of nymph PRMs fed plasma from chickens immunized with recombinant histamine release factors from different species of mites (Experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=84)							
No. of dead PRMs post-feeding	2	3	3	3	4	4	6
Mortality (%)	2.38	3.57	3.57	3.57	4.76	4.76	7.14
Immunized group (rHRF PRM, *n*=144)							
No. of dead PRMs post-feeding	8	11	11	12	19	25	31
Mortality (%)	5.56	7.64	7.64	8.33	13.19	17.36	21.53
p value (Fisher's exact)	0.331	0.264	0.264	0.267	0.043*	0.007*	0.005*
Odds ratio	2.40	2.23	2.23	2.45	3.03	4.18	3.55
95% confidence interval	0.46–23.77	0.57–12.79	0.57–12.79	0.63–13.92	0.96–12.68	1.36–17.15	1.37–10.91
Immunized group (rHRF TFM, *n*=70)							
No. of dead PRMs post-feeding	4	8	8	10	12	16	19
Mortality (%)	5.71	11.43	11.43	14.29	17.14	22.86	27.14
p value (Fisher's exact)	0.412	0.113	0.113	0.021*	0.016*	0.001*	0.001*
Odds ratio	2.47	3.46	3.46	4.46	4.10	5.86	4.79
95% confidence interval	0.34–28.11	0.78–21.05	0.78–21.05	1.08–26.28	1.16–18.33	1.76–25.40	1.69–15.69
Immunized group (rHRF NFM, *n*=123)							
No. of dead PRMs post-feeding	4	7	8	11	13	16	28
Mortality (%)	3.25	5.69	6.50	8.94	10.57	13.01	22.76
p value (Fisher's exact)	1	0.743	0.531	0.165	0.197	0.057	0.004*
Odds ratio	1.38	1.63	1.87	2.64	2.35	2.98	3.81
95% confidence interval	0.19–15.55	0.35–10.03	0.43–11.29	0.67–15.21	0.69–10.28	0.91–12.71	1.45–11.84

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table I-7. Mortality of adult PRMs fed with plasma from chickens immunized with recombinant histamine release factors from different mite species (Experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=49)							
No. of dead PRMs post-feeding	0	1	3	3	5	5	5
Mortality (%)	0	2.04	6.12	6.12	10.20	10.20	10.20
Immunized group (rHRF PRM, *n*=37)							
No. of dead PRMs post-feeding	0	1	1	3	4	4	9
Mortality (%)	0	2.70	2.70	8.11	10.81	10.81	24.32
p value (Fisher's exact)	-	1	0.631	1	1	1	0.138
Odds ratio	-	1.33	0.43	1.35	1.07	1.07	2.79
95% confidence interval	-	0.02–106.72	0.01–5.61	0.17–10.69	0.19–5.38	0.19–5.38	0.74–11.76
Immunized group (rHRF TFM, *n*=31)							
No. of dead PRMs post-feeding	2	3	5	5	5	6	6
Mortality (%)	6.45	9.68	16.13	16.13	16.13	19.35	19.35
p value (Fisher's exact)	0.147	0.293	0.250	0.250	0.498	0.322	0.322
Odds ratio	-	5.04	2.91	2.91	1.68	2.09	2.09
95% confidence interval	-	0.38–275.03	0.52–20.24	0.52–20.24	0.35–8.07	0.48–9.63	0.48–9.63
Immunized group (rHRF NFM, *n*=44)							
No. of dead PRMs post-feeding	1	2	4	4	5	6	7
Mortality (%)	2.27	4.55	9.09	9.09	11.36	13.64	15.91
p value (Fisher's exact)	0.473	0.601	0.704	0.704	1	0.751	0.539
Odds ratio	-	2.27	1.53	1.53	1.13	1.38	1.66
95% confidence interval	-	0.11–137.49	0.24–11.05	0.24–11.05	0.24–5.29	0.32–6.23	0.41–7.20

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

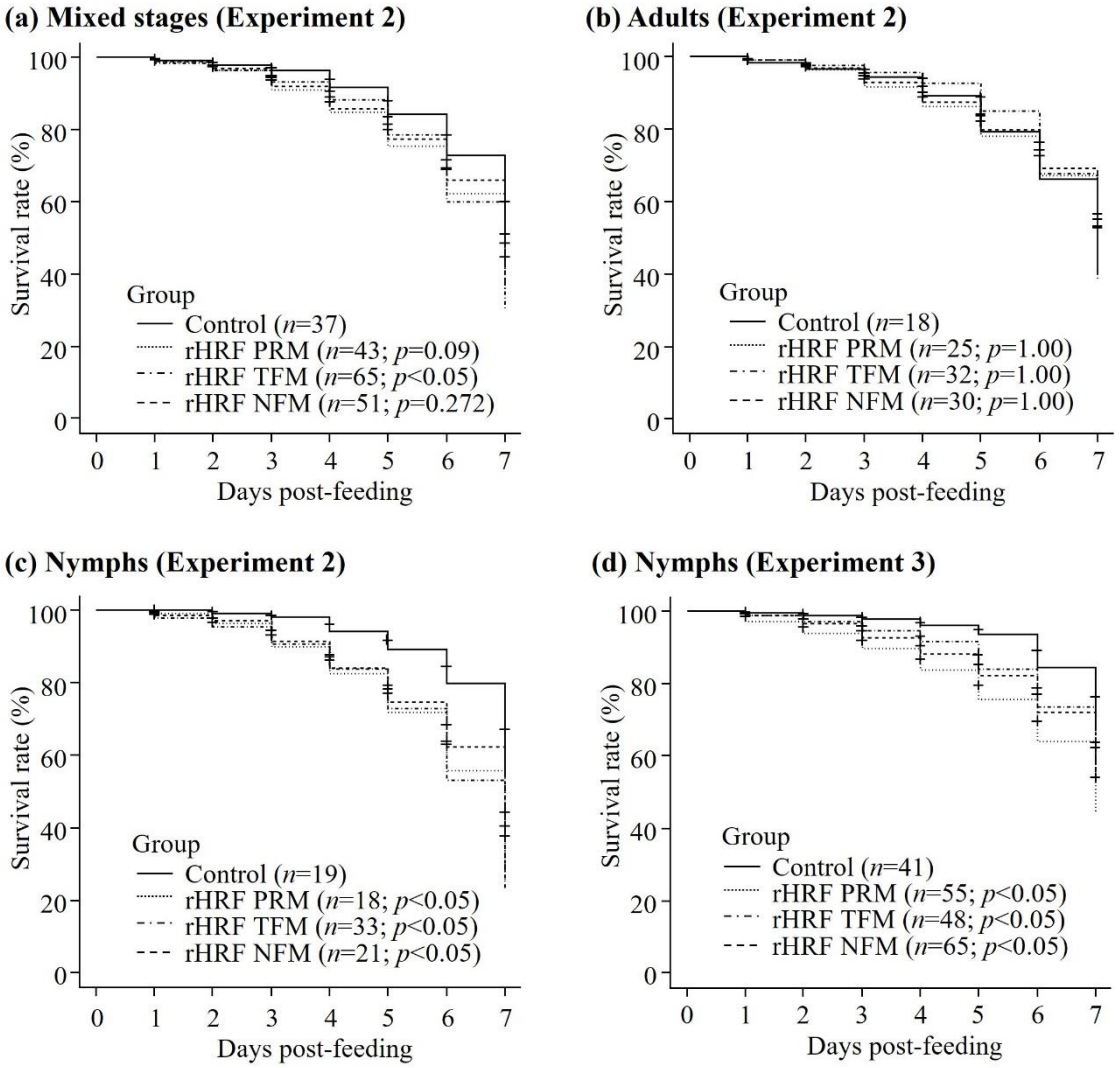

Figure I-6. Assessment of the acaricidal effects on the mixed stages of PRMs, adults, and nymphs (Experiments 2 and 3).

The mixed stages of PRMs (Experiment 2) or nymphs (Experiment 3) were artificially fed with immune plasmas containing antibodies against each rHRF. In Experiment 2, the survival rates of mixed stages of PRMs (a), adults (b), and nymphs (c) were evaluated. In Experiment 3, the survival rate of nymphs (d) was evaluated. The total numbers of mixed stage or each stage of PRMs are as follows; (a) Mixed stage (Experiment 2): Control (n=37), rHRF PRM (n=43), rHRF TFM (n=65), rHRF NFM (n=51); (b) Adults (Experiment 2): Control (n=18, rHRF PRM (n=25), rHRF TFM (n=32), rHRF NFM (n=30); (c) Nymphs (Experiment 2): Control (n=19), rHRF PRM (n=18), rHRF TFM (n=33), rHRF NFM (n=21); (d) Nymphs (Experiment 3): Control (n=41), rHRF PRM (n=55), rHRF TFM (n=48), rHRF NFM (n=65). Statistical test was conducted using a log-rank test. Results with p<0.05 were considered statistically significant.

Table I-8. Mortality in mixed stages of PRMs fed with plasma from chickens immunized with recombinant histamine release factors from different mite species (Experiment 2)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=37)							
No. of dead PRMs post-feeding	2	3	3	7	9	10	13
Mortality (%)	5.41	8.11	8.11	18.92	24.32	27.03	35.14
Immunized group (rHRF PRM, *n*=43)							
No. of dead PRMs post-feeding	4	6	12	12	14	15	19
Mortality (%)	9.30	13.95	27.91	27.91	32.56	34.88	44.19
p value (Fisher's exact)	0.681	0.494	0.042*	0.433	0.466	0.479	0.260
Odds ratio	1.78	1.82	4.31	1.65	1.49	1.44	1.74
95% confidence interval	0.23–20.83	0.35–12.15	1.03–26.02	0.51–5.66	0.50–4.59	0.51–4.26	0.65–4.81
Immunized group (rHRF TFM, *n*=65)							
No. of dead PRMs post-feeding	7	8	11	14	21	31	33
Mortality (%)	10.77	12.31	16.92	21.54	32.31	47.69	50.77
p value (Fisher's exact)	0.482	0.742	0.249	0.805	0.499	0.058*	0.151
Odds ratio	2.09	1.58	2.29	1.17	1.48	2.44	1.89
95% confidence interval	0.37–21.81	0.35–9.89	0.55–13.71	0.39–3.84	0.55–4.22	0.96–6.61	0.77–4.80
Immunized group (rHRF NFM, *n*=51)							
No. of dead PRMs post-feeding	4	6	13	14	15	15	23
Mortality (%)	7.84	11.76	25.49	27.45	29.41	29.41	45.09
p value (Fisher's exact)	1	0.728	0.050*	0.450	0.636	1	0.386
Odds ratio	1.48	1.50	3.82	1.61	1.29	1.12	1.51
95% confidence interval	0.19–17.26	0.29–9.96	0.94–22.69	0.53–5.36	0.45–3.88	0.39–3.27	0.58–4.0

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table I-9. Mortality of adult PRMs fed with plasma from chickens immunized with recombinant histamine release factors from different mite species (Experiment 2)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=18)							
No. of dead PRMs post-feeding	2	2	2	4	6	6	7
Mortality (%)	11.11	11.11	11.11	22.22	33.33	33.33	38.89
Immunized group (rHRF PRM, *n*=25)							
No. of dead PRMs post-feeding	3	3	6	6	7	7	9
Mortality (%)	12.0	12.0	24.0	24.0	28.0	28.0	36.0
p value (Fisher's exact)	1	1	0.434	1	0.747	0.747	1
Odds ratio	1.09	1.09	2.48	1.10	0.78	0.78	0.89
95% confidence interval	0.11–14.45	0.11–14.45	0.37–28.40	0.21–6.37	0.17–3.59	0.17–3.59	0.21–3.74
Immunized group (rHRF TFM, *n*=32)							
No. of dead PRMs post-feeding	2	3	3	4	8	13	14
Mortality (%)	6.25	9.38	9.38	12.50	25.0	40.63	43.75
p value (Fisher's exact)	0.612	1	1	0.436	0.533	0.764	0.774
Odds ratio	0.54	0.83	0.83	0.51	0.67	1.36	1.22
95% confidence interval	0.04–8.11	0.09–10.92	0.09–10.92	0.08–3.16	0.16–2.93	0.35–5.62	0.33–4.75
Immunized group (rHRF NFM, *n*=30)							
No. of dead PRMs post-feeding	2	4	6	7	8	8	11
Mortality (%)	6.67	13.33	20.0	23.33	26.67	26.67	36.67
p value (Fisher's exact)	0.624	1	0.692	1	0.746	0.746	1
Odds ratio	0.58	1.23	1.97	1.06	0.73	0.73	0.91
95% confidence interval	0.04–8.69	0.15–15.01	0.30–22.38	0.22–5.89	0.17–3.21	0.17–3.21	0.23–3.65

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table I- 10. Mortality of nymph PRMs fed with plasma from chickens immunized with recombinant histamine release factors from different mite species (Experiment 2)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=19)							
No. of dead PRMs post-feeding	0	1	1	3	3	4	6
Mortality (%)		5.26	5.26	15.79	15.79	21.05	31.58
Immunized group (rHRF PRM, *n*=18)							
No. of dead PRMs post-feeding	1	3	6	6	7	8	10
Mortality (%)	5.56	16.67	33.33	33.33	38.89	44.44	55.56
p value (Fisher's exact)	0.486	0.340	0.042*	0.269	0.151	0.170	0.191
Odds ratio	-	3.48	8.51	2.59	3.28	2.91	2.63
95% confidence interval	-	0.25–199.04	0.87–435.28	0.44–19.38	0.59–24.07	0.58–17.03	0.59–12.84
Immunized group (rHRF TFM, *n*=33)							
No. of dead PRMs post-feeding	5	5	8	10	13	18	19
Mortality (%)	15.15	15.15	24.24	30.30	39.39	54.55	57.58
p value (Fisher's exact)	0.145	0.397	0.130	0.328	0.119	0.023*	0.089
Odds ratio	-	3.15	5.61	2.28	3.39	4.37	2.88
95% confidence interval	-	0.31–160.20	0.65–269.16	0.48–14.95	0.75–21.73	1.08–22.06	0.78–11.73
Immunized group (rHRF NFM, *n*=21)							
No. of dead PRMs post-feeding	2	2	6	7	7	7	12
Mortality (%)	9.52	9.52	28.57	33.33	33.33	33.33	57.14
p value (Fisher's exact)	0.488	1	0.095	0.281	0.281	0.488	0.125
Odds ratio	-	1.87	6.89	2.60	2.60	1.85	2.81
95% confidence interval	-	0.09–117.81	0.71–348.49	0.48–18.61	0.48–18.61	0.37–10.59	0.67–13.0

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table I-11. Mortality of nymph PRMs fed with plasma from chickens immunized with recombinant histamine release factors from different mite species (Experiment 3)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=41)							
No. of dead PRMs post-feeding	1	2	2	3	3	8	8
Mortality (%)	2.44	4.88	4.88	7.32	7.32	19.51	19.51
Immunized group (rHRF PRM, *n*=55)							
No. of dead PRMs post-feeding	11	11	12	15	16	17	17
Mortality (%)	20.0	20.0	21.82	27.27	29.09	30.91	30.91
p value (Fisher's exact)	0.012*	0.038*	0.022*	0.017*	0.009*	0.246	0.246
Odds ratio	9.82	4.81	5.36	4.68	5.12	1.83	1.83
95% confidence interval	1.32–440.04	0.96–47.28	1.09–52.29	1.19–27.21	1.31–29.62	0.65–5.58	0.65–5.58
Immunized group (rHRF TFM, *n*=48)							
No. of dead PRMs post-feeding	4	5	6	6	12	12	14
Mortality (%)	8.33	10.42	12.50	12.50	25.0	25.0	29.17
p value (Fisher's exact)	0.369	0.445	0.279	0.498	0.044*	0.615	0.333
Odds ratio	3.59	2.25	2.76	1.79	4.16	1.37	1.68
95% confidence interval	0.34–183.30	0.34–24.89	0.46–29.50	0.35–11.88	1.01–24.84	0.45–4.39	0.57–5.30
Immunized group (rHRF NFM, *n*=65)							
No. of dead PRMs post-feeding	5	9	13	13	13	16	18
Mortality (%)	7.69	13.85	20.0	20.0	20.0	24.62	27.69
p value (Fisher's exact)	0.402	0.197	0.043*	0.097	0.097	0.637	0.366
Odds ratio	3.30	3.10	4.82	3.14	3.14	1.34	1.57
95% confidence interval	0.35–161.41	0.59–31.07	1.0–46.42	0.78–18.34	0.78–18.34	0.48–4.06	0.57–4.70

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

DISCUSSION

In this study, HRFs identified from TFMs and NFMs lacked a signal peptide similar to those of PRMs, ticks, mammals, and invertebrates [Mulenga *et al*., 2003; Bartley *et al*., 2009; Dai *et al*., 2010; Kawakami *et al*., 2019; Xu *et al*., 2022; Zhong *et al*., 2022]. Generally, the typical secretory proteins include a short signal peptide sequence at the N terminus and are secreted via the endoplasmic reticulum/Golgi-dependent pathway [Gao *et al*., 2022]. Despite the absence of signal peptides, however, unconventional proteins, such as TCTP/HRF, are secreted in exosomes (extracellular vesicles, EVs) in the endosomal compartment in most eukaryotic cells [Gao *et al*., 2022]. In humans, HRF/TCTP has cytokine-like functions such as histamine release and cytokine production, activating mast cells and basophils in an IgE-dependent manner, resulting in allergy and inflammatory cascades [Kawakami *et al*., 2014]. Additionally, human HRF/TCTP induced by exposure to house dust mites elicit airway inflammation [Kasakura *et al*., 2022]. In ticks, HRF/TCTP triggered histamine releases from rat basophils, suggesting its role in controlling local inflammatory responses at sites bitten by ticks [Mulenga *et al*., 2003]. On the contrary, HRF secreted via the EVs of *P. berghei* was shown to be essential for the EV-mediated suppression of host immunity by inhibiting T-cell responses [Demarta-Gatsi *et al*., 2019]. Thus, HRFs/TCTPs in ticks, mites, and humans have been confirmed as secretory molecules with extracellular functions [Mulenga *et al*., 2003; Bartley *et al*., 2009; Dai *et al*., 2010; Kawakami *et al*., 2019; Xu *et al*., 2022]. In addition to its extracellular functions, HRF/TCTP is a multifunctional molecule that contributes to intracellular functions, such as promoting disease progression, growth, and development, and antioxidants in various organisms, including parasites [Bommer and Telerman, 2020; Amson *et al*., 2024]. TCTP/HRF is critical in EV-mediated intracellular signaling during tumorigenicity in sarcoma and human breast cancer models [Amson *et al*., 2024]. Moreover, HRF/TCTP acts as an antioxidant mplecule in *B. malayi* [Gnanasekar and Ramaswamy, 2007] and is important for the development and reproduction of *S. mansoni* [Zhong *et al*., 2022]. These observations indicate that the HRFs/TCTPs of parasites have characteristics similar to those of mammals and other vertebrates as molecules that function extracellularly and intracellularly. In a previous study, the HRF of PRMs induces the granulation of RPMCs, suggesting that the HRF PRM has a histamine-releasing function as a secretory protein conserved among multiple species [Bartley *et al.*, 2009]. In this study, the deduced amino acid sequences of HRFs identified from TFMs and NFMs were shorter by 12 amino acids than those of PRMs, whereas their sequence indicated high similarity and belonged to the group of mites, including PRMs and other mites in the phylogenetic analysis. Therefore, HRFs of TFMs and NFMs may exhibit functions similar to those of PRMs. In order to investigate whether the HRFs of avian mites are secretory proteins exposed to the host

during blood feeding, profiling of proteins present in the saliva is necessary. However, unlike ticks, collecting saliva from avian mites is challenging because of their small size. Therefore, developing a technique for collecting salivary glands or saliva is required to further investigate the physiological roles of HRFs in avian mites. Additionally, it is necessary to explore how the HRFs of avian mites signal physiological functions and whether it is an extracellular or intracellular activity.

In this study, the cross-reactivity and acaricidal effects of TFMs and NFMs were evaluated by using the immune plasma generated by immunization with each rHRF. When recombinant proteins were produced, the molecular weight differences between the rHRF TFM/NFM and rHRF PRM were observed. Although the deduced amino acid sequences of the HRFs of TFMs/NFMs were shorter than those of PRMs, the molecular weight of the rHRF PRM reflected on SDS-PAGE was lower than that of the rHRF TFM/NFM. It is unclear whether post-translational modifications of proteins or other underlying properties are reflected in its molecular weight. However, the immune plasma, including antibodies specific to the rHRFs of TFMs and NFMs, was cross-reacted with the rHRF of PRMs and exhibited acaricidal effects on nymph PRMs. Therefore, the HRFs of TFMs and NFMs seem to possess physiological functions similar to those of PRMs. The antibodies generated by immunization presumably impair the physiological function by binding to naïve HRF expressed in avian mites, causing damage to their tissues, including in the midgut. However, this study did not analyze the expression of HRFs in the tissues of TFMs and NFMs, as well as their binding to and cross-reactivity with HRFs in the tissues of each mite. Therefore, in order to confirm the cross-reactivity of immune plasmas with HRFs of different mites, an immunohistochemical analysis using immune plasma or purified IgY is required to verify which tissues express HRFs in TFMs and NFMs and whether the antibodies against each rHRF recognize HRFs of PRMs, TFMs, and NFMs.

The potentials of HRFs/TCTPs as vaccine antigens for controlling ticks and tick-borne pathogen transmission [Dai *et al.*, 2010] and *S. japonicum* [Zhong *et al.*, 2022] have been evaluated. In addition, the increased mortality of PRMs was observed after PRMs were fed with fresh heparinized chicken blood containing antibodies against the rHRF of PRMs [Bartley *et al*., 2009], suggesting that PRM HRF has crucial physiological functions in PRMs and can be a vaccine antigen candidate. In this study, immune plasma against the rHRFs of all avian mites showed acaricidal effects on nymph PRMs. However, increased mortality was not observed in adults fed immune plasma, suggesting that the expression levels of *HRF* genes may vary at different developmental stages in avian mites. Therefore, expression analysis at each developmental stage is required to determine the range of efficacy of vaccine antigens. In addition, it is necessary to analyze the fecundity of female adults fed with immune plasma to further clarify the effects of immune plasma on adults. In addition, HRFs are conserved among various organisms, although chicken

HRF/TCTP shows low homology with those of avian mites; therefore, careful monitoring for longer periods is required to ensure that the antibodies induced by immunization do not negatively affect the host. Thus, HRF has limitations in its application as a universal vaccine antigen. However, the findings of this study indicate that HRF may be effective against nymphs across mite species, showing the potential of HRF as a candidate universal vaccine antigen to reduce economic losses and improve animal welfare in the poultry industry.

SUMMARY

Hematophagous avian mites are of serious concern to the poultry industry because they are distributed worldwide and negatively impact poultry production and welfare. Vaccines represent a promising approach for controlling avian mites, and the identification of antigens with broad-spectrum efficacy against multiple avian mite species provides advantages for vaccine approaches.

In Chapter I, HRF was identified from TFMs and NFMs, and the cross-reactivities and acaricidal effects on different avian mite species were analyzed. HRF, which is highly conserved among multiple organisms and is ubiquitously expressed in multiple cell types, was previously demonstrated as a vaccine antigen candidate against PRMs. The deduced amino acid sequences of HRFs from TFMs and NFMs indicated high homology with that of PRMs. The immune plasma against each rHRF was produced by the immunization with rHRF of TFMs, NFMs, and PRMs, and these immune plasma containing antibodies specific to each rHRF were cross-reacted with rHRFs from different avian mites. Importantly, each immune plasma exhibited high mortality rates to nymph PRMs compared with the control plasma. Therefore, HRF could be applied as a candidate antigen for a universal vaccine with broad efficacy across avian mites.

CHAPTER II

Assessment of glutathione S-transferase as a universal vaccine antigen against avian mites using an *in vitro* mite-feeding model

INTRODUCTION

Glutathione S-transferases (GSTs) are multifunctional enzymes crucial for protecting against oxidative stress, aging, and cancer, and for metabolic detoxification of endogenous and xenobiotic compounds such as drugs, herbicides, and insecticides [Hayes *et al*., 1995; Ketterman *et al*., 2011]. GSTs are expressed in various tissues and catalyze several kinds of chemical substances in the presence of glutathione (GSH) to generate less harmful substances [Sheehan *et al*., 2001]. GSTs from various species of parasites and pests, including house dust mites, parasitic nematodes, and cockroaches, trigger allergic inflammation in animals and humans [Sookrung *et al*., 2018; Zakzuk *et al*., 2021]. Several classes of GSTs, such as alpha, mu, theta, and sigma, have been identified in chickens, and these heterologously expressed proteins have unique properties as avian GSTs [Hsieh *et al*., 1999]. The effectiveness of acaricides relies on the detoxification process of GST in ticks [da Silva Vaz Jr *et al*., 2004; Hernandez *et al*., 2018]. Due to the significant roles of GST in detoxification and drug metabolism, they are of great interest as targets for innovative control strategies against several ecto- and endoparasites [Goud *et al*., 2012; Parizi *et al*., 2012; Bourke *et al*., 2014]. Moreover, GSTs are potential antigen candidates for anti-tick vaccines [Sabadin *et al*., 2017; Ndawula *et al*., 2019]. Furthermore, a specific B-cell epitope-based GST vaccine has been developed to investigate its cross-protective potential against tick species [Ndawula *et al*., 2020]. In PRMs, two classes of GSTs, delta and mu, have been identified, and their enzymatic activities for acaricide detoxification have been reported [Bartley *et al*., 2015a]. However, GSTs from TFMs and NFMs have not been identified, and their potential as universal vaccine antigens across avian mites remains to be determined. In Chapter I, the immune plasmas against rHRFs of avian mites showed cross-reactivities with rHRFs from different mites; however, acaricidal effects were limited on nymph PRMs. Therefore, to enhance the efficacy of universal vaccines, GSTs may be candidate vaccine antigens for future application as cocktail vaccines against avian mites.

In Chapter II, the utilization of avian mite GSTs as potential vaccine antigens was evaluated *in vitro*. *GST* genes from TFMs and NFMs were identified and indicated high homology when compared to those of PRMs. The recombinant GSTs (rGSTs) of each avian mite were prepared, and their catalytic enzyme activities in conjugation with GSH to substrates were analyzed. Immune plasmas containing specific antibodies were produced by immunization with each rGST, and their cross-reactivities with rGSTs from different avian mites were evaluated. Finally, the acaricidal effects of immune plasmas on PRMs were assessed *in vitro*.

MATERIALS AND METHODS

Ethics statement

1. Genetic recombination experiment

All genetic recombination experiments were performed in accordance with the Manual for Safety Management on Genetic Recombination Experiment in Hokkaido University (Approval number: 2019-024).

2. Animal experiments

The chickens used in this study were housed in the animal facility at the Faculty of Veterinary Medicine, Hokkaido University, with the Institutional Animal Care and Use Committee of Hokkaido University (approval number 22-0051). The animal experiments were performed in accordance with the regulations fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Mite samples and RNA extraction

PRM samples were obtained from egg-laying farms contaminated with PRMs in Japan. PRMs were collected in a TubeSpin bioreactor 600 bottle and transferred to the laboratory at 4 °C. For further analysis, PRMs were stored at 25 °C and 70% humidity for a week without blood feeding to starve PRMs and produce newborn nymphs. The mixed stages of starved PRMs were stored at 5 °C. RNA samples from TFMs and NFMs that were morphologically and genetically characterized in a previous study [Takehara *et al*., 2019] were used. Total RNA from each mite species was isolated using the RNeasy RNA isolation kit according to the manufacturer's protocol. 1 μg of RNA was used to synthesize cDNA as described in Chapter I.

Amplification of *GST* genes from TFMs and NFMs

The *GST* genes from TFMs and NFMs were amplified using the primers listed in Table II-1. The partial gene of each *GST* was amplified using the primers designed based on the sequences of PRMs (*D. gallinae*; accession no. KR337505.1), *V. jacobsoni* (accession no. XM022854296.1), and *V. destructor* (accession no. XM022792712.1). The partial gene products were cloned into the pMD20 vector, and transformed into competent DH5α *E. coli* cells. The nucleotide sequence analysis was performed with a Beckman CEQ GeXP automated sequencer. To determine ORFs of GSTs, the RACE analysis was performed. The ORF of *GST* gene of PRMs from Japan was determined using primers designed based on the ORF regions of *GST* (*D. gallinae*; accession no. KR337505.1) (Table II-1). The amplified products were purified, cloned into pGEMT easy vector (Promega) and transformed into competent DH5α *E. coli* cells.

Genetic characterization and structure prediction of GSTs from avian mites

The ORF sequences of the *GST* genes of PRMs, TFMs, and NFMs were determined and assigned as accession nos. LC776937, LC776938, and LC776939, respectively. A homology of GSTs was searched using the BLAST of the NCBI. The InterPro95.0 (https://www.ebi.ac.uk/interpro/) and Conserved Domain search programs (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) of NCBI were used to identify the functional domains and active residues from the translated sequences of each *GST* from avian mites. For the 3D structural analysis of GSTs, the SWISS-MODEL (https://swissmodel.expasy.org/), an automated homology modeling server was used. The protein structures of GSTs from TFMs and NFMs were generated by using the alpha-fold monomeric structure of PRM GST (A0A0H4FNI7) as a template. The 3D structures of GSTs from PRMs, TFMs, and NFMs were manipulated using the PyMOL program (version 2.5.5). The maximum likelihood phylogeny was constructed using the MEGA software (version X) [Kumar *et al*., 2018], with 1,000 bootstrap values, a discrete gamma distribution (+G), and assuming that a certain fraction of sites was evolutionarily invariable (+I) to improve tree topology.

Generation of rGST proteins

The rGSTs were expressed and purified as described in Chapter I. The rGST proteins from PRMs, TFMs, and NFMs were expressed as fusion proteins with a histidine tag using an *E. coli* expression system and termed rGST PRM, rGST TFM, and rGST NFM, respectively. The coding regions of *GST* genes from PRMs, TFMs, and NFMs were amplified using specific primers including the NdeI and XhoI restriction sites (Table II-1). The products were cloned into the pET19b vector and transformed into *E. coli* (DE3). The inclusion body fractions containing target proteins were fractionated using BugBuster solution. The proteins were solubilized in a buffer containing 0.3% N-lauroylsarcosine and 50 mM CAPS. The rGST proteins were purified using the Ni Sepharose™ 6 Fast Flow resin and were eluted with buffer containing 0.3% N-lauroylsarcosine and 50 mM CAPS (pH 11.0) and 250 mM imidazole. The proteins were refolded by dialysis against 10 mM Tris-HCL (pH 8.5) buffer containing 0.1 mM DTT at 4 ℃ overnight. The expression and purity of rGSTs were confirmed by SDS-PAGE and western blotting. The protein concentration was measured using a Pierce™ Bicinchoninic Acid Protein Assay Kit.

Enzyme activity assay

The enzyme activity of rGSTs of PRMs, TFMs, and NFMs was assessed as described previously [Bartley *et al*., 2015a]. In this study, the enzyme activity was measured at different concentrations of each rGST in the presence of a constant concentration of GSH and the 1-chloro-2,4-dinitrobenzene (CDNB) substrate. Briefly, 20

µL of each rGST (1, 2, and 4 µg) in Tris/NaCl buffer (10 mM Tris, 0.5 M NaCl, pH 7.4) were placed in the 96-well microtiter plate in triplicate, and the same amount of Tris/NaCl buffer was placed in the control wells. One hundred µL of 1 mM CDNB in substrate buffer (100 mM potassium dihydrogen phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 6.5, and 2 mM GSH) was added in all the wells. Immediately, the absorbance at 340 nm was measured every minute using a microplate reader MTP 900 for 20 min. To adjust the spontaneous hydrolysis of CDNB, the mean absorbance of the control wells was subtracted from that of the test wells. The specific enzyme activities (µmol/min/mg protein) at different concentrations of each rGST were calculated using the following formula [Bartley *et al*., 2015b].

$$\frac{(At2-At1)\times 1000}{A\varepsilon\times(t2-t1)\times b\times m}\times Vtot \qquad (1)$$

At1 - initial time point
At2 - final time point
t1 - starting time (min)
t2 - end time (min)
Aε - the molar extinction coefficient of CDNB (Aε = 9.6)
b - path length of the spectrophotometer (0.286 cm)
m - the quantity of rGST per well (mg)
Vtot- total volume per well (L)

Production of anti-rGST antibody

To produce immune plasma against rGSTs of PRMs, TFMs, and NFMs, chickens were subcutaneously injected with 20 µg of each rGST emulsified with Freund's incomplete adjuvant at 3 and 7 weeks with 21-G needles. For the control group, chickens were injected with PBS with the same adjuvant. Four chickens were used for each immunized group. Three weeks after the second administration, chickens were euthanized by collecting heparinized whole blood from the hearts of each immunized chicken separately, under deep general anesthesia with Isoflurane inhalation. The immune plasmas were isolated for further experiments. Throughout the experimental period, the health status of chickens was recorded, and there were no instances of mortality, reduction in body weight, or any other noticeable symptoms in all immunized groups.

ELISA

The antibody titers in the plasma isolated from each chicken were analyzed by ELISA as described in Chapter I. Briefly, 100 ng/well of rGST of PRMs, TFMs, and

NFMs was coated onto a 96-well plate using carbon-bicarbonate buffer (pH 9.8) at 4 °C overnight. After blocking the plate with 1 % BSA, the plate was reacted with 100μL of diluted immune plasma (×2,000, ×4,000, ×8,000, ×16,000, ×32,000, and ×64,000) obtained from each chicken as the primary antibody. After the plate was incubated at 25°C for 1 h, the reaction complex was labeled with a 10,000-fold dilution of anti-chicken IgY[IgG](H+L)-goat HRP, and the TMB one component HRP microwell substrate was added and incubated for 20 min at RT in the dark. The absorbance was measured at 450 nm using an ELISA plate reader MTP 900. The cutoff value was set as the absorbance values of the control plasma at a 2,000-fold dilution.

Western blotting

The production of specific antibodies against each rGST and the cross-reactivity of each anti-rGST antibody with rGSTs from different avian mites were analyzed by western blotting as described in Chapter I. Briefly, 1 μg of each rGST was resolved by 13 % SDS-PAGE and transferred to PVDF membranes. After the membranes were blocked with 1 % skim milk in PBS-T, they were incubated with each anti-GST chicken plasma (1:1,000). Membranes were washed with PBS-T three times and labeled with anti-chicken IgY peroxidase rabbit antibody (1:10,000). Signal detection was performed by using Immobilon Western Chemiluminescent HRP Substrate. To confirm His-tagged rGSTs purification, each rGST was incubated with HRP-conjugated anti-6-his, mouse monoclonal antibody and labeled with goat anti-mouse IgG antibody.

Assessment of acaricidal potential by using immune plasma

To assess the acaricidal efficacy of each GST on PRMs, *in vitro* feeding assays were conducted as described in Chapter I, and the mortality rate of blood-fed PRMs was monitored. Briefly, plasma of fresh blood from healthy chickens housed at the Field Science Center for the Northern Biosphere, Hokkaido University, was replaced with pooled plasma from each immunized or control group. After placing the starved PRMs in the devices, PRMs fed with blood containing immune plasma for 4 h at 40 ℃. Blood-fed PRMs from each feeding group were collected, and their mortality was monitored. The mortalities of the blood-fed PRMs were recorded daily for 7 days. For each assay, the same batch of PRMs was used. *In vitro* feeding assays were conducted in duplicate.

Statistical analysis

The EZR statistical software was used for statistical analysis [Kanda, 2013]. To compare the daily mortality rate of PRMs between each immunized and control group, Fisher's exact test and ORs with 95% CI were analyzed. A log-rank test was conducted, and Kaplan–Meier survival curves were generated to compare the mortality of PRMs

between the immunized and control groups for 7 days after *in vitro* feeding. The significant difference with the control group was set at $p<0.05$.

Table II-1. List of primers used for the amplification of *glutathione S-transferase* genes in this study

Primer	Mite species	Intended use	Sequences (5´-3´)
GST outer -F	NFM & TFM	Partial gene amplification	TACTGGAACATCCGCGGMTT
GST outer- R			TGCCARTCGAGGAAYTCGWACA
GST inner -F			TTATCGAGAAGCAGGCYGATG
GST inner-R			AAGCGCTCRAGGAAKGCCTTKA
GSP1	NFM & TFM	3′RACE	AGCACAGCCCATTCGTAATCT
GSP2			TGGCGAGGAATATGAAGACCG
GSP3			AGCGATTGCCAACTACTGCT
GSP1	NFM & TFM	5′RACE	GAAGTCAACGTAGGTC
GSP2			AATCGCTTGCTTGAGGTCCA
GSP3			GGAAGATTGGGGAAGTCCAT
GST ORF-F	PRM, TFM & NFM	ORF confirmation	CGAGAGAAGTGGAGACAGCT
GST ORF-R			TGCTTGGAACGAGTTTGCTGA
rGST PRM-F	PRM	Construction for expression plasmids	AAGCATATGATGGCTCCGTCCACGACGTTC
rGST PRM-R			ATCCTCGAGTCAGTTGCCCTTGGTCCAGCC
rGST TFM-F	NFM & TFM		AAGCATATGATGGCACCTTCAATTATTCTTGGTTACTGG
rGST TFM-R			ATCCTCGAGTCACTTATAGTAACCATATTTCTTGGCGAAGGG

*The NdeI and XhoI sites are underlined.

PRM, poultry red mite; NFM, northern fowl mite; TFM, tropical fowl mite; GST, glutathione S-transferase; rGST, recombinant glutathione S-transferase

Table II-2 List of *glutathione S-transferase* genes used for phylogenetic analysis in Figure 2

Accession no.	GenBank	Organism
KR337505.1	Glutathione S-transferases-1 (GST-1)	*Dermanyssus gallinae*
XM022854296.1	Glutathione S-transferase Mu 1-like (LOC111272717) mRNA	*Varroa jacobsoni*
XM022792712.1	Glutathione S-transferase Mu 1-like (LOC111245011) mRNA	*Varroa destructor*
XM003747361.1	Glutathione S-transferase Mu 1 (LOC100904919) mRNA	*Metaseiulus occidentalis*
KR337507.1	Glutathione S-transferases-3 (GST-3) mRNA complete cds	*Dermanyssus gallinae*
XM029970261.3	Glutathione S-transferase (LOC8032802) mRNA	*Ixodes scapularis*
XM037657656.2	Glutathione S-transferase (LOC119390107) mRNA	*Rhipicephalus sanguineus*
XM037727032.2	Glutathione S-transferase-like (LOC119466514) mRNA	*Dermacentor silvarum*
XM037424660.1	Glutathione S-transferase-like (LOC119173874) mRNA	*Rhipicephalus microplus*
XM037412779.1	Glutathione S-transferase Mu 1-like (LOC119160027) mRNA	*Rhipicephalus microplus*
MT665977.1	Mu class glutathione S-transferase protein 4 (GSTM4) mRNA complete cds	*Dermacentor marginatus*
XM050192192.1	Glutathione S-transferase Mu 1-like (LOC126544729) transcript variant X1 mRNA	*Dermacentor andersoni*
XM027349261.1	Glutathione S-transferase D7-like (LOC113798691) mRNA	*Dermatophagoides pteronyssinus*
XM037722444.2	Glutathione S-transferase omega-1 (LOC119461218) mRNA	*Dermacentor silvarum*
NM001396288.1	Glutathione S-transferase theta 1 (GSTT1) transcript variant 2 mRNA	*Gallus gallus*
U13676.1	Liver theta glutathione-S-transferase mRNA complete cds	*Gallus gallus*
MT665978.1	Zeta class glutathione S-transferase protein 1 (GSTZ1) mRNA complete cds	*Dermacentor marginatus*
MT799206.1	GST zeta protein mRNA complete cds	*Diaphanosoma celebensis*
L15386.1	Glutathione S-transferase (GST) mRNA complete cds	Chicken
NM204818.2	Glutathione S-transferase alpha 4 (GSTA4) mRNA	*Gallus gallus*
MT665976.1	Epsilon class glutathione S-transferase protein 1 (GSTE1) mRNA complete cds	*Dermacentor marginatus*
XM029978184.3	Glutathione S-transferase 1-like (LOC8029517) mRNA	*Ixodes scapularis*
AB443867.1	CqGSTd1 gene for glutathione transferase delta complete cds	*Culex quinquefasciatus*
JN251103.1	Glutathione S-transferase delta (GSTd1) mRNA complete cds	*Culex pipiens*
KR337506.1	Glutathione S-transferases-2 (GST-2)	*Dermanyssus gallinae*
GQ214698	Glutathione S-transferase delta class 2 mRNA partial cds	*Sarcoptes scabiei*
XM027349261.1	Glutathione S-transferase D7-like (LOC113798691) mRNA	*Dermatophagoides pteronyssinus*

RESULTS

Genetic characterization and structure prediction of GSTs from avian mites

The PRM *GST* gene identified from Japan matched the reported *GST* sequence of PRMs (KR337505) with 100% identity. The nucleotide and deduced amino acid sequences of GSTs from TFMs and NFMs were 75% and 80% identical to those of PRMs, respectively. The homology of GSTs between TFMs and NFMs was 100% (Table II-3).

The alpha-fold monomeric structures of GSTs from PRMs, TFMs, and NFMs were characterized (Figure II-1-a-d). The predicted monomer form of GSTs of PRMs, TFMs, and NFMs consists of two domains. The N-terminal Domain I consists of 1–4 β strands and 1–4 α helices, and the C-terminal Domain II consists of 5–8 alpha helices. Domains I and II are connected with the linker loop region. As a typical feature of GSTs, these domains have representative active residues that are responsible for two ligand-binding sites, G- and H-sites. The G-site residues, Tyr9, Tyr10, Met42, Pro46 (Leu46 in GSTs of TFMs and NFMs), Asn75, Leu76, Gln93, and Ser94, and the H-site residues, Met126, Lys129, Gln130, Ala133, Gly134 (Asn134 in GSTs of TFMs and NFMs), Tyr137, Phe185, Trp188, Pro229, Leu230, and Val23, are expected to bind multiple substrates and capable for catalytic functions. As shown in the multiple sequence alignment, GSTs from TFMs and NFMs were conserved among PRMs (Figure II-1-d,e). Furthermore, the active residues included in each domain were conserved among avian mites. Therefore, the GSTs of avian mites appear to have similar functions.

The phylogenetic tree was constructed using different classes of *GST* genes from arthropods, including other mites and ticks (Figure II-2 and Table II-2). The tree was divided into seven clusters: mu, alpha, zeta, delta, omega, epsilon, and theta. The *GST* genes of avian mites belonged to the mu class. The mu cluster was grouped into two subclusters, 1 and 2, representing the mu class GST of mites and ticks, respectively. Therefore, the GSTs of avian mites seem to be similar to the mu-class GSTs of other mites and ticks.

Enzyme activity of rGSTs from PRMs, TFMs, and NFMs

To analyze the function of GSTs, rGST proteins from PRMs, TFMs, and NFMs were prepared and termed rGST PRM, rGST TFM, and rGST NFM, respectively. Although the GST sequences of the TFMs and NFMs were 100 % identical, rGST proteins were separately produced from the sequence of TFMs and NFMs to improve the reproducibility of the following analysis. As expected, the purified proteins were expressed at 27 kDa (Figure II- 3-a,b).

The CDNB-based colorimetric assay demonstrated that the GSTs of the PRMs, TFMs, and NFMs were enzymatically active and capable of catalyzing the conjugation

of GSH to CDNB substrate. The enzymatic activity of each GST was shown in a dose-dependent manner (Figure II-4).

Cross-reactivities of immune plasmas with rGSTs of different mites

Chickens were immunized with each rGST, and immune plasma was isolated. Increased antibody production was confirmed three weeks after the second immunization with each rGST (Table II-4). The presence of specific antibodies in the plasma against each rGST was confirmed by western blotting. In addition, the immune plasma from chickens immunized with each GST cross-reacted with rGSTs from different mite species, and vice versa (Figure II-5). There were slight differences in signals when the immune plasma against rGST PRM reacted with rGSTs from TFMs and NFMs, compared with that of rGST from PRMs. On the other hand, the signal intensity was different when the plasmas from chickens immunized with rGST TFM and NFM were reacted with rGST PRM, although no difference was observed when reacted with rGST TFM and NFM. This difference may be due to slight differences in epitopes present in the GSTs of TFMs and NFMs compared to those of PRMs. Another signal was found at higher molecular weights in each lane than the predicted size. In addition, this signal was faint in the PRM immune plasma when tested against rGST PRM. This signal may be attributed to residual protein dimers or aggregates, but it is unclear why it was generated.

***In vitro* acaricidal effect of immune plasma**

To investigate the potential of rGSTs for vaccine application, PRMs were fed with fresh blood containing specific immune plasma against each rGST *in vitro*. The *in vitro* feeding assays were performed in duplicate. In Experiment 1, the mortality rate of PRMs gradually increased in each immunized group and reached 28.8, 38.9, and 23.6% in rGST PRM, rGST TFM, and rGST NFM, respectively, at 7 days post-feeding (Table II-5). In addition, at 2–7 days post-feeding, the mortality rate of PRMs in all immunized groups was significantly higher than in the control group. A log-rank test and Kaplan–Meier analysis revealed that the survival rate of PRMs significantly decreased after feeding with immune plasma against rGST TFM or rGST NFM, in addition to rGST PRM (Figure II-6-a). Similarly, in Experiment 2, the survival rates of PRMs fed with plasma from each immunized group were significantly lower than those of the control group (Figure II-6-b). Significant differences in mortality rates were observed at 7 days post-feeding in the rGST PRM and rGST NFM immunized groups and at 5–7 days post-feeding in the immunized group of rGST TFM (Table II-6). The acaricidal efficacy was assessed on the developmental stages in Experiment 1 (Tables II-7,8 and Figures II-7-a,b). The significant differences were observed in both adults and nymphs. Experiment 2 was not analyzed, because most of the PRMs used in this assay were nymphs. These results indicated that immune plasma, including specific antibodies against each rGST,

showed acaricidal effects on PRMs. Therefore, GST has the potential to be applicable as an antigen in the development of vaccines against avian mites.

Table II-3. Comparison of sequences of glutathione S-transferase from poultry red mites (PRMs), northern fowl mites (NFMs) and tropical fowl mites (TFMs)

		Amino acid sequence (%)		
		PRM	TFM	NFM
Nucleotide sequence (%)	PRM	-	80	80
	TFM	75	-	100
	NFM	75	100	-

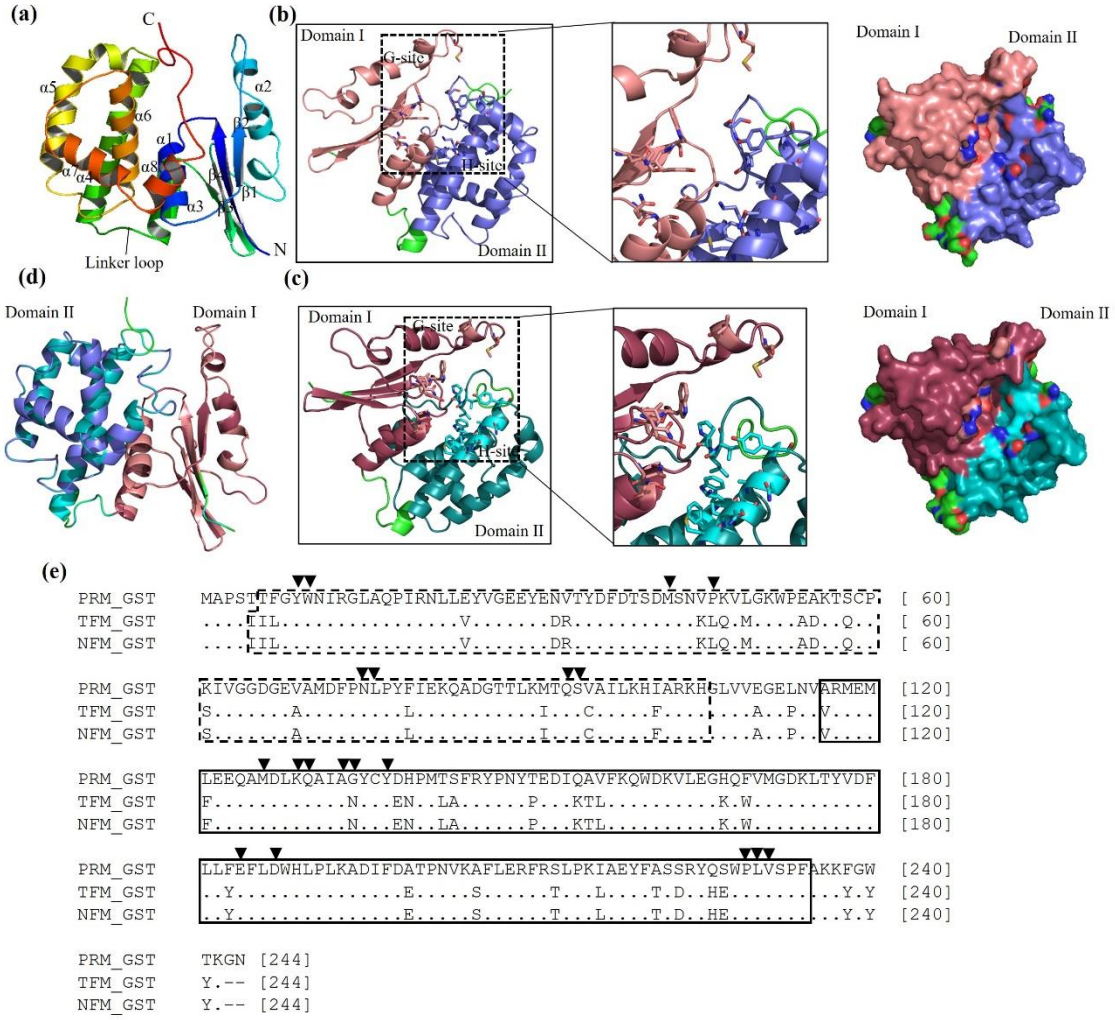

Figure II-1. Characterization of GSTs from avian mites.

(a) Cartoon views of PRM GST labeled with the monomer structure (α1–α8, β1–β4, and linker loop). Cartoon views of GSTs from (b) PRMs and (c) TFMs/NFMs. The right panels indicate the surface structure of GSTs locating their binding sites. The monomer structure of GSTs contains N-terminal Domain I and C-terminal Domain II connected with a linker loop. The ligand-binding sites (G- and H-sites) for binding multiple substrates and catalytic function are shown in stick format. Domains I and II indicate salmon and slate colors in PRMs and raspberry and teal colors in TFMs/NFMs. (d) 3D structure alignment of GSTs from PRMs, TFMs, and NFMs. (e) Multiple sequence alignments of GSTs where the monomer structure, functional domains, and residues in binding sites are labeled. The structure of GST contains Domain I (dashed box) at positions of 6–105 in PRMs and 5–105 in TFMs and NFMs and Domain II (white box) at positions of 116–234 in PRMs, TFMs, and NFMs. The black arrowhead indicates the active residues of G- (Domain I) and H-sites (Domain II).

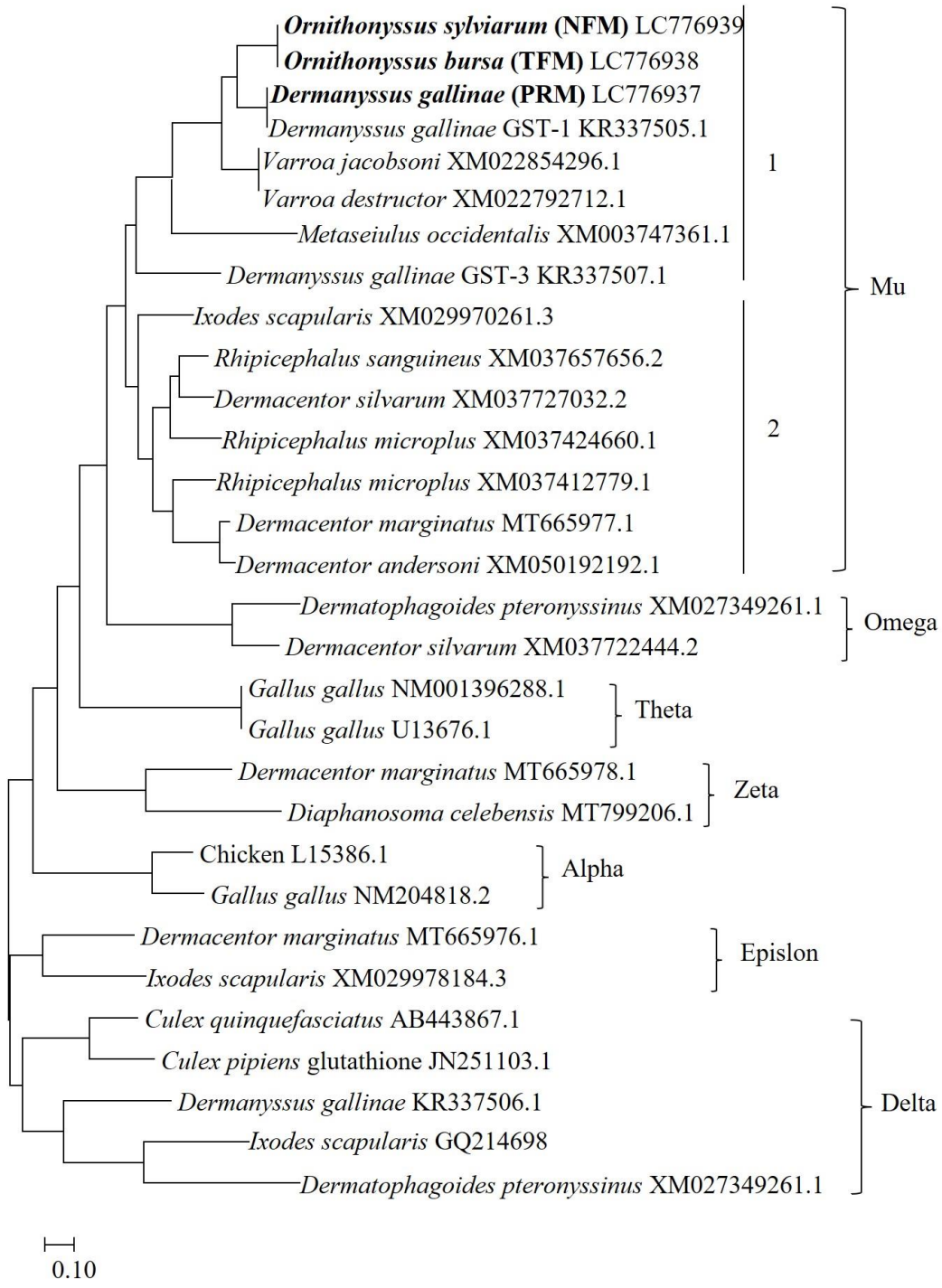

Figure II-2. Phylogenetic analysis of *GST* nucleotide sequences derived from avian mites.

The assigned *GST* classes are labeled. Sub-clusters 1 and 2 of the mu clade indicate the mu class *GST* of mites and ticks, respectively. The *GST* genes of PRMs, TFMs, and NFMs are shown in bold.

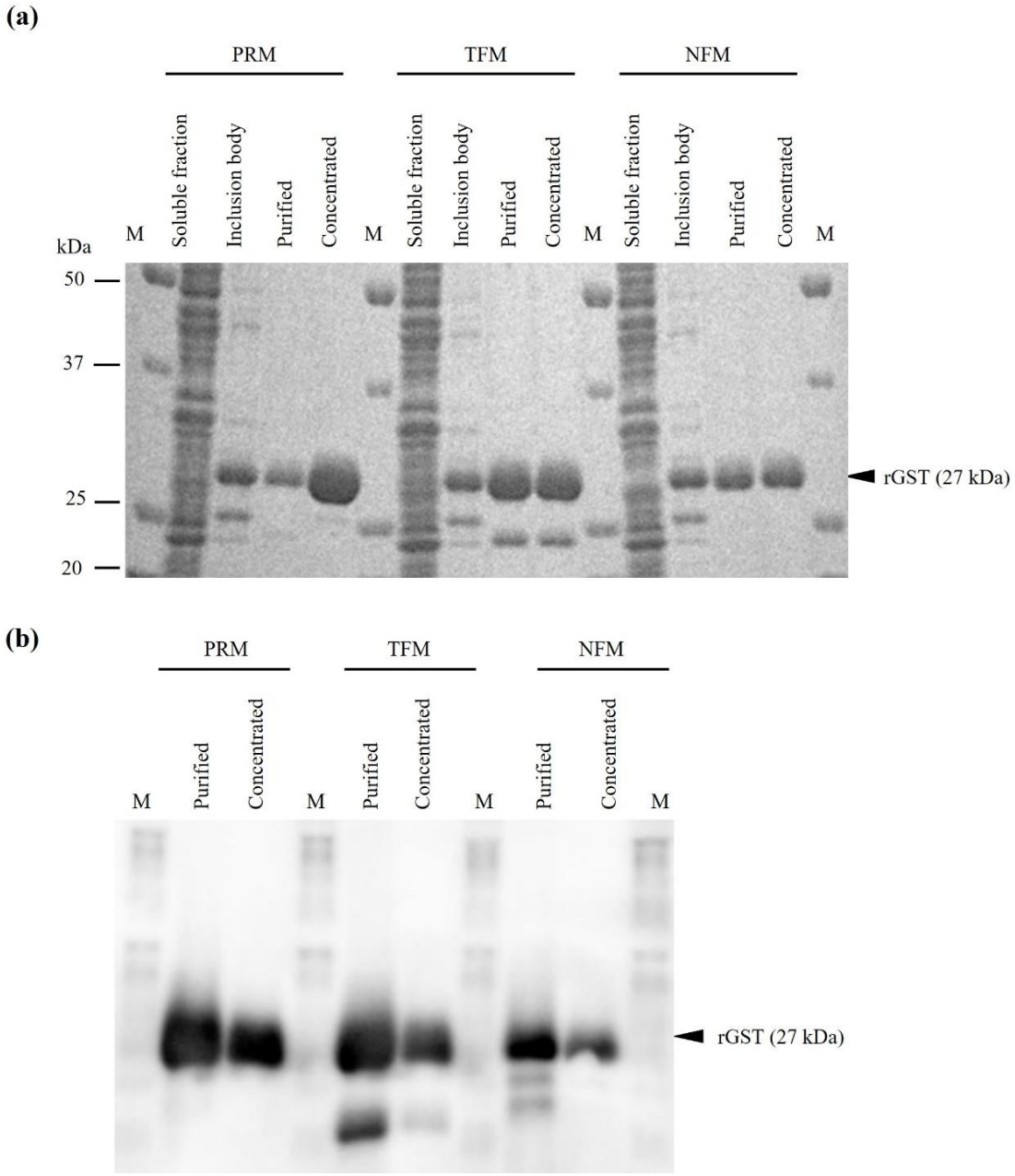

Figure II-3. Expression and purification of rGST proteins of avian mites.
The rGSTs were purified and designated as rGST PRM, rGST TFM, and rNFM NFM. The expressions of rGSTs were confirmed by (a) SDS-PAGE and (b) western blotting. M, Marker.

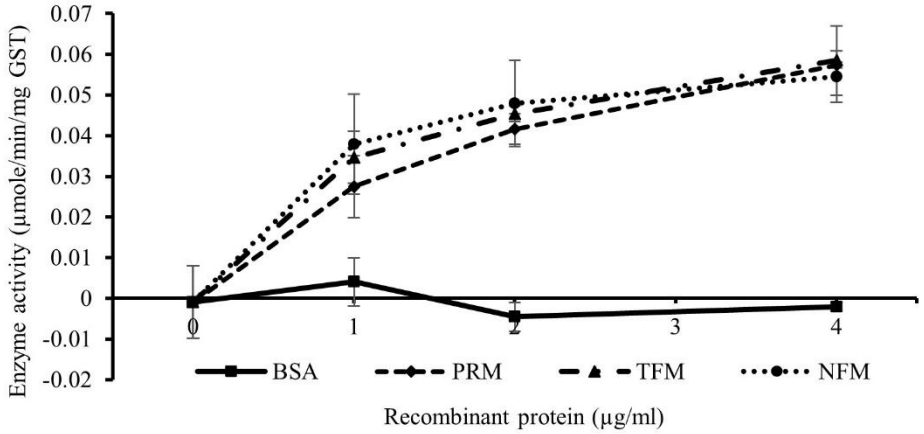

Figure II-4. Enzyme activity of rGSTs.
The colorimetric-based enzyme activities of rGSTs from PRMs, TFMs, and NFMs were conducted in triplicate. The specific enzyme activities (μmol/min/mg protein) upon the different concentrations of each rGST were calculated. The x-axis indicates the concentration of rGSTs used in these assays. The error bar indicates the standard deviations. BSA, bovine serum albumin.

Table II-4. Antibody titers in plasma samples from chickens immunized with recombinant glutathione S-transferase

Group	Chicken	Antibody titer
Control	C1	1:2,000
	C2	1:2,000
	C3	1:2,000
	C4	1:2,000
Immunized- rGST PRM	PG1	1:32,000
	PG2	1:64,000
	PG3	1:64,000
	PG4	1:64,000
Immunized- rGST TFM	TG1	1:64,000
	TG2	1:64,000
	TG3	1:64,000
	TG4	1:64,000
Immunized- rGST NFM	NG1	1:64,000
	NG2	1:64,000
	NG3	1:64,000
	NG4	1:64,000

PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite; rGST, recombinant glutathione S-transferase

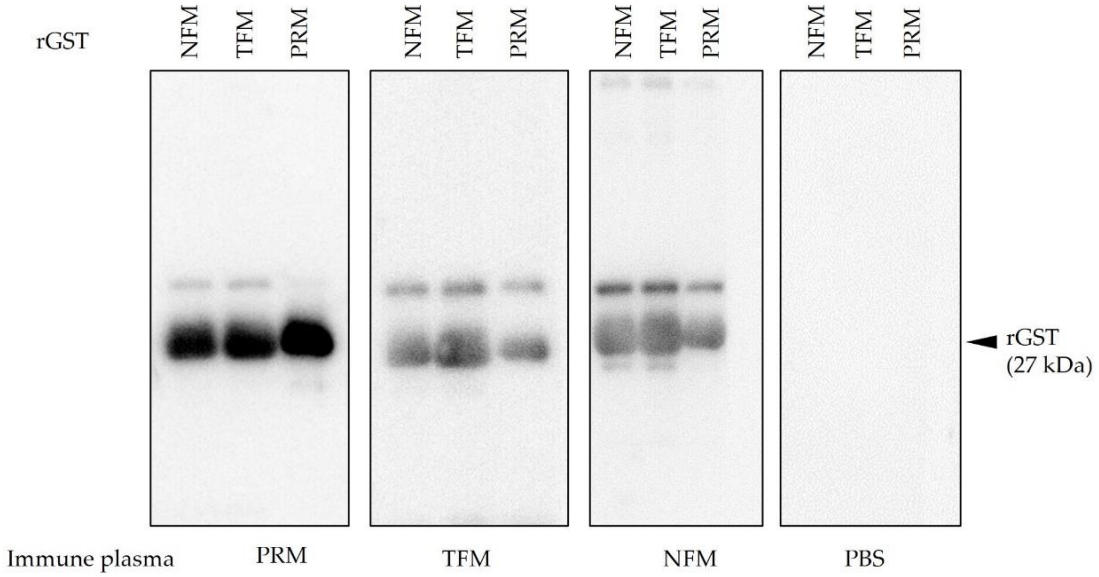

Figure II-5. The production of antibodies specific to each rGST in the plasma from immunized chickens.

The plasma was isolated from chickens immunized with each rGST. The production of antibodies specific to each rGST in the plasma and cross-reactivities of immune plasmas with rGSTs were analyzed by western blotting. The black arrowhead indicates the predicted molecular weight of rGSTs (27 kDa).

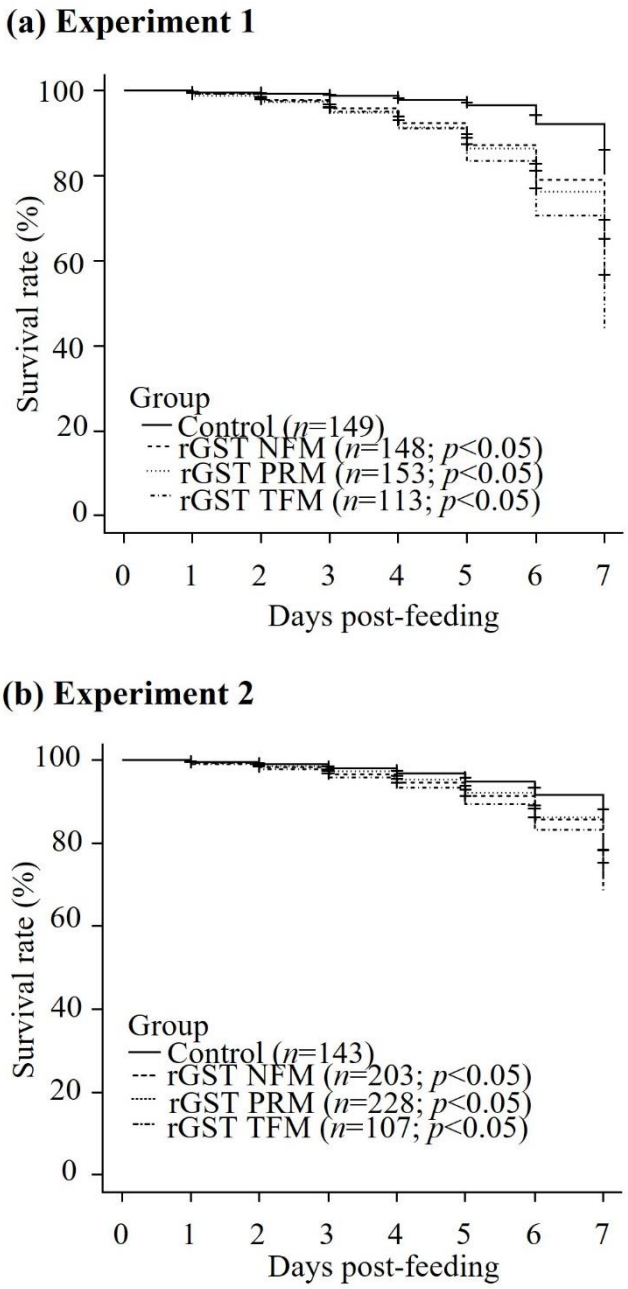

Figure II-6. Evaluation of the acaricidal potential of GSTs by *in vitro* feeding with PRMs.

The survival rate of PRMs fed with plasmas obtained from each immunized group was assessed daily for a week. Number of PRMs for these analyses are (a) Experiment 1: immune plasma: n=153 (rGST PRM), n=113 (rGST TFM), n=148 (rGST NFM), control plasma: n=149, (b) Experiment 2: immune plasma: n=228 (rGST PRM), n=107 (rGST TFM), n=203 (rGST NFM), control plasma: n=143. The number of dead PRMs was recorded. The statistical analysis was calculated by a log-rank test. p<0.05 were considered statistically significant.

Table II-5. Mortality of mixed stages of PRMs fed plasma from chickens immunized with recombinant glutathione S-transferase from different species of mites (Experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=149)							
No. of dead PRMs post-feeding	3	3	3	6	6	14	19
Mortality (%)	2.01	2.01	2.01	4.03	4.03	9.49	12.75
Immunized group (rGST PRM, *n*=153)							
No. of dead PRMs post-feeding	11	15	19	23	25	36	44
Mortality (%)	7.19	9.80	12.42	15.03	16.34	23.53	28.76
p value (Fisher's exact)	0.052	6.01E-3*	5.74E-4*	1.43E-3*	4.78E-4*	1.06E-3*	6.55E-4*
Odds ratio	3.76	5.26	6.86	4.19	4.63	2.96	2.75
95% confidence interval	0.96–21.39	1.44–29.01	1.95–37.02	1.59–13.01	1.78–14.25	1.47–6.24	1.47–5.31
Immunized group (rGST TFM, *n*=113)							
No. of dead PRMs post-feeding	6	11	15	18	29	35	44
Mortality (%)	5.31	9.73	13.27	15.93	25.66	30.97	38.94
p value (Fisher's exact)	0.180	0.010*	7.85E-4*	1.89E-3*	5.63E-7*	2.2E-5*	2.05E-6*
Odds ratio	2.68	5.13	7.27	4.41	8.01	4.22	4.24
95% confidence interval	0.55–16.89	1.31–29.36	1.98–40.21	1.60–14.08	3.10–24.58	2.06–9.04	2.23–8.33
Immunized group (rGST NFM, *n*=148)							
No. of dead PRMs post-feeding	7	12	16	21	25	28	35
Mortality (%)	4.73	8.11	10.81	14.19	16.89	18.92	23.65
p value (Fisher's exact)	0.335	0.031*	3.32E-3*	3.95E-3*	4.5E-4*	0.029*	0.023*
Odds ratio	2.39	4.24	5.83	3.89	4.78	2.23	2.09
95% confidence interval	0.53–14.62	1.11–23.94	1.61–31.91	1.46–12.18	1.84–14.74	1.08–4.81	1.09–4.11

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table II-6. Mortality of mixed stages of PRMs fed plasma from chickens immunized with glutathione S-transferase from different species of mites (Experiment 2)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=143)							
No. of dead PRMs post-feeding	4	5	6	8	8	10	11
Mortality (%)	2.79	3.49	4.19	5.59	5.59	6.99	7.69
Immunized group (rGST PRM, *n*=228)							
No. of dead PRMs post-feeding	9	11	15	18	23	29	41
Mortality (%)	3.95	4.82	6.58	7.89	10.09	12.72	17.98
p value (Fisher's exact)	0.773	0.423	0.368	0.531	0.176	0.085	5.49E-3*
Odds ratio	1.43	1.76	1.61	1.45	1.89	1.93	2.62
95% confidence interval	0.39–6.46	0.51–7.73	0.57–5.18	0.58–3.95	0.79–5.04	0.88–4.60	1.27–5.88
Immunized group (rGST TFM, *n*=107)							
No. of dead PRMs post-feeding	6	9	10	11	14	15	20
Mortality (%)	5.61	8.41	9.35	10.28	13.08	14.02	18.69
p value (Fisher's exact)	0.334	0.104	0.120	0.228	0.044*	0.088	0.011*
Odds ratio	2.06	2.53	2.35	1.93	2.53	2.16	2.75
95% confidence interval	0.47–10.18	0.73–9.89	0.74–8.13	0.68–5.75	0.95–7.26	0.86–5.64	1.19–6.68
Immunized group (rGST NFM, *n*=203)							
No. of dead PRMs post-feeding	9	12	17	18	21	25	35
Mortality (%)	4.43	5.91	8.37	8.87	10.34	12.32	17.24
p value (Fisher's exact)	0.570	0.450	0.187	0.304	0.167	0.147	0.010*
Odds ratio	1.61	1.73	2.08	1.64	1.94	1.86	2.49
95% confidence interval	0.44–7.29	0.55–6.42	0.76–6.63	0.65–4.49	0.79–5.23	0.83–4.51	1.18–5.66

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table II-7. Mortality of adult PRMs fed with plasma from chickens immunized with glutathione S-transferase from different species of mites (Experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=50)							
No. of dead PRMs post-feeding	2	2	2	4	4	8	11
Mortality (%)	4.0	4.0	4.0	8.0	8.0	16.0	22.0
Immunized group (rGST PRM, *n*=76)							
No. of dead PRMs post-feeding	7	11	14	15	16	19	23
Mortality (%)	9.21	14.47	18.42	19.74	21.05	25.0	30.26
p value (Fisher's exact)	0.316	0.075	0.026*	0.081	0.079	0.272	0.412
Odds ratio	2.42	4.02	5.36	2.81	3.04	1.74	1.53
95% confidence interval	0.43–24.85	0.82–39.03	1.15–50.84	0.82–12.39	0.89–13.35	0.65–5.06	0.63–3.92
Immunized group (rGST TFM, *n*=58)							
No. of dead PRMs post-feeding	3	6	10	10	11	15	23
Mortality (%)	5.17	10.34	17.24	17.24	18.97	25.86	39.66
p value (Fisher's exact)	1	0.282	0.034*	0.25	0.162	0.245	0.062
Odds ratio	1.31	2.75	4.93	2.38	0.72	1.82	2.311
95% confidence interval	0.14–16.24	0.46–29.09	0.97–48.67	0.63–11.13	0.72–12.33	0.64–5.52	0.92–6.06
Immunized group (rGST NFM, *n*=69)							
No. of dead PRMs post-feeding	3	8	12	14	15	16	18
Mortality (%)	4.35	11.59	17.39	20.29	21.74	23.19	26.09
p value (Fisher's exact)	1	0.189	0.041*	0.075	0.047*	0.365	0.669
Odds ratio	1.09	3.12	4.99	2.90	3.17	1.58	1.25
95% confidence interval	0.12–13.52	0.58–31.49	1.03–48.14	0.83–12.95	0.92–14.03	0.57–4.69	0.49–3.29

*$p<0.05$ was considered statistically significant.

PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table II-8. Mortality of nymph PRMs fed with plasma from chickens immunized with glutathione S-transferase from different species of mites (Experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=99)							
No. of dead PRMs post-feeding	1	1	1	2	2	6	8
Mortality (%)	1.01	1.01	1.01	2.02	2.02	6.06	8.08
Immunized group (rGST PRM, *n*=77)							
No. of dead PRMs post-feeding	4	4	5	8	9	17	21
Mortality (%)	5.19	5.19	6.49	10.39	11.69	22.08	27.27
p value (Fisher's exact)	0.170	0.170	0.088	0.022*	0.011*	0.003*	8.92E-4*
Odds ratio	5.32	5.32	6.74	5.57	6.36	4.35	4.23
95% confidence interval	0.51–266.7	0.51–266.71	0.73–324.55	1.06–55.40	1.25–62.32	1.53–14.27	1.66–11.82
Immunized group (rGST TFM, *n*=55)							
No. of dead PRMs post-feeding	3	5	5	8	18	20	21
Mortality (%)	5.45	9.09	9.09	14.55	32.73	36.36	38.18
p value (Fisher's exact)	0.130	0.022*	0.022*	0.004*	1.14E-7*	5.78E-06*	9.4E-06*
Odds ratio	5.59	9.66	9.66	8.14	23.09	8.71	6.92
95% confidence interval	0.43–299.24	1.04–466.93	1.04–466.93	1.54–81.82	5.13–214.21	3.05–28.78	2.64–19.88
Immunized group (rGST NFM, *n*=79)							
No. of dead PRMs post-feeding	4	4	4	7	10	12	17
Mortality (%)	5.06	5.06	5.06	8.86	12.66	15.19	21.52
p value (Fisher's exact)	0.173	0.173	0.173	0.079	0.006*	0.077	0.016*
Odds ratio	5.18	5.18	5.18	4.68	6.96	2.76	3.09
95% confidence interval	0.49–259.61	0.49–259.61	0.49–259.61	0.85–47.43	1.42–67.39	0.90–9.43	1.18–8.83

*$p<0.05$ was considered statistically significant.

PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

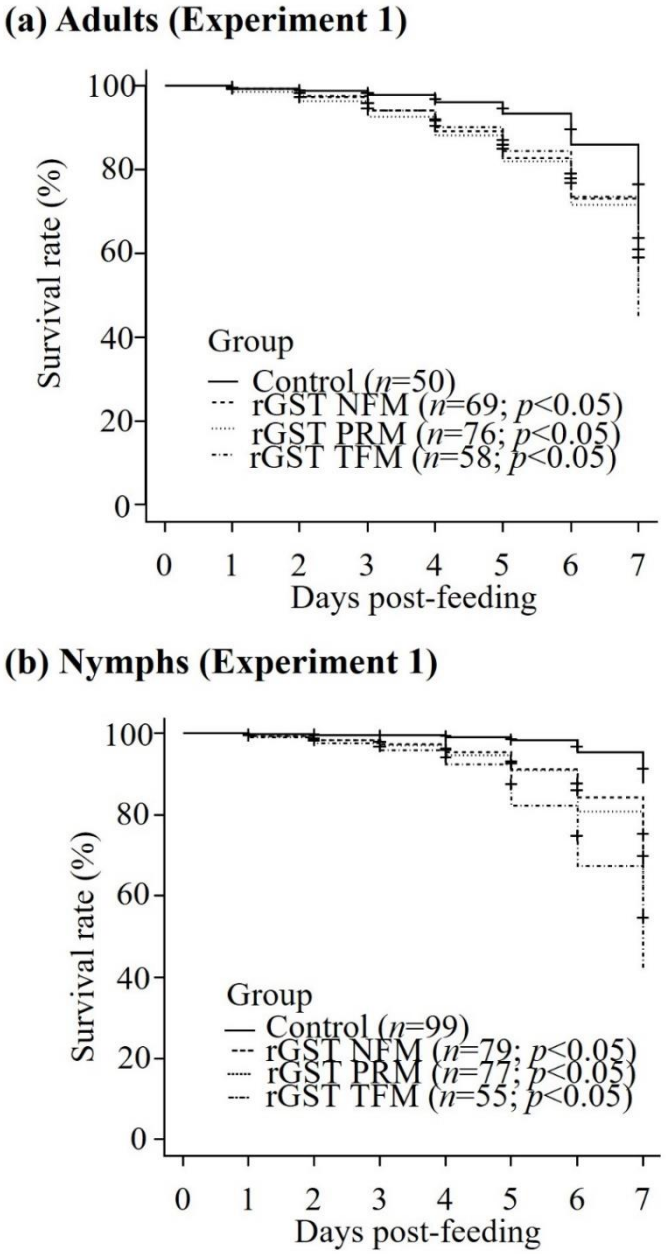

Figure II-7. Evaluation of the acaricidal potential of GSTs by *in vitro* feeding with developmental stages of PRMs (Experiment 1).

The survival rates of PRMs fed with plasmas obtained from each immunized group were assessed daily for a week. Number of PRMs for these analyses are (a) adult PRMs: immune plasma: n=76 (rGST PRM), n=58 (rGST TFM), n=69 (rGST NFM), control plasma: n=50, (b) nymph PRMs: immune plasma: n=77 (rGST PRM), n=55 (rGST TFM), n=79 (rGST NFM), control plasma: n=99. The number of dead PRMs was recorded. The statistical analysis was calculated by a log-rank test. p<0.05 were considered statistically significant.

DISCUSSION

GSTs belong to a large superfamily of versatile proteins and are classified into various classes, such as cytosolic, mitochondrial, and microsomal GSTs, based on class-specific motifs and amino acid residues in the active sites involved in binding to their substrates [Mashiyama *et al*., 2014]. Generally, cytosolic GSTs are classified into alpha, mu, pi, theta, and sigma classes in mammals [Le Gall *et al*., 2018]. Among cytosolic GST classes, the amino acid sequence similarities are high (~70%) in the N-terminal domains, whereas they are low (~30%) or vary considerably in the C-terminal domains [Sinning *et al*., 1993; Armstrong, 2010; Deponte *et al*., 2013]. The mu-class GSTs identified in this study from avian mites are closely related to the same class of GSTs from other mite species. Avian mite GSTs consist of two important domains: I at the N-terminus and II at the C-terminus, which are similar to the general structures of vertebrate GSTs and are necessary for enzymatic activities and detoxification to reduce the toxicity of acaricides. In *R. microplus*, the active form of GSTs is a dimer, and each monomer contains eight helixes (α1–α8) and four beta-strands (β1–β4) [Rangubpit *et al*., 2022]. Moreover, GSTs identified as allergens from *Dermatophagoides pteronyssinus* (house dust mite) and *Tyrophagus putrescentiae* mites form the dimer structure with eight helixes (α1−α8) and four beta-strands (β1−β4) [Liao *et al*., 2013]. In this study, GSTs identified from avian mites were characterized into alpha-fold monomeric structures containing eight helixes (α1−α8), four beta-strands (β1−β4), two active sites (G-and H-sites), and a mu loop. These characteristics were similar to those of the monomer GST of *R. microplus* and allergens of other mite species [Zhan *et al*., 2010; Liao *et al*., 2013], and their sequence homology indicated 46–53%. In addition, the GSTs of avian mites were shown to have a function in catalyzing the conjugation of GSH to CDNB, similar to those in a previous report [Bartley *et al*., 2015a]. Furthermore, mu-class GSTs of PRMs contributed to the detoxification of several classes of acaricides in the presence of GSH [Bartley *et al*., 2015a]. Therefore, the avian mite GSTs identified in this study seem to be cytosolic mu-class GST of vertebrates, which are required for catalytic activities and are involved in the detoxification of acaricides.

GST is considered as a potential vaccine antigen candidate against several parasites in animals and humans. In hamsters and dogs, immunization with recombinant *Necator americanus* GST (Na-GST-1) and *Ancylostoma caninum* GST-1 (*Ac*-GST-1), respectively, decreased worm burden and fecal egg counts [Xiao *et al*., 2008; Zhan *et al*., 2010;]. Moreover, the World Health Organization recommended GST as a vaccine antigen against Schistosomiasis, resulting in a reduced worm burden and egg counts in experimental mice [Tang *et al*., 2017]. Thus, GST could be a promising vaccine antigen candidate. Furthermore, the potential of homologous GST to induce cross-immunity has been reported, and the results of immunization with *Haemaphysalis longicornis* GST

showed large differences in the cross-protective efficacy; immunization was 57% effective against *R. microplus* [Ndawula *et al*., 2019] and 67% effective against *R. appendiculatus,* but ineffective against *R. sanguineus* [Parizi *et al*., 2011, 2012]. In addition, vaccination with *Wuchereria bancrofti* rGST (rWbGST) induced higher levels of WbGST-specific IgG1 and IgG2a antibodies, as well as IFN-γ, IL-10 and IL-4 cytokine production in immunized animals, exhibiting 65% in situ cytotoxicity to *Brugia malayi* (lymphatic filaria) [Andure *et al*., 2016]. Furthermore, the elevated mRNA levels of delta and mu class GSTs were detected in *Sarcoptes scabies* samples collected from a patient with recurrent crusted scabies over the course of their ivermectin treatment [Mounsey *et al*., 2010]. These findings strongly suggest that GSTs could be potentially utilized in the development of universal vaccines. In this study, the utility of avian mite GSTs as vaccine antigens was evaluated, and the immunogenicity of each rGST and high antibody production by immunization in chickens were confirmed. In addition, the immune plasma obtained from chickens immunized with each rGST cross-reacted with rGSTs of different avian mites, and immune plasma against rGSTs from TFMs and NFMs showed acaricidal effects on PRMs. These observations show that immunization with GSTs of avian mites induces cross-immunity to protect against multiple avian mites. In this regard, immune plasma against rGSTs may trap the naïve GSTs of mites, potentially inhibiting various mechanisms of metabolism and detoxification. To determine if vaccines against GSTs affect mite metabolism and detoxification, further assessment is needed to evaluate their acaricidal effects on acaricide-resistant mites. Moreover, this study did not determine whether immune plasma containing cross-reactive antibodies can induce cytotoxicity across mite species. In addition, it should be considered that other factors induced by immunization may affect its acaricidal effects on PRMs. To properly confirm the efficiency of these cross-reactive vaccine antigens, their acaricidal effects must be examined using IgY purified from immune plasma.

The mu-class GST is expressed in a variety of tissues and is critical for the detoxification of harmful organic compounds [Mounsey *et al*., 2010]. Moreover, the GST superfamily protein is conserved among various organisms and potentially shares similar epitopes across species. However, in previous reports, there were no autoimmune responses associated with GST administration [Sabadin *et al*., 2017]. Additionally, the phase 1 safety trial of Na-GST-1 immunization induced antigen-specific IgG antibodies in humans, and vaccine-related adverse effects were not observed [van de Wetering *et al*., 2021]. However, the presence of long-lasting anti-GST antibodies in hosts may impair the crucial functions of GST. In addition to detoxification, some types of GSTs are known to be engaged in several signaling cascades, including those involved in inflammation [Diemert *et al*., 2017]. Therefore, the long-term presence of anti-GST antibodies may increase host susceptibility to infection. In this study, any abnormalities in the health status of chickens were not observed during the experimental period; however, the long-

term safety of GST immunization and its effects on immune responses need to be carefully evaluated.

The possible application of a single antigen as a universal vaccine for controlling avian mites can encourage commercial production. In the present study, the potential application of GST as a broad-spectrum vaccine antigen against avian mites was assessed. Previously, GSTs were identified and characterized as molecules involved in acaricide detoxification in PRMs [Bartley *et al*., 2015a]. However, PRM GSTs have not been assessed as vaccine antigens. In this study, the immune plasma showed cross-reactivity with rGSTs from other mite species, and *in vitro* feeding assays revealed the acaricidal effects of immune plasma against the GSTs of TFMs/NFMs, in addition to PRMs. Furthermore, acaricidal effects were observed on both adult and nymph stages in Experiment 1. Although significant mortality rates of PRMs were observed in the immunized groups compared to the control groups, more than 70% of PRMs in immunized groups were alive after 7 days post-feeding. During the *in vitro* feeding in the present study, each PRM had an opportunity for blood feeding. Avian mites require blood feeding for development beyond the larval stage, and adult mites feed on blood up to 8 times during their egg-producing lifespan [Oliver *et al*., 1996]. Therefore, multiple feedings of anti-GST-containing blood may induce an additive effect on the survival of avian mites. Thus, GSTs could be vaccine antigen candidates against avian mites. However, as the efficacy of vaccines needs to be improved for practical application on poultry farms [Bartley *et al*., 2017], the identification of other common antigens and the combined use of multiple antigens as a cocktail vaccine are required to further enhance the efficacy of universal vaccines using GST.

SUMMARY

Several antigens with anti-PRM effects have been discovered to develop a novel control strategy by vaccine approaches against PRMs. However, the efficacy of vaccines needs to be improved for practical use on poultry farms, and the combined use of multiple antigens as a cocktail vaccine is required. In addition, the application of common antigens conserved among avian mites has the potential to be applicable for developing a universal vaccine with broad protective efficacy against multiple avian mites. Therefore, the identification of highly conserved antigens with crucial physiological functions would provide a significant breakthrough for this vaccine strategy.

Chapter II aimed to characterize avian mite GSTs, which are multifunctional enzymes crucial for metabolisms and detoxification, to evaluate their efficacy as universal vaccine antigen candidates. In the present study, the GSTs of TFMs and NFMs were identified, and they were highly conserved among mite species, including PRMs. The rGSTs of PRMs, TFMs, and NFMs exhibit catalytic activity in the conjugation of GSH to substrates. Immunization with rGSTs induced specific antibodies in chickens, and immune plasma against each rGST showed cross-reactivity with rGSTs from different avian mites. Moreover, the immune plasma against TFMs and NFMs exhibited acaricidal effects on PRMs. These results suggest the utility of GSTs as universal vaccine antigens against avian mites. To further enhance the efficacy, the combined use of multiple antigens, including GSTs, as a cocktail vaccine is required.

CHAPTER III

Feasibility of cysteine proteases from poultry red mites, tropical fowl mites, and northern fowl mites as a universal vaccine antigen with broad efficacy against multiple avian mite species

INTRODUCTION

In Chapters I and II, the utility of HRFs and GSTs, which are highly conserved in various organisms, including avian mites, as universal vaccine antigens, was demonstrated. However, to enhance the efficacy of vaccines, more effective vaccine antigens should be identified, and their application as multivalent vaccines should be considered. As a common feature among hematophagous avian mites, nutrient acquisition depends on the host blood, and blood digestion by proteases produced in the midguts is an essential process for their growth and reproduction. Cathepsin L protease, an enzyme involved in hemoglobin digestion in ticks, has been identified as an intriguing antigen candidate for the development of an anti-tick vaccine [Saidi *et al*., 2016]. In PRMs, the potentials of cysteine proteases (CPs) 1 and 2 [Bartley *et al*., 2015b; Murata *et al*., 2021; Ariizumi *et al*., 2022] and cathepsin D- and L-like proteases [Bartley *et al*., 2012], which are possibly associated with blood digestion, as vaccine antigens have been evaluated. Previous studies have reported the potential usefulness of cysteine protease 1 as a vaccine antigen [Bartley *et al*., 2015b; Murata *et al*., 2021]. Thus, proteases could be vaccine antigen candidates to effectively control PRMs and may be applied for the development of universal vaccines.

CPs are molecules conserved in a variety of organisms, including mammals, vertebrates, and invertebrates. CPs are proteolytic enzymes that play crucial roles in biological processes, including catabolism and protein processing. According to a bioinformatics investigation of the human genome, humans have 11 CPs, which include cathepsins B, C, F, H, K, L, O, S, V, X, and W [Rossi *et al*., 2004]. CPs have been identified in some parasites and are involved in hemoglobin digestion, parasite egress, and surface protein processing in *P. falciparum* [Verma *et al*., 2016]. In addition, cathepsins B, C, and L are known to be associated with hemoglobin digestion in ticks [Sojka *et al*., 2013], and consequently, these molecules are considered potentially effective antigens for the development of anti-tick vaccines [Saidi *et al*., 2016]. Blood feeding is a common feature of PRMs, TFMs, and NFMs and is necessary for the growth of mites. Proteases with similar characteristics are potentially conserved among these avian mite species; therefore, the application of CPs as vaccine antigens may provide prospective antigens for the development of universal vaccines against PRMs, TFMs, and NFMs.

In Chapter III, the potential usefulness of CPs as vaccine antigens for developing universal vaccines against avian mites was assessed. The genetic information of the *CP* gene on PRMs was reported [Bartley *et al*., 2015b, Murata *et al*., 2021]. However, there is still a lack of genetic information on TFMs and NFMs, although the transcriptomes of NFMs were recently reported [Bhowmick *et al*., 2022]. In this chapter, therefore, the *CP* genes were identified from TFMs and NFMs, and the genetic features of *CP*s were

analyzed. Based on the determined sequences, the recombinant proteins of the peptidase domains (PDs) of CPs (rCP-PDs) from PRMs, TFMs, and NFMs were generated, and their enzymatic activities were investigated. In addition, the cross-reactivities of the plasma from chickens immunized with each rCP-PD with the rCP-PDs from different mite-species were investigated. Finally, the mortality of PRMs fed on each immunized plasma was evaluated by artificial feeding to assess the potential whether these CPs can be applied for effective vaccine antigens to broadly control avian mites.

MATERIALS AND METHODS

Ethics statements

1. Genetic recombination experiment

All genetic recombination experiments were performed in accordance with the Manual for Safety Management on Genetic Recombination Experiment in Hokkaido University (Approval number: 2019-024).

2. Animal experiments

Immunizations with rCP-PDs of PRMs, TFMs and NFMs were approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval number 20–0051) in accordance with the guidelines and regulations of the Faculty of Veterinary Medicine, Hokkaido University, which is fully accredited by the AAALAC.

Sample availability, RNA isolation, and cDNA synthesis

The samples that were morphologically and genetically identified as TFMs and NFMs collected in Myanmar were used [Takehara *et al*., 2019]. PRMs were collected from egg-laying farms contaminated with PRMs in Japan and transported to the laboratory as described in Chapter I. Total RNA was isolated from each mite species, and cDNA was synthesized as described in Chapter I.

RACE and molecular cloning of *CP* genes

To determine the ORFs of *CP* genes of TFMs and NFMs, the partial segments of *CP* genes were amplified using nested PCR. The primers used to amplify *CP* genes were designed using the *CP* sequences of *D. gallinae* (HZ459284, KR697573) and *V. destructor* (XM 022808259, XM 022835169) and *M. occidentalis* (XM 018640260). The primers used in this study are listed in Table III-1. The amplified fragments were cloned into a pGEMT easy vector, and nucleotide sequences were analyzed using a Beckman CEQ GeXP automated sequencer. To amplify ORFs of *CP* genes, 3′ and 5′ RACE analysis (Invitrogen) was carried out according to the manufacturer's protocol. The primers for 3′ and 5′ RACE PCR were designed using the partial sequences of *CP* genes amplified from TFMs and NFMs. The 5′ and 3′ RACE PCR products were separated using agarose gel electrophoresis, purified, cloned into the pGEMT-Easy vector, and transformed into competent DH5α *E. coli* cells.

Genetic analysis

ORFs of *CP* genes from PRMs, TFMs, and NFMs were genetically characterized using the BLAST to determine homology with other species. For phylogenetic analysis, nucleotide sequences of *CP* genes of arthropods, including other mites and ticks, chickens,

and other species (Table III-2), were aligned using the MUSCLE (codon) function of the MEGA X program [Kumar *et al*., 2018]. A maximum-likelihood phylogenetic tree was constructed using the same software with 1,000 bootstrap replicates and a discrete gamma distribution (+G) to improve tree topology.

Expression of rCP-PD proteins

N-terminal His-tagged rCP-PD proteins were generated using *E. coli* expression systems, named as rCP-PD PRM, rCP-PD TFM, and rCP-PD NFM, respectively. The PD region from the *CP* sequence (HZ459284) reported from Japan was used to prepare rCP-PD PRM. The coding regions of CP-PDs were amplified using primers containing NdeI and XhoI restriction sites (Table III-1) and were cloned into a pET19b vector and transformed into *E. coli* (DE3, pLysS). Recombinant protein expression and purification were conducted as described in Chapter I. The refolding process was carried out overnight at a temperature of 4°C. The eluted recombinant proteins were subjected to dialysis using a buffer containing 10 mM Tris-HCL (pH 8.5) and 0.1 mM DTT. After dialysis, recombinant proteins were concentrated using a 10 K centrifugal filter unit. The rCP-PD concentration was measured using a Pierce™ Bicinchoninic Acid Protein Assay Kit according to the manufacturer's instructions. The expression and purification of each rCP-PD were confirmed by 13% SDS-PAGE as described in Chapter I.

Generation of immune plasmas via immunization with rCP-PD proteins

To generate immune plasma, chickens were immunized with each rCP-PD. Four chickens per group were subcutaneously immunized with 20 μg of each rCP-PD mixed with Freund's incomplete adjuvant at 3 weeks of age. The second immunization was performed with recombinant proteins mixed with the same adjuvant at 4 weeks after the first immunization. Chickens from the control group were immunized with PBS mixed with the same adjuvant. Three weeks after the second immunization, chickens were euthanized by collecting heparinized whole blood from the hearts of each immunized chicken separately, under deep general anesthesia with Isoflurane inhalation. The immune plasmas were isolated for further experiments. During the whole experimental trial, the health status of chickens was recorded, and there were no instances of mortality, reduction in body weight, or any other noticeable symptoms in all immunized groups.

Western blotting

Western blotting assay was used to validate the generation of specific antibodies against rCP-PD PRM, rCP-PD TFM, and rCP-PD NFM and to examine the cross-reactivity of immune plasma, as described in Chapter I. Briefly, the rCP-PD proteins were separated on 13% polyacrylamide gel and transferred onto PVDF membranes. After the membranes were blocked with 3% skim milk and were incubated with immune plasma

(1:1,000). The membranes were incubated with anti-chicken IgY peroxidase rabbit antibody. Finally, the peroxidase signals were visualized using Immobilon Western Chemiluminescent Horseradish Peroxidase (HRP) Substrate.

ELISA

ELISA was performed to determine the antibody titers in the plasmas of chickens as described in Chapter I. Briefly, the 96-well ELISA plates were coated with each rCP-PD (100 ng/well) using carbon-bicarbonate buffer (pH 9.8) at 4°C overnight. The coated wells were blocked with 1% BSA. The wells were reacted with immune plasmas (×8,000, ×16,000, and ×32,000) and incubated at 25°C for 30 min. Thereafter, anti-chicken IgY[IgG](H+L)-goat HRP was added into each well and incubated at 37°C for 1 h. The reaction was detected by adding TMB One Component HRP Microwell Substrate. After the reaction was stopped by adding 0.18 M H_2SO_4 to each well. The absorbance was measured at 450 nm using a microplate reader.

Enzyme activity assay

The enzymatic activity of rCP-PDs was analyzed using 1, 5, and 10 μg of recombinant proteins, and substrates and CP inhibitors (Cathepsin L Inhibitor) obtained from a commercial SensoLyte Rh110 Cathepsin L Assay kit, following the manufacturer's instructions. The fluorescence excitation and emission values were measured at 490 nm and 520 nm, respectively.

***In vitro* feeding assay**

In vitro feeding assays were conducted as described in Chapter I. Briefly, plasmas from each immunized group and control group were pooled separately, and the plasma from fresh blood was replaced with pooled plasma. Starved PRMs of mixed developmental stages were used for *in vitro* feeding. After blood feeding, only blood-fed PRMs were collected using Pasteur pipettes and kept at 25℃ and 70% humidity. The mortality of blood-fed PRMs in each group was observed daily for one week. The anti-PRM effects were assessed by calculating the survival rates of PRMs.

Statistical analysis

The statistical analysis was conducted using the EZR [Kanda, 2013]. Kaplan–Meier curves were generated and a log-rank test was performed to compare the mortality of immunized and control groups after *in vitro* feeding. In addition, daily mortality between groups was compared using Fisher’s exact test. The ORs and 95% CI were calculated. The statistical significance was set at $p<0.05$.

Table III-1. Primers used for the amplification of *cysteine protease* genes

Primer	Mite species	Intended use	Sequences (5′-3′)
CP outer -F	NFM & TFM	Partial gene amplification	TACRAHGGMGAGATGAARACMTTC
CP outer- R			TCKAGTKMCGTCGARCTGCA
CP inner -F			CTCYAARTAYACSTTCTGGGC
CP inner-R			TCCTTGCAYTTRCCRTCAATGCC
GSP1	NFM & TFM	3′RACE	GAGGGAGATGATTCGCATGT
GSP2			GCAACCTTTCGTACGCTCTC
GSP1	NFM & TFM	5′RACE	TTTCACATCGAAAATA
GSP2			GAGTCGGCATCACAGGGTTC
GSP3			AAATCCATTCGCGATTTCTG
rCP-PD PRM-F	PRM	Construction for expression plasmids	AAGCATATGCCGGACTACGTCGACTGGCG
rCP-PD PRM-R			ATCCTCGAGCTACAGCTCGACGTAGGTTGCCTG
rCP-PD TFM-F	NFM & TFM		AAGCATATGCTTCCGGACTACGTAGACTGGCG
rCP-PD TFM-R			ATCCTCGAGTTAGAGTTCAACATAGGTCGCCTGTGAAG

PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table III-2. List of *cysteine protease* genes used for the phylogenetic analysis in Figure III-2

Accession No.	GenBank	Species
XM 029111664	Digestive cysteine proteinase 2	*Metaseiulus occidentalis*
XM 018640217	Cathepsin S (LOC108864476)	*Metaseiulus occidentalis*
XM 018640260	Digestive cysteine proteinase 2	*Metaseiulus occidentalis*
HZ 459284	Peptidase C1A cysteine proteinase	*Dermanyssus gallinae*
KR 697573	Peptidase C1A cysteine proteinase-1	*Dermanyssus gallinae*
XM 022808259	Digestive cysteine proteinase 2 like	*Varroa destructor*
XM 022835169	Digestive cysteine proteinase 2 like	*Varroa destructor*
NM 001281488	Cathepsin L1-like	*Gallus gallus*
NM 001281489	Cathepsin L1-like	*Gallus gallus*
XR 001470428	Cathepsin L1-like (LOC420160) transcript	*Gallus gallus*
XM 425038	Cathepsin V (CTSV) mRNA	*Gallus gallus*
NM 001168009	Cathepsin V	*Gallus gallus*
NM 204971	Cathepsin K	*Gallus gallus*
XM 015280007	Cathepsin S (CTSS) transcript variant X1	*Gallus gallus*
XM 015280008	Cathepsin S	*Gallus gallus*
EF 428205	Cathepsin L-like cysteine protease mRNA	*Ixodes ricinus*
AB 490783	HlCPL-A mRNA for cysteine protease	*Haemaphysalis longicornis*
AF 227957	Cathepsin L-like proteinase precursor (Cl1) mRNA	*Rhipicephalus microplus*
JX 502826	Voucher Pra-10-1 cathepsin L mRNA complete	*Rhipicephalus microplus*
AB 255051	HlCath mRNA for Longipain complete cds	*Haemaphysalis longicornis*
EU 128750	Cathepsin C precursor mRNA	*Ixodes ricinus*
XM 040688345	Cathepsin Z (CTSZ) mRNA	*Gallus gallus*
XM 417483	Cathepsin Z (CTSZ)	*Gallus gallus*
NM 205177	Cathepsin D (CTSD) mRNA	*Gallus gallus*
XM 002403608	Midgut cysteine proteinase	*Ixodes scapularis*
XM 029972398	Digestive cysteine proteinase 2	*Ixodes scapularis*
XM 037727070	Digestive cysteine proteinase	*Dermacentor silvarum*
XM 037646015	Digestive cysteine proteinase	*Rhipicephalus sanguineus*
XM 040651895	Digestive cysteine proteinase 1-like	Gallus gallus
XM 040690025	Digestive cysteine proteinase 2-like	*Gallus gallus*
XM 014811466	Digestive cysteine proteinase 1-like	*Priapulus caudatus*
XM 017622247	Digestive cysteine proteinase 1	*Rhagoletis zephyria*
XM 041502300	Digestive cysteine proteinase 2-like	*Gigantopelta aegis*

Table III-2. List of *cysteine protease* genes used for the phylogenetic analysis in Figure III-2 (continued)

Accession no.	GenBank	Species
XM 016073987	Digestive cysteine proteinase	*Parasteatoda tepidariorum*
XM 003494096	Digestive cysteine proteinase 1	*Bombus impatiens*
XM 027344408	Digestive cysteine	*Dermatophagoides pteronyssinus*
XM 015932208	Digestive cysteine proteinase 2-like	*Tetranychus urticae*
XM 015930175	Digestive cysteine proteinase 2	*Tetranychus urticae*

RESULTS

Identification and genetic characterization of *CP* genes of TFMs and NFMs

The ORFs of the *CP* genes from TFMs and NFMs, which were obtained from poultry farms in Myanmar [Takehara *et al*., 2019], were identified. The nucleotide sequences of ORFs of TFMs and NFMs were 100% identical, and the nucleotide and deduced amino acid sequences showed high similarities to those of PRMs (Table III-3). The deduced amino acid sequences of CPs of TFMs and NFMs consist of a signal peptide at 1–16 positions, an inhibitor domain at 246–302 positions, and a peptidase C1 domain at 331–544 positions. Among avian mites, the peptidase C1 domains, which are required to exhibit enzymatic activities of CPs, were highly conserved, and these domains of TFMs and NFMs showed 89.5% similarity to that of PRMs. Although differences in the CP amino acid sequences between TFMs/NFMs and PRMs were observed, four putative active sites were conserved (Figure III-1). Therefore, proteins from TFMs and NFMs were assumed to possess enzymatic activity of CPs, similar to that of PRMs, which act by the cleavage of the inhibitor domain.

To examine the genetic features of the *CP* genes of TFMs and NFMs, a phylogenetic analysis was conducted by comparing with those of PRMs, arthropods, chickens, and other species (Figure III-2). The *CP* genes of TFMs and NFMs were classified into cluster 1 with those from PRMs and other mite species including *M. occidentalis* and *Varroa* mites. Cluster 2 included cathepsin L-like *CP* genes, which are associated with hemoglobin digestion in some ticks, and other *CP* genes, such as cathepsins D, K, L, S, V, and Z from chickens. Clusters 1 and 2 originated from the same rooted clade. Aside from those in clusters 1 and 2, other digestive *CP* genes in ticks and insects belonged to cluster 3. Thus, *CP* genes of mites, including those of TFMs and NFMs, appear to be involved in digestive activity in various species.

Enzymatic activities of rCP-PDs of PRMs, TFMs, and NFMs

The PDs of CPs from PRMs, TFMs, and NFMs were generated as recombinant proteins fused with a His-tag at the N terminus, termed rCP-PD PRM, rCP-PD TFM, and rCP-PD NFM, respectively. Although the sequences of CP-PDs from TFMs and NFMs were fully identical, the recombinant proteins were generated separately from TFMs and NFMs to ensure the reproducibility of the following experiments. All rCP-PDs were purified from insoluble fractions through affinity chromatography. Protein purity was confirmed by SDS-PAGE and western blotting (Figures III-3a,b). The cathepsin-L-like enzyme activity for rCP-PDs of TFMs and NFMs was examined using a commercial fluorescent substrate (Figure III-4), similar to that in PRMs [Murata *et al*., 2021]. The rCP-PDs of PRMs, TFMs, and NFMs exhibited dose-dependent cathepsin L-like enzyme activity, whereas enzymatic activities were inhibited by the addition of a cathepsin L

inhibitor. These data suggest that rCP-PDs have cathepsin L-like CP activities similar to that of the CP of PRMs.

Cross-reactivities of immune plasmas with each rCP-PD

In order to assess the potential of CPs as common antigens against avian mites, plasmas were obtained from chickens that were vaccinated with each rCP-PD. The elevated antibody titers in the plasmas of chickens from each immunized group were confirmed by ELISA (Table III-4). In addition, the production of specific antibodies in the plasma of chickens against each rCP-PD was confirmed by western blot analysis (Figure III-5). Moreover, plasmas from chickens immunized with rCP-PD PRM, rCP-PD TFM, or rCP-PD NFM recognized rCP-PDs from different mite species (Figure III-5), indicating that CPs are potentially useful as antigens for universal vaccine across avian mites.

Evaluation of the acaricidal effects of plasma of chickens immunized with rCP-PDs

To assess the acaricidal effects of immunization with rCP-PDs, the mortality rates of PRMs fed on the plasmas derived from each immunized group were compared with those of the control group. In experiment 1, the mortality rates of PRMs fed plasma against each rCP-PD-immunized group were increased throughout the experimental period (Table III-5). The survival rates of PRMs in the immunized groups versus control group were significantly different; specifically, on 5 days post-feeding in the rCP-PD PRM-immunized group, on 5 and 7 days post-feeding in the rCP-PD TFM-immunized group, and on 2–5 and 7 days post-feeding in the rCP-PD NFM-immunized group. Kaplan–Meier curves showed that the survival rates of PRMs fed with immune plasma against each rCP-PD were significantly lower than that of PRMs fed with plasma from the control group (Figure III-6-a). Similarly, the second experiment revealed that the survival rates of PRMs fed with plasmas from all immunized groups were significantly decreased than that of the control group (Figure III-6-b). In addition, Fisher's exact tests and odds ratio analyses showed that the mortality rates of PRMs fed with immune plasma were significantly increased. A significant increase in mortality was observed on days 6 and 7 in the rCP-PD PRM group, days 2 and 5–7 in the rCP-PD TFM group, and days 1, 2, and 7 in the rCP-PD TFM group (Table III-6). Collectively, these findings suggest that immunization against all rCP-PDs induces acaricidal effects on PRMs. Thus, CPs are potentially ideal antigens for universal vaccine development targeting a wide range of avian mites.

Table III-3. Sequence homology of cysteine proteases from poultry red mites (PRMs), northern fowl mites (NFMs) and tropical fowl mites (TFMs)

		Amino acid sequence (%)		
		PRM	TFM	NFM
Nucleotide sequence similarity (%)	PRM	-	74.0	74.0
	TFM	73.0	-	100
	NFM	73.0	100	-

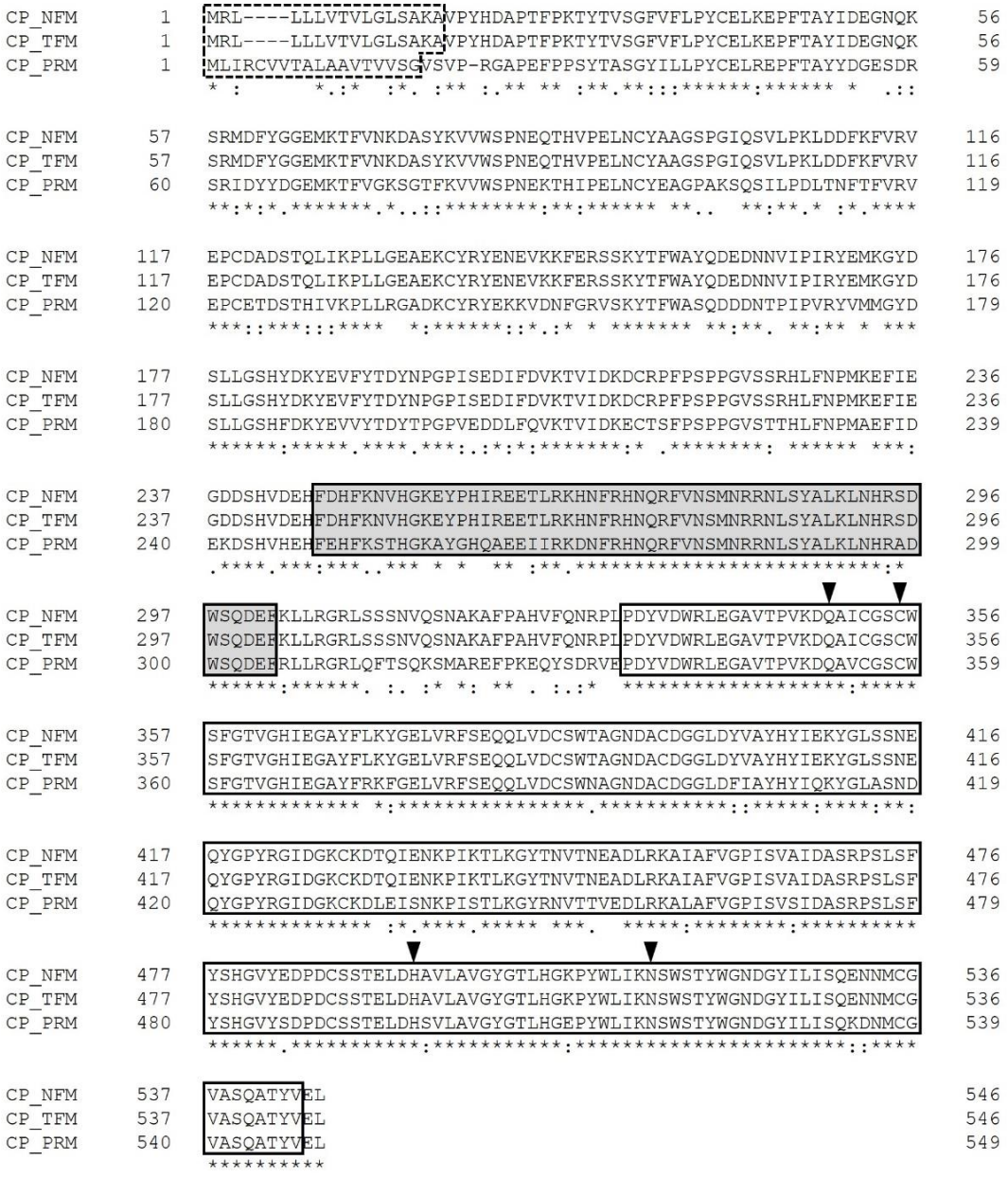

Figure III-1. Multiple alignments of deduced amino acid sequences of CPs from PRMs, TFMs, and NFMs.

The deduced amino acid sequences of CPs include signal peptides (dashed white box) at positions of 1–18 in PRMs and 1–16 in TFMs and NFMs, peptidase inhibitor domains (grey box) at 249–305 in PRMs and 246–302 in TFMs and NFMs and peptidase domains (white box) at 335–547 in PRMs and 331–544 in TFMs and NFMs. The black arrowhead indicates predicted active sites for peptidase activities.

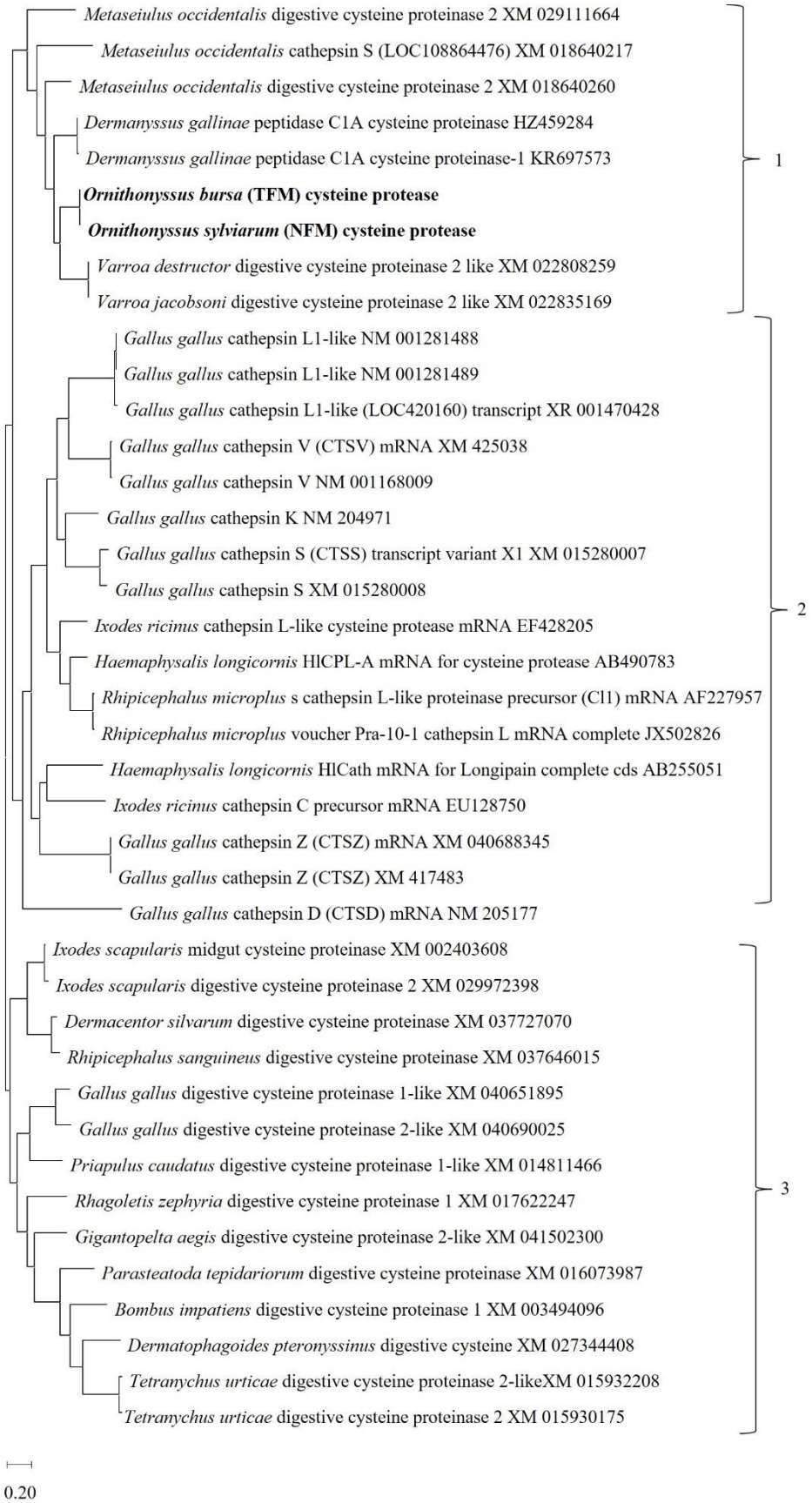
Metaseiulus occidentalis digestive cysteine proteinase 2 XM 029111664
Metaseiulus occidentalis cathepsin S (LOC108864476) XM 018640217
Metaseiulus occidentalis digestive cysteine proteinase 2 XM 018640260
Dermanyssus gallinae peptidase C1A cysteine proteinase HZ459284
Dermanyssus gallinae peptidase C1A cysteine proteinase-1 KR697573
***Ornithonyssus bursa* (TFM) cysteine protease**
***Ornithonyssus sylviarum* (NFM) cysteine protease**
Varroa destructor digestive cysteine proteinase 2 like XM 022808259
Varroa jacobsoni digestive cysteine proteinase 2 like XM 022835169
1
Gallus gallus cathepsin L1-like NM 001281488
Gallus gallus cathepsin L1-like NM 001281489
Gallus gallus cathepsin L1-like (LOC420160) transcript XR 001470428
Gallus gallus cathepsin V (CTSV) mRNA XM 425038
Gallus gallus cathepsin V NM 001168009
Gallus gallus cathepsin K NM 204971
Gallus gallus cathepsin S (CTSS) transcript variant X1 XM 015280007
Gallus gallus cathepsin S XM 015280008
Ixodes ricinus cathepsin L-like cysteine protease mRNA EF428205
Haemaphysalis longicornis HlCPL-A mRNA for cysteine protease AB490783
Rhipicephalus microplus s cathepsin L-like proteinase precursor (Cl1) mRNA AF227957
Rhipicephalus microplus voucher Pra-10-1 cathepsin L mRNA complete JX502826
Haemaphysalis longicornis HlCath mRNA for Longipain complete cds AB255051
Ixodes ricinus cathepsin C precursor mRNA EU128750
Gallus gallus cathepsin Z (CTSZ) mRNA XM 040688345
Gallus gallus cathepsin Z (CTSZ) XM 417483
2
Gallus gallus cathepsin D (CTSD) mRNA NM 205177
Ixodes scapularis midgut cysteine proteinase XM 002403608
Ixodes scapularis digestive cysteine proteinase 2 XM 029972398
Dermacentor silvarum digestive cysteine proteinase XM 037727070
Rhipicephalus sanguineus digestive cysteine proteinase XM 037646015
Gallus gallus digestive cysteine proteinase 1-like XM 040651895
Gallus gallus digestive cysteine proteinase 2-like XM 040690025
Priapulus caudatus digestive cysteine proteinase 1-like XM 014811466
Rhagoletis zephyria digestive cysteine proteinase 1 XM 017622247
Gigantopelta aegis digestive cysteine proteinase 2-like XM 041502300
Parasteatoda tepidariorum digestive cysteine proteinase XM 016073987
Bombus impatiens digestive cysteine proteinase 1 XM 003494096
Dermatophagoides pteronyssinus digestive cysteine XM 027344408
Tetranychus urticae digestive cysteine proteinase 2-likeXM 015932208
Tetranychus urticae digestive cysteine proteinase 2 XM 015930175
3
0.20

Figure III-2. Phylogenetic analysis of *CP* genes.

The *CP* genes of PRMs, TFMs, NFMs, other arthropods (including other mites and ticks), chickens, and other species such as insects and invertebrates were aligned. The phylogenetic tree was constructed using the maximum-likelihood method with MEGA X software. The numbers on the right indicate clusters. Cluster 1 contains digestive *CP* genes from TFMs and NFMs (bold) along with those of PRMs and other mites. Cluster 2 consists of *CP* genes and *cathepsin D*, *L*, *K*, *S*, *V,* and *Z* genes of chickens and *CP* genes of some ticks. Cluster 3 contains *CP* genes of mites other than those of Cluster 1.

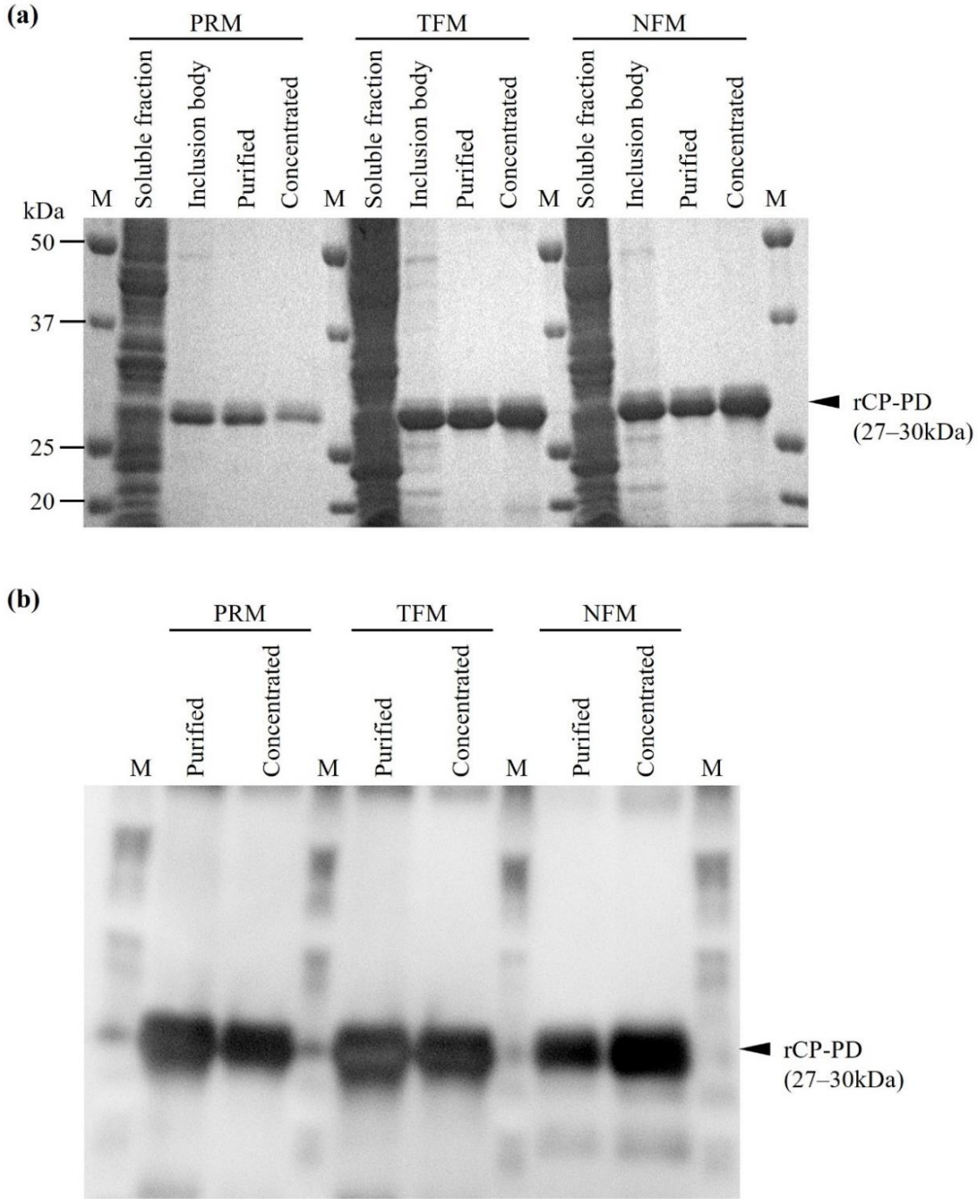

Figure III-3. Expression and purification of rCP-PD proteins of avian mites.
The PD regions of CPs from PRMs, TFMs, and NFMs were prepared as recombinant proteins and termed rCP-PD PRM, rCP-PD TFM, and rCP-PD NFM, respectively. rCP-PDs were expressed using an *E. coli* expression system, and the expressions were confirmed by (a) SDS-PAGE and (b) western blotting. M, Marker.

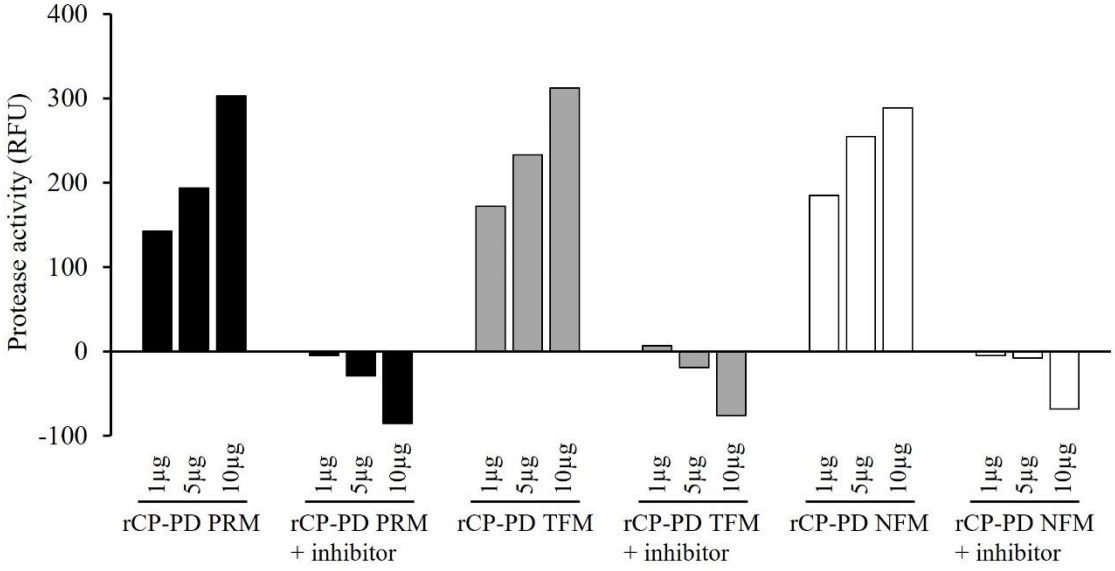

Figure III-4. Enzyme activity of rCP-PDs.

The enzyme activity of rCP-PDs from PRMs, TFMs, and NFMs was assessed using a commercial SensoLyte Rh110 Cathepsin L Assay Kit. In addition, enzymatic activities were assessed in the presence of a cysteine protease inhibitor. The x-axis indicates the rCP-PD quantities used in assays. Protease activity is indicated using relative fluorescence units (RFU).

Table III-4. Antibody titers of plasmas from chickens immunized with recombinant cysteine protease peptidase domains (rCP-PDs)

Group	Chicken	Antibody titer
Control	C1	1:2,000
	C2	1:2,000
	C3	1:2,000
	C4	1:2,000
Immunized against rCP-PD PRM	PRM1	1: 32,000
	PRM2	1: 32,000
	PRM3	1: 32,000
	PRM4	1:16,000
Immunized against rCP-PD TFM	TFM1	1:16,000
	TFM2	1: 32,000
	TFM3	1:16,000
	TFM4	1: 32,000
Immunized against rCP-PD NFM	NFM1	1: 32,000
	NFM2	1:16,000
	NFM3	1: 16,000
	NFM4	1: 32,000

PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

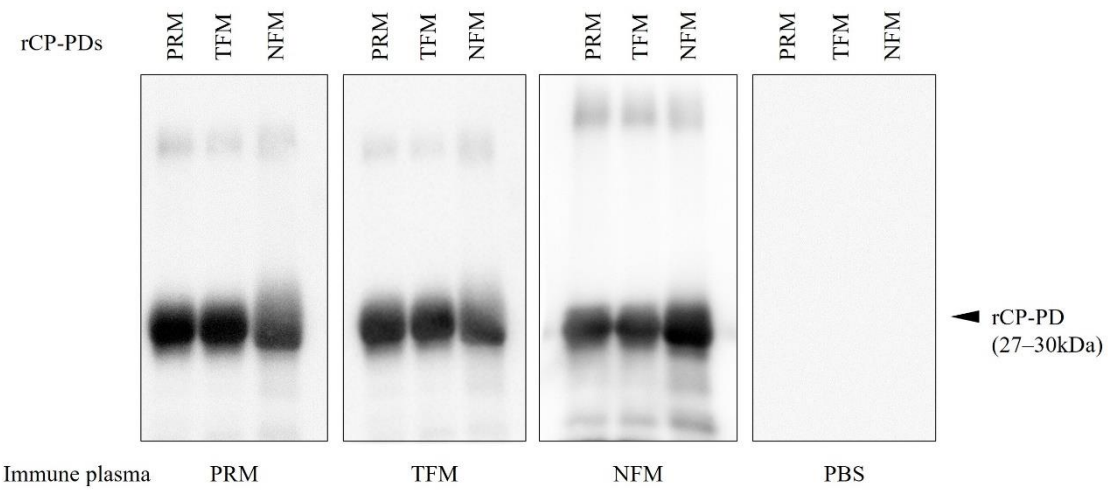

Figure III-5. Production of antibodies specific to each rCP-PD in the plasma of chickens via immunization with rCP-PDs.

Four chickens per group were immunized with rCP-PDs. Plasma was then isolated from each immunized chicken. The presence of antibodies specific to rCP-PDs in the plasmas of chickens was detected by western blotting. The cross-reactivities of the immune plasmas of each immunized group against rCP-PDs were detected. The arrowhead indicates the predicted molecular weight of rCP-PDs (27–30 kDa).

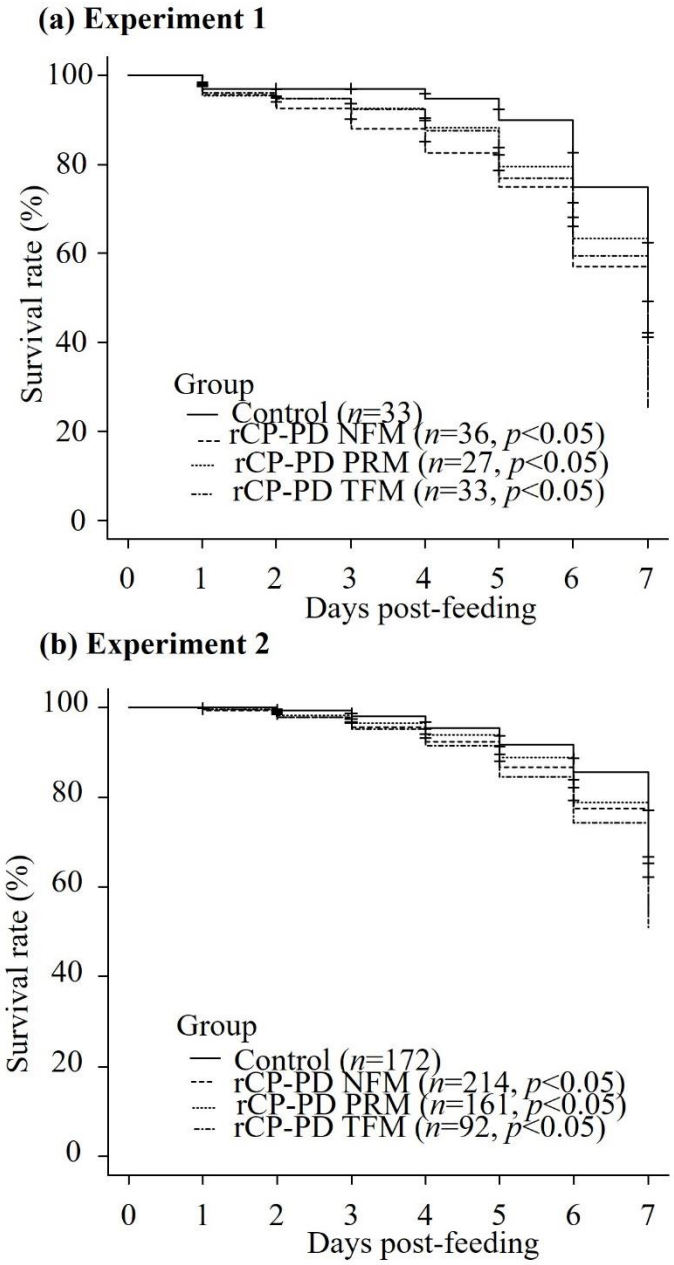

Figure III-6. Acaricidal effects of plasmas from chickens immunized with rCP-PDs. PRMs were fed with immune plasmas against rCP-PDs using *in vitro* feeding assays. The survival rates of PRMs from each immunized and control group were recorded daily for one week. The *in vitro* feeding assays were conducted twice. The total numbers of PRMs used in this study are as follows: (a) Experiment 1: fed immune plasma, *n*=27 (rCP-PD PRM), *n*=33 (rCP-PD TFM), *n*=36 (rCP-PD NFM), fed control plasma: *n*=33; (b) Experiment 2: fed immune plasma: *n*=161 (rCP-PD PRM), *n*=92 (rCP-PD TFM), *n*=214 (rCP-PD NFM), fed control plasma: *n*=172. Kaplan–Meier survival curves were generated, and statistical analysis was performed by the log-rank test was performed. $p<0.05$ indicates statistically significant.

Table III-5. Mortality of PRMs fed with plasmas chickens immunized with rCP-PDs from different mite species (Experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=33)							
No. of dead PRMs post-feeding	7	7	7	9	11	18	18
Mortality (%)	21.21	21.21	21.21	27.27	33.33	54.55	54.55
Immunized group (rCP-PD PRM) (*n*=27)							
No. of dead PRMs post-feeding	9	10	12	14	17	20	21
Mortality (%)	26.09	30.43	39.13	47.83	56.52	69.67	73.91
p value (Fisher's exact)	0.382	0.251	0.093	0.065	0.036*	0.178	0.102
Odds ratio	1.83	2.15	2.91	2.81	3.32	2.34	2.86
95% confidence interval	0.50–7.00	0.60–8.12	0.84–10.85	0.86–9.69	1.03–11.31	0.70–8.46	0.83–11.02
Immunized group (rCP-PD TFM) (*n*=33)							
No. of dead PRMs post-feeding	9	12	13	16	20	24	28
Mortality (%)	17.86	28.57	32.14	42.86	57.14	67.86	82.14
p value (Fisher's exact)	0.775	0.277	0.18	0.127	0.048*	0.20	0.015*
Odds ratio	1.39	2.09	2.38	2.47	3.02	2.19	4.55
95% confidence interval	0.39–5.14	0.63–7.51	0.72–8.47	0.81–8.02	1.01–9.51	0.71–7.12	1.29–18.92
Immunized group (rCP-PD NFM) (*n*=36)							
No. of dead PRMs post-feeding	11	18	20	20	21	28	31
Mortality (%)	29.03	45.16	51.61	51.61	54.84	77.42	83.87
p value (Fisher's exact)	0.422	0.023*	0.006*	0.028*	0.053*	0.072	0.007*
Odds ratio	1.62	3.64	4.53	3.27	2.76	2.87	5.04
95% confidence interval	0.48–5.79	1.16–12.61	1.44–15.77	1.09–10.44	0.95–8.41	0.92–9.57	1.44–20.82

*Statistically significant based on a significance level set at $p<0.05$

rCP-PD, recombinant peptidase domains of cysteine protease; PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table III-6. Mortality of PRMs fed with plasmas of chickens immunized with rCP-PDs from different mite species (Experiment 2)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=172)							
No. of dead PRMs post-feeding	2	6	11	17	20	23	34
Mortality (%)	1.16	3.48	6.39	9.88	11.63	13.37	19.77
Immunized group (rCP-PD PRM, *n*=161)							
No. of dead PRMs post-feeding	7	11	14	18	26	36	50
Mortality (%)	4.35	6.83	8.69	11.18	16.15	22.36	31.06
p value (Fisher's exact)	0.095	0.214	0.533	0.724	0.267	0.043*	0.022*
Odds ratio	3.84	2.02	1.39	1.14	1.46	1.86	1.82
95% confidence interval	0.71–38.53	0.66–6.83	0.56–3.50	0.53–2.47	0.74–2.89	1.01–3.47	1.07–3.12
Immunized group (rCP-PD TFM, *n*=92)							
No. of dead PRMs post-feeding	4	9	12	15	21	22	30
Mortality (%)	4.35	9.78	13.04	16.30	22.83	23.91	32.61
p value (Fisher's exact)	0.187	0.049*	0.107	0.165	0.020*	0.038*	0.024*
Odds ratio	3.84	2.98	2.18	1.77	2.24	2.03	1.95
95% confidence interval	0.53–43.25	0.91–10.56	0.84–5.74	0.77–4.00	1.07–4.66	1.00–4.10	1.05–3.62
Immunized group (rCP-PD NFM, *n*=214)							
No. of dead PRMs post-feeding	12	20	23	29	39	45	68
Mortality (%)	5.61	9.35	10.75	13.55	18.22	21.03	31.78
p value (Fisher's exact)	0.026*	0.024*	0.151	0.343	0.087	0.059	0.010*
Odds ratio	5.03	0.84	1.75	1.42	1.69	1.72	1.88
95% confidence interval	1.09–46.91	1.06–8.86	0.79–4.12	0.72–2.88	0.91–3.20	0.96–3.13	1.15–3.13

*Statistically significant based on a significance level set at $p<0.05$

rCP-PD, recombinant peptidase domains of cysteine protease; PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

DISCUSSION

Genetic analysis showed that the *CP* genes from TFMs and NFMs are included in a cluster of genes encoding digestive CPs of mite species, including the CP of PRMs; these genes are obviously different from *CP* genes of other species. The cathepsin L subfamily, consisting of cathepsins L, V, K, S, F, and H, is characterized by the presence of a CP peptidase inhibitor-I29 domain (prodomain) at the N-terminus and a peptidase C1A superfamily domain (mature) at the C-terminus [Verma *et al*., 2016]. These domains were also predicted to be included in the CPs of PRMs, TFMs, and NFMs. In addition, the PDs were highly conserved among avian mites. According to the transcriptome analysis of PRMs, approximately 62% of the reads encoding proteases were attributed to *CP* genes, and among them, 80% were cathepsin L-like proteases in the midgut [Ribeiro *et al*., 2023]. Furthermore, the expression of *CP* mRNA (Deg-CPR-1) was detected in the midgut by a laser capture microdissection and RT-PCR [Murata *et al*., 2021]. Taken together, these findings suggest that the CP of PRMs (Deg-CPR-1) is a digestive enzyme involved in blood digestion. In this study, the *CP* gene expression in the TFMs and NFMs was not analyzed because of the limited availability of TFMs and NFMs. However, similar to the rCP-PD of PRMs, the rCP-PDs of TFMs and NFMs exhibited enzymatic activity. In addition, the plasma from chickens immunized with rCP-PD TFM and rCP-PD NFM showed acaricidal activities on PRMs, similar to that of immune plasma against rCP-PD PRM. Therefore, the CPs identified in TFMs and NFMs in this study may operate similar functions to those of PRMs, although further research on expression patterns and functions of CPs in TFMs and NFMs is required.

In this study, the immune plasma against rCP-PDs of each of the three mite species exhibited cross-reactivity with rCP-PDs from the other two species, suggesting that the CPs of hematophagous avian mites could be suitable antigens for developing a vaccine that provides broad protective efficacy against multiple mite species. To determine the efficacy of CPs as vaccine antigens, the acaricidal effects of rCP-PDs via *in vitro* feeding assays using PRMs were analyzed. A remarkable decrease in the survival rate of PRMs fed with immune plasma was observed, even though the plasma was derived from chickens immunized with rCP-PDs of different mites. Cathepsin L proteases have been shown to be involved in the digestion of hemoglobin in blood-feeding arthropods, including some tick species such as *R. microplus*, *H. longicornis*, and *I. ricinus* [Sojka *et al*., 2013]. Thus, these proteases are considered potential antigens for the development of vaccines against multiple tick species [Saidi *et al*. 2016]. However, the present study has some limitations regarding TFM and NFM availability in Japan, and therefore, *in vitro* feeding assays assessed only PRMs but not TFMs or NFMs survival. To clearly indicate the potential of CPs as antigens with broad-protective efficacy across hematophagous avian mites, it is required to assess the efficacy as vaccine antigens in TFMs and NFMs.

In Chapter III, the usefulness of CPs from avian mites for universal vaccine development was assessed. *CP* genes identified in TFMs and NFMs were highly similar to that of PRMs, and rCP-PDs from PRMs, TFMs, and NFMs showed enzymatic activities. In addition, the immune plasma from chickens immunized with each rCP-PD cross-reacted with rCP-PDs from different mites. Importantly, immune plasmas against rCP-PDs of TFMs and NFMs exerted acaricidal effects on PRMs. Taken together, these findings will help in the development of an effective universal vaccine for future practical use.

SUMMARY

Infestation with avian mites such as PRMs, TFMs, and NFMs causes significant economic losses to the poultry industry, particularly egg-laying farms. Vaccine approaches have been focused on as a novel strategy for the control of PRMs, and several vaccine candidates against PRMs have been discovered. On the other hand, vaccines against TFMs and NFMs have not been studied to date, although they cause similar problems in poultry farms. Therefore, the ideal antigens for anti-PRM vaccines are molecules that can cross-react with those of TFMs and NFMs and exert acaricidal effects on TFMs and NFMs, similar to those against PRMs.

CPs are proteolytic enzymes that play crucial roles in hemoglobin digestion in blood-feeding arthropods. Since blood-sucking is a characteristic shared among PRMs, TFMs, and NFMs, CPs may be applicable as vaccine antigens with broad-spectrum efficacy against avian mites. In Chapter III, therefore, *CP* genes were identified from TFMs and NFMs, and the potential utility of CPs as common antigens for the development of universal vaccines against multiple avian mites was evaluated. The *CP* genes of TFMs and NFMs were highly conserved with that of PRMs, and they belonged to the cluster that includes digestive CPs, similar to that of PRMs. In addition, the recombinant proteins of the peptidase domain of each CP exhibited cathepsin-L-like enzymatic activities. To investigate the potential of CPs as common antigens against avian mites, immune plasmas against each rCP-PD were generated. Immune plasmas from chickens immunized with rCP-PDs of PRMs, TFMs, and NFMs cross-reacted with rCP-PDs from different mite species. Furthermore, *in vitro* feeding assays revealed that immune plasmas against rCP-PDs reduced the survival rate of PRMs, even when the plasma from chickens immunized with rCP-PDs from TFMs and NFMs was utilized. Therefore, CP could be an effective antigen for developing a vaccine with broad-spectrum efficacy across avian mite species.

CHAPTER IV

Evaluation of ferritin 2 as an antigen for the development of a universal vaccine against poultry red mites, tropical fowl mites, and northern fowl mites

INTRODUCTION

Iron is a vital nutrient for blood-sucking parasites. However, excessive intake of iron could be harmful to blood-feeding ectoparasites. Therefore, blood-feeding ectoparasites possess fundamental mechanisms for controlling iron homeostasis that mainly rely on ferritin (FER), which is an iron-binding protein conserved among various organisms, for iron homeostasis [Arosio *et al*., 2009]. Two types of FER, FER1 and FER2, were identified in ticks, similar to the other blood-sucking arthropods. Both FERs were essential for blood-feeding, reproduction, iron transport, and antioxidant activities in ticks and were utilized as anti-tick vaccine antigen candidates [Hajdusek *et al*., 2009, 2010; Galay *et al*., 2013]. FER1 is an intracellular ferritin that serves as an antioxidant by preserving excess intracellular iron [Hajdusek et al., 2009], whereas FER2 is a secretory molecule that transports iron to peripheral tissues [Hajdusek *et al*., 2009]. Moreover, gene silencing of *FER2* in *I. ricinus* is related to losses of *FER1* gene expression in the salivary glands and ovaries and dramatically impaired blood-feeding ability and hatching rate, indicating that FER2 is critical for iron homeostasis [Hajdusek *et al*., 2009]. Thus, among FERs, FER2 was suggested as a promising antigen for an anti-tick vaccine [Hajdusek *et al*., 2009, 2010]. FER1 and FER2 have been tested for anti-tick vaccine efficacy, and immunization of rabbits with recombinant FER2 (rFER2) of *H. longicornis* reduced the engorged weight and reproductive capacity of ticks ingested the anti-FER2 antibodies [Galay *et al*., 2013]. Thus, FER2 of several tick species has been focused for anti-tick vaccine development [Hajdusek *et al*., 2010; Galay *et al*., 2013; Knorr *et al*., 2018; Githaka *et al*., 2020]. Two FERs were identified in PRMs, and RNAi assays for *FER1* and *FER2* revealed that both FERs have negative impacts on the survival, reproduction, and physiology of PRMs [Xu *et al*., 2021]. Furthermore, both FERs showed the acaricidal potential as vaccine antigens against PRMs, and importantly, the survival rate of PRMs fed with immune plasma from chickens vaccinated with rFER2 (rDg-FER-1 in the original research) was lower than that of the rFER1-immunized group (rDgFER-2 in the original study) [Xu *et al*., 2021]. However, FER2 from TFMs and NFMs have not been identified and characterized. In Chapters III, the usefulness of CPs, which are considered molecules critical for blood digestion, as vaccine antigens against multiple avian mites was assessed using an *in vitro* evaluation system. The blood meal obtained from the host is a nutrient source for blood-feeding arthropods, including avian mites, and therefore, absorption of essential nutrients could be desirable candidates for vaccine antigens, which have a wide protective range among hematophagous avian mites. In recent years, FER2 has been considered as a universal vaccine candidate for the protection of cross-tick species [Xavier *et al*., 2021; Zeb *et al*., 2024]. *In vitro* blood feeding of anti-*I. persulcatus*-FER2 antibodies to *R. micropus* led to a decrease in engorged weight [Xaiver *et al*., 2021]. Moreover, vaccination with rFER2 from *Hyalomma anatolicum* showed cross-

immunoprotection against *R. sanguineus* infestation, indicating 95.9% vaccine efficacy [Zeb *et al*., 2024]. Thus, FER2 is an intriguing target for investigating its potential as a common antigen to provide broad-spectrum efficacies across avian mites.

In Chapter IV, the feasibility of FER2 as a common antigen to develop a universal vaccine against avian mites was evaluated. To assess the potential of FEF2 as a common antigen, *FER2* genes were identified from TFMs and NFMs. The rFER2 of each mite was generated, and iron-binding activities were analyzed. The plasmas were isolated from chickens immunized with each rFER2, and the cross-reactivities of immune plasmas with the rFER2 of different mites were analyzed. In addition, the acaricidal effects of immune plasmas against rFER2 of TFMs and NFMs on PRMs were assessed.

MATERIALS AND METHODS

Ethics statements

1. Genetic recombination experiment

All genetic recombination experiments were performed in accordance with the Manual for Safety Management on Genetic Recombination Experiment in Hokkaido University (Approval number: 2019-024).

2. Animal experiments

All experimental chickens were housed at the animal facility of the Faculty of Veterinary Medicine, Hokkaido University. The animal experiment was conducted in full accordance with the rules and regulations of the Faculty of Veterinary Medicine, Hokkaido University, which is fully accredited by AAALAC and approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval number: 20–0051).

Sample availability, RNA isolation, and cDNA synthesis

Blood-fed PRMs were collected from PRMs-contaminated egg-laying farms in Japan, as described in Chapter I. Starved PRMs were prepared by keeping at 25°C and 70% humidity for a week refraining from blood consumption, then stored at 5°C and 70% humidity for further use. The samples of TFMs and NFMs, as described in Chapter I, were used for the analysis. The isolation of the total RNA of each mite and cDNA synthesis were performed as described in Chapter I.

Amplification of ORF sequences of *FER2* of TFMs and NFMs

To amplify ORF of *FER2* genes from TFMs and NFMs, the RACE system (Invitrogen) was performed. The partial segments of *FER2* genes from TFMs and NFMs were amplified using the primers established on the conserved region of *FER2* from *D. gallinae* (HZ459285) and *V. destructor* (XM022808086). The primers utilized throughout this experiment are provided in Table IV-1. The amplified DNA was cloned into a pMD20 vector. The nucleotide sequences were analyzed using the CEQ GeXP automated sequencer. Based on the partial sequences of *FER2* genes, primers for 3′ and 5′ RACE were designed, and the ORF sequences of *FER2* genes were determined using the RACE system as described in Chapter I.

Genetic characterization of *FER2* of TFMs and NFMs

The sequence homologies of *FER2* genes of TFMs and NFMs were compared with those of PRMs and other organisms, which were reported in the NCBI gene bank using the BLAST program. To analyze the genetic relationship of *FER2* genes of TFMs

and NFMs with other *FER* genes, including *FER1* and *FER2* genes from mites, ticks, and chickens (Table IV-2), the phylogenetic tree was constructed. The sequences were aligned using the MUSCLE (codon) of MEGA X software [Kumar *et al*., 2018]. A maximum-likelihood phylogenetic tree was generated using the same software with 1,000 bootstrap repeats and a discrete gamma distribution (+G) to improve the tree topology.

Production of recombinant proteins

The coding regions of *FER2* genes were amplified using primers containing NdeI and XhoI sites (Table IV-1) with Taq polymerase to introduce into the pET19b vector. The amplified fragments were purified and inserted into the pET19b vector and transformed into *E. coli* strain *Rosetta-gami* B (DE3, pLysS). N terminal His-tagged rFER2 proteins of PRMs, TFMs, and NFMs were generated, termed as rFER2 PRM, rFER2 TFM, and rFER2 NFM, respectively. The sequence of the *FER2* gene (HZ459284), which was previously identified in Japan, was used for the generation of rFER2 PRM. The expression and purification of recombinant proteins were carried out as described in Chapter I. The rFER2 proteins were eluted with 0.3% N-lauroylsarcosine, 50 mM CAPS (pH 11.0) buffer containing 250 mM imidazole. The purified rFER2 proteins were dialyzed against a 10 mM Tris-HCL (pH 8.5) buffer containing 0.1 mM DDT at 4°C overnight. The expression and purity of the recombinant proteins were determined using 13% SDS-PAGE and Coomassie brilliant blue staining. The Pierce™ BCA Protein Assay Kit was used to determine the concentration of recombinant proteins, according to the manufacturer's instructions.

Iron binding assay

The capacity of each rFER2 protein for iron-binding was assessed using a ferrozine-based iron-binding assay [Galay *et al*., 2013]. Different concentrations of rFER2 (2.5, 5, and 10 μg/mL) were suspended in 954 μL of double distilled water (DDW) and mixed with 20 μL of 1 M HEPES (pH 7.0) and 1 μL of 40 mM $Fe_2(NH_4)_2(SO_4)_2$. After the mixture was incubated at 30°C for 30 min., 20 μL of 10 mM ferrozine (Sigma-Aldrich) was added and incubated for 30 min. Each mixture (300 μL) was added to 3 wells of a microplate. The absorbance value was determined at 570 nm using a microplate reader. The apoferritin from the equine spleen (Sigma-Aldrich) and BSA were used as the positive control and negative control, respectively. All data were presented as means, and error bars indicate standard deviations.

Generation of immune plasma against each rFER2

To produce immune plasma, four chickens were subcutaneously immunized with 20 μg of each rFER2 mixed with the Freund incomplete adjuvant at 3 weeks of age. Four weeks after the initial immunization, the chickens were boosted with 20 μg of each rFER2

with the same adjuvant. The chickens in the control group were injected with PBS mixed with the same adjuvant. Three weeks after the second immunization, the heparinized blood was collected from chickens in each group. The immune plasma from each chicken was isolated. During the experimental trial, the health status of the chickens was monitored. No instances of mortality, reduction in body weight, or any other noticeable symptoms were observed in any of the immunized groups.

Western blotting

Western blotting was conducted to analyze the production of antibodies specific to each rFER2 and the cross-reactivity of each immune plasma with rFER2 of different mites as described in Chapter I. Briefly, the rFER2 was separated on 13% polyacrylamide gel and transferred onto PVDF membranes. The membrane was blocked with 3% skim milk PBS-T and reacted with immune plasma (1:1,000) at 25℃ for 1 h. After the membranes were reacted with an anti-chicken IgY peroxidase rabbit antibody (1:10,000), the signal was visualized using the Immobilon Western Chemiluminescent HRP Substrate.

ELISA

The antibody titers in the plasma of immunized chickens were analyzed by ELISA as described in Chapter I. Briefly, each rFER2 protein (100 ng/well) was coated onto an ELISA plate using a carbon-bicarbonate buffer (pH 9.8) at 4°C overnight. After blocking with PBST-containing 1% BSA, each well was incubated with immune plasma (×2,000, ×4,000, ×8,000, ×16,000, ×32,000) at 25°C for 30 min. The wells were incubated with anti-chicken IgY[IgG](H+L)-goat HRP and incubated at 37°C for 1 h. The reaction was detected by adding TMB One Component HRP Microwell Substrate. After stopping the reaction with 0.18 M H_2SO_4, the absorbance was measured at 450 nm using a microplate reader.

***In vitro* feeding assay**

To assess the acaricidal effects of each immune plasma on PRMs, *in vitro* feeding assays were performed as described in Chapter I. Briefly, plasmas isolated from chickens of an unimmunized group or each immunized group were pooled separately. Each pooled plasma was replaced with the plasma from heparinized fresh blood. Starved PRMs of mixed developmental stages were used for the *in vitro* feeding. After blood feeding, blood-fed PRMs were collected using Pasteur pipettes and maintained at 25℃ in 70% humidity. The numbers of dead PRMs from each group were counted daily for a week. The acaricidal effects of immune plasma against rFER2 were evaluated by the survival rate of PRMs.

Statistical analysis

The EZR software was used to calculate all statistical analyses [Kanda, 2013]. The Kruskal–Wallis test with the Steel–Dwass comparison was used to analyze the iron-binding abilities. The asterisks indicate significant differences (*p<0.05 and **p<0.01). The daily mortality rate of PRMs between the groups was evaluated by Fisher's exact test, and the ORs and their 95% CI were calculated. In addition, to evaluate the survival rate after *in vitro* feeding, a log-rank test with Bonferroni multiple comparisons was conducted, and a Kaplan–Meier curve was generated. p<0.05 was considered statistically significant.

Table IV-1. Primers used for the amplification of *ferritin 2* genes.

Primer	Mite species	Intended use	Sequences (5′-3′)
FER2 outer-F	NFMs & TFMs	Partial gene amplification	TCGTRCCSCTGCTSTTCGC
FER2 outer-R			TCGGCATCCSDAYGTTGAKRT
FER2 inner-F			ACCCTCGARATGCAYGCYTC
FER2 inner-R			AGGWCWAGVGCRGCYTGVAG
GSP1	NFMs & TFMs	3′RACE	GTGAACACGCACAGAAGCTC
GSP2			ATGTCTGCGCACTTTGACAC
GSP3			GAGATGCACGCCTCTCTGAT
GSP1	NFMs & TFMs	5′RACE	CTCATTGTTGACTTTG
GSP2			TGGCACTGCTCCACGAACTA
GSP3			CTTGGCAAATCCTGGACGAG
rFER2 PRM-F	PRMs	Construction for expression plasmids	AAGCATATGCGCCAAGATAATGCCACCCCATC
rFER2 PRM-R			ATCCTCGAGCTAGAGAAGGTCCTTGTTGACAAAGTATTCTCC
rFER2 TFM-F	NFMs & TFMs		AAGCATATGCGTCAGAGCAATGACACGCCT
rFER2 TFM-R			ATCCTCGAGCTAAGCGAGATCCTTGTCGAGGAGATA

The NdeI and XhoI sites are underlined.
PRM, poultry red mite; NFM, northern fowl mite; TFM, tropical fowl mite; FER2, ferritin 2; rFER2, recombinant ferritin 2

Table IV-2. List of *ferritin* genes used for the phylogenetic analysis in Figure IV-1b

Accession No.	GenBank	Species
XM 003737350	PREDICTED: soma ferritin-like	*Metaseiulus occidentalis*
XM 003737351	PREDICTED: soma ferritin-like	*Metaseiulus occidentalis*
MNPL01001122	Ferritin	*Metaseiulus occidentalis*
HZ459285	Ferritin 2	*Dermanyssus gallinae*
MW798797	Ferritin 2	*Dermanyssus gallinae*
XM 022808087	PREDICTED: soma ferritin-like	*Varroa destructor*
XM 022808086	PREDICTED: soma ferritin-like	*Varroa destructor*
JQ922403	Ferritin 2	*Rhipicephalus microplus*
MW346659	XJ-ZS-41 ferritin 2	*Dermacentor marginatus*
MZ332506	Ferritin 2	*Ornithodoros moubata*
EU885951	Secreted ferritin	*Ixodes ricinus*
KF311110	Ferritin 2	*Ixodes persulcatus*
XM 029988441	PREDICTED: ferritin heavy chain	*Ixodes scapularis*
MW798798	Ferritin 1 mRNA	*Dermanyssus gallinae*
XM 022787705	PREDICTED: soma ferritin-like	*Varroa destructor*
XM 018638352	PREDICTED: ferritin heavy chain	*Metaseiulus occidentalis*
XM 050182915	PREDICTED: soma ferritin-like	*Dermacentor andersoni*
AY277902	Ferritin mRNA	*Boophilus microplus*
MN937442	Ferritin 1 mRNA	*Haemaphysalis flava*
AY277905	Ferritin mRNA	*Haemaphysalis longicornis*
BM486522	Ferritin	*Gallus gallus*

RESULTS

Characterization of *FER2* genes of TFMs and NFMs

The ORFs of *FER2* genes amplified from TFMs (LC752110) and NFMs (LC752111) contained 588 bp with 195 amino acids. The *FER2* genes of TFMs and NFMs showed 65.0% homology with that of PRMs and indicated 99.0% homology with each other (Table IV-3). Signal peptides were detected at positions 1–17 at the N terminus of FER2 of TFMs and NFMs and at positions 1–18 for PRMs (Figure IV-1a). Furthermore, ferroxidase centers, which are iron-binding sites for the oxidation of Fe(II) in heavy-chain (H) ferritin, were conserved among avian mites (Figure IV-1a). To analyze the genetic relationship of *FER2* genes of TFMs and NFMs, a phylogenetic tree using the *FER* sequences of PRMs, other mites, ticks, and chickens was generated (Figure IV-1b). The *FER2* genes of TFMs and NFMs were classified into cluster 1, which included secreted *FER* (*FER2*) genes, and were closely related to the secreted *FER2* genes of PRMs, *V. destructor*, and *T. mercedesae* in subcluster 1-1, which included secreted *FER* genes of mites. Secreted *FER* genes from ticks were classified into subcluster 1-2. The intracellular *FER1* genes of PRMs, as well as those of other mites, ticks, and chickens, belonged to cluster 2. Thus, *FER2* genes of mites, including TFMs and NFMs, are definitely classified into the cluster of secretory FERs.

Iron-binding ability of rFER2 proteins of PRMs, TFMs, and NFMs

The N terminal his-tagged rFER2 from PRMs, TFMs, and NFMs, without the signal peptides, was generated. The rFER2 proteins were purified with metal affinity chromatography and validated by SDS-PAGE and western blotting (Figures IV-2a,b). To assess the function of rFER2, a ferrozine-based colorimetric iron-binding assay was conducted. The absorbance of rFER2 PRM, rFER2 TFM, and rFER2 NFM, was decreased in a dose-dependent manner, similar to that of the positive control, horse apoferritin (Figure IV-3). Thus, these data demonstrated that rFER2 proteins of PRMs, TFMs, and NFMs have iron-binding abilities.

Cross-reactivities of immune plasmas against rFER2 of different mites

To investigate the potential use of rFER2 as a universal vaccine antigen against avian mites, immune plasma was isolated from chickens immunized with each rFER2. The high antibody titers were verified in the immunized groups by ELISA (Table IV-4), and specific antibodies against each rFER2 present in the immune plasma were confirmed by western blotting (Figure IV-4). Moreover, the immune plasmas cross-reacted with rFER2 proteins from different avian mites (Figure IV-4). However, the intensity of the signals was slightly weak, when the cross-reactivity of immune plasma against rFER2 PRM with rFER2 proteins of TFMs and NFMs was compared to that with rFER2 PRM,

and vice versa. These findings indicated the potential of rFER2s as a common vaccine antigen across avian mites.

Evaluation of acaricidal effects of immune plasmas against rFER2 proteins

To evaluate the acaricidal activities of FER2, immune plasma against each rFER2 was artificially fed to PRMs using an *in vitro* system, and the mortality of PRMs was monitored. Due to the limited prevalence of NFMs and TFMs in Japan, PRMs were used for *in vitro* feeding assays. The *in vitro* feeding assays were conducted two times. In experiment 1, Kaplan–Meier survival curves showed that the survival rate of PRMs fed with the immune plasma against each rFER2 was significantly decreased compared to that of the control group (Figure IV-5a). In addition, at 7 days post-feeding, immune plasma against rFER2 PRM, rFER2 TFM, and rFER2 NFM, exerted the 40.55%, 32.03%, and 39.34% mortality rate of PRMs, respectively. According to Fisher's exact test, significant differences in the mortality of PRMs fed the immune plasma against rFER2 PRM at 2–7 days post-feeding were observed when compared to those of the control group (Table IV-5). In addition, in the immunized groups of rFER2 TFM and rFER2 NFM, the mortality was significantly increased at 3–7 days post-feeding (Table IV-5). Similar results were obtained in experiment 2. Significant differences in the mortality rates of PRMs were observed at 3–7 days post-feeding in the immunized groups of rFER2 PRM and rFER2 NFM, and 4–7 days post-feeding in the immunized group of rFER2 TFM (Table IV-6). Furthermore, the survival rate of PRMs fed with immune plasma against each rFER2 was significantly decreased (Figure IV-5b). Thus, the immune plasma against each rFER2 showed acaricidal effects on PRMs. Therefore, rFER2 could be a candidate antigen for the development of a universal vaccine across avian mites.

Table IV-3. Sequence homology of ferritin 2 from poultry red mites (PRMs), northern fowl mites (NFMs), and tropical fowl mites (TFMs)

		Amino acid sequence (%)		
		PRM	TFM	NFM
Nucleotide sequence (%)	PRM	-	65.0	66.0
	TFM	67.0	-	99.0
	NFM	68.0	99.0	-

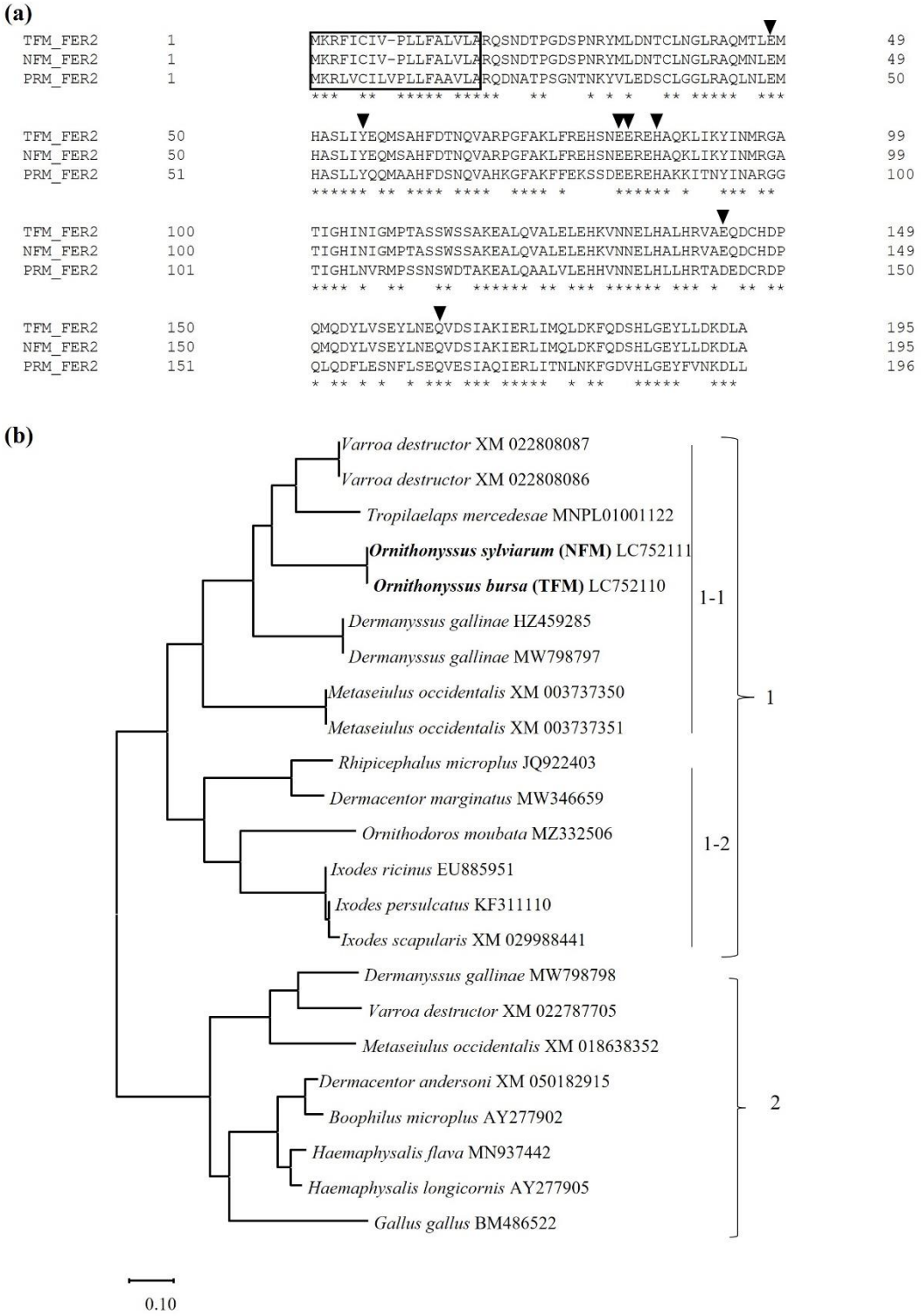

Figure IV-1. Genetic characterization of *FER2* genes of avian mites.

(a) The multiple alignment of FER2 of PRMs, TFMs, and NFMs. The deduced amino acid sequences of FER2 from TFMs and NFMs were aligned with that of PRM FER2. FER2 proteins have the signal peptides (box) at the positions of 1–17 in TFMs and NFMs and 1–18 in PRMs. The black arrowheads indicate the ferroxidase centers of heavy chain

subunits of FER2. (b) Phylogenetic analysis of the *FER* genes from PRMs, TFMs, NFMs, arthropods, including other mites and ticks, and chickens. The maximum-likelihood phylogenetic tree was constructed using MEGA X software. The numbers on the right indicate the clusters. Cluster 1 includes secretory types of *FER2* genes, and is divided into 2 subclusters. Subcluster 1-1: The secretory types of *FER2* genes of mites, including *FER2* genes of PRMs, TFMs (bold), and NFMs (bold), are classified into this subcluster. Subcluster 1-2: This cluster consists of secretory types of *FER2* genes from ticks. Cluster 2: The intracellular *FER* genes of mites, ticks, and chickens are classified into this cluster.

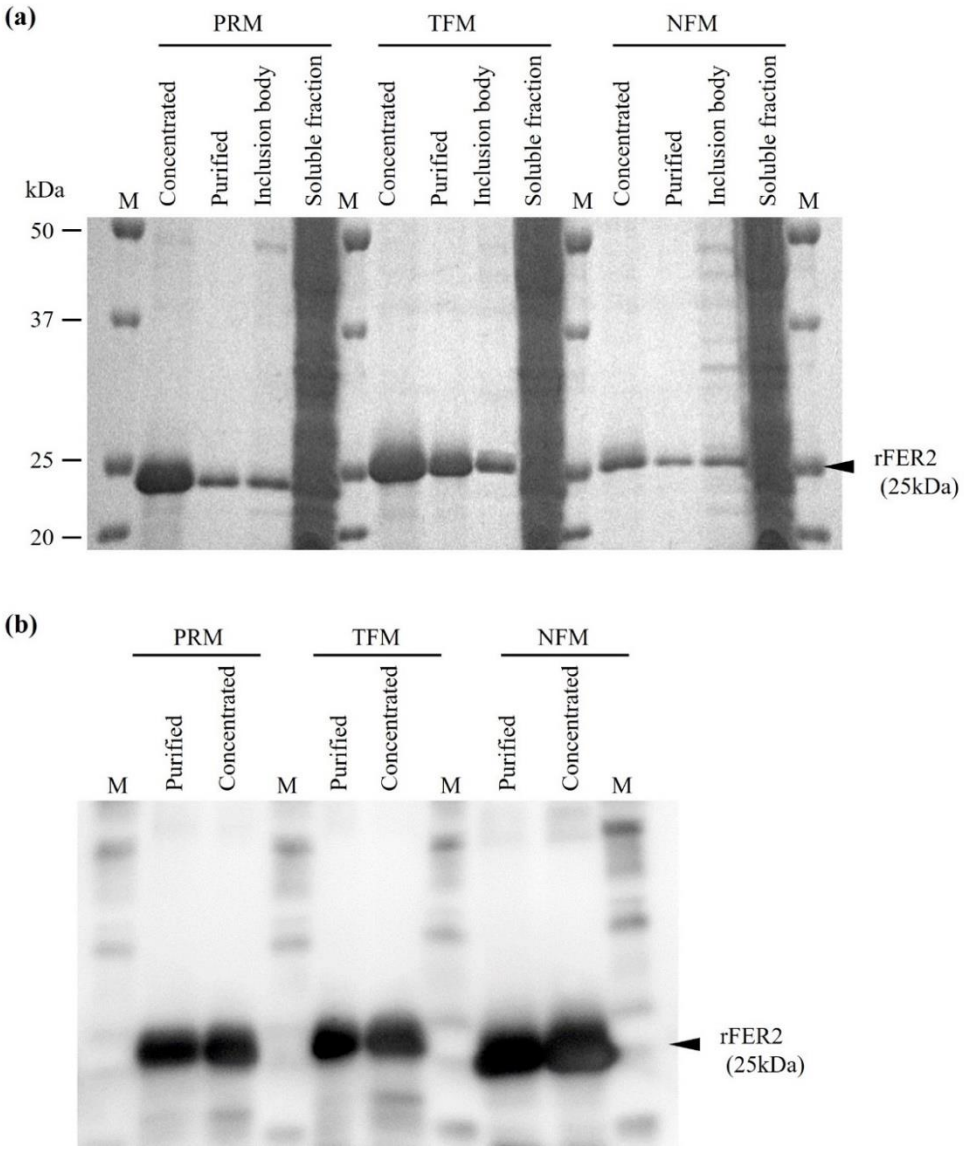

Figure IV-2. Expression and purification of rFER2 proteins of avian mites.
The FER2 from PRMs, TFMs, and NFMs were expressed and purified as recombinant proteins fused with his-tag, and termed as rFER2 PRM, rFER2 TFM, and rFER2 NFM, respectively. rFER2 from each mite was expressed in *E. coli* and purified from insoluble fractions using affinity chromatography. The purity of rFER2 was confirmed by (a) SDS-PAGE and (b) western blotting. M, Marker.

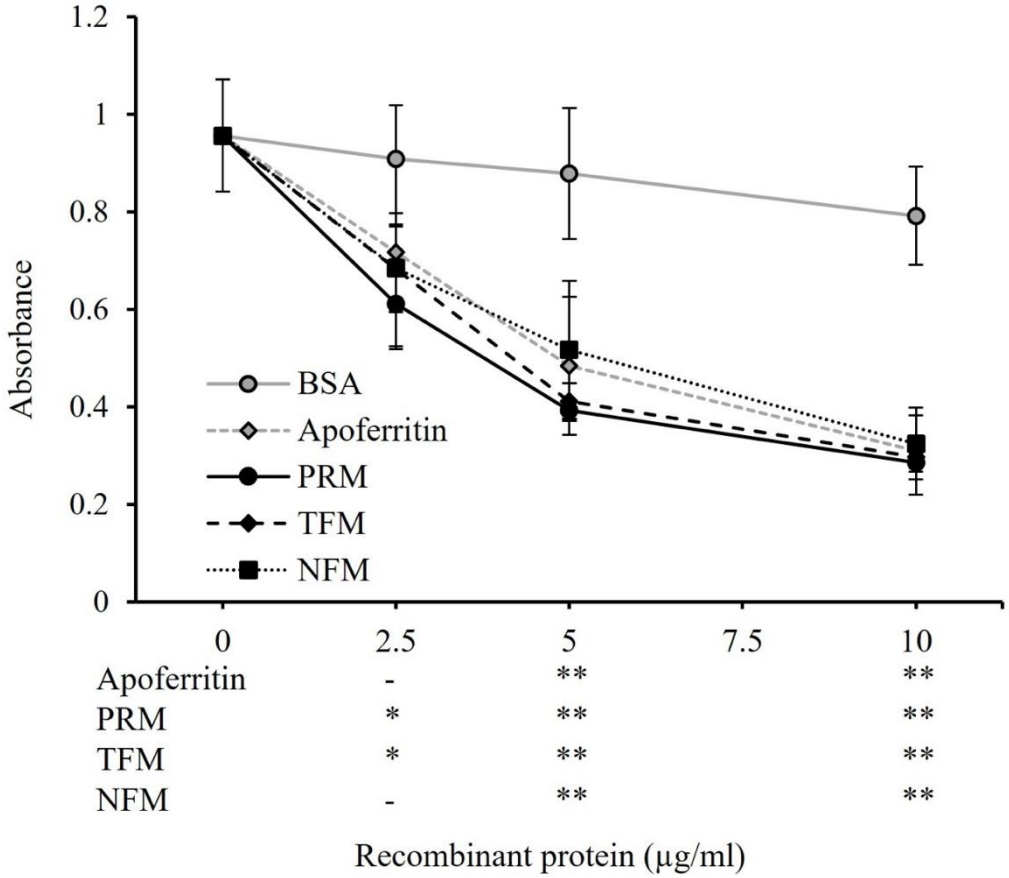

Figure IV-3. Iron-binding ability of rFER2 proteins.

The iron-binding ability of different concentrations of rFER2 proteins from PRMs, TFMs, NFMs was assessed by a ferrozine-based colorimetric assay. Ferrozine was used as an indicator agent. BSA and horse apoferritin were used as the negative and positive controls, respectively. The x-axis indicates the concentrations of rFER2 proteins used in this assay. Error bars indicate standard deviations. Statistical differences are shown in comparison to BSA. Asterisks indicate significant differences (*p<0.05 and **p<0.01).

Table IV-4. Antibody titers in plasma samples of chickens immunized with each recombinant ferritin 2

Group	Chicken	Antibody titer
Control	C1	1:2,000
	C2	1:2,000
	C3	1:2,000
	C4	1:2,000
Immunized- rFER2 PRM	PRM1	1:32,000
	PRM2	1:128,000
	PRM3	1:128,000
	PRM4	1:64,000
Immunized- rFER2 TFM	TFM1	1:128,000
	TFM2	1:128,000
	TFM3	1:64,000
	TFM4	1:64,000
Immunized- rFER2 NFM	NFM1	1:64,000
	NFM2	1:32,000
	NFM3	1:16,000
	NFM4	1:32,000

PRM, poultry red mite; NFM, northern fowl mite; TFM, tropical fowl mite; rFER2, recombinant ferritin 2

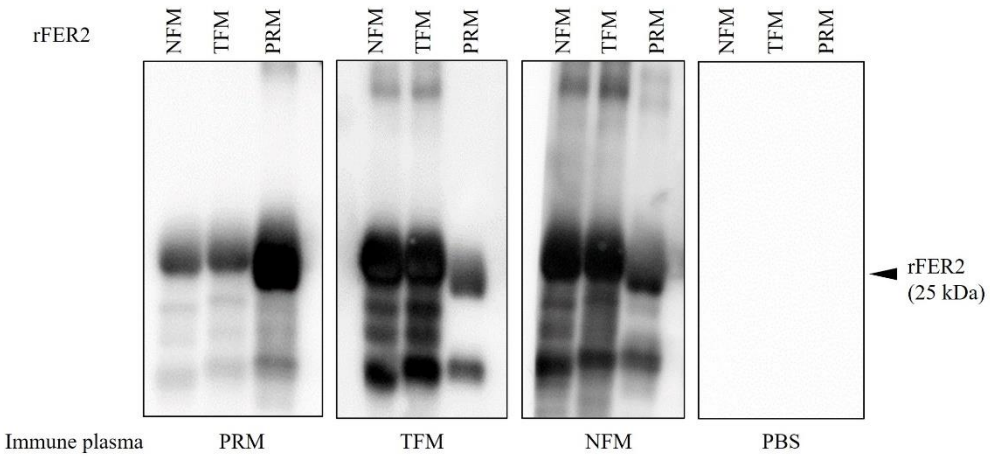

Figure IV-4. Production of specific antibodies in the plasmas from chickens immunized with rFER2 proteins.

The plasmas were isolated from chickens immunized with each rFER2. The production of antibodies specific to each rFER2 in the plasma and the cross-reactivities of immune plasmas with rFER2 proteins of different avian mites were detected by western blotting. The arrowhead indicates the predicted molecular weight of rFER2 (approximately 25 kDa).

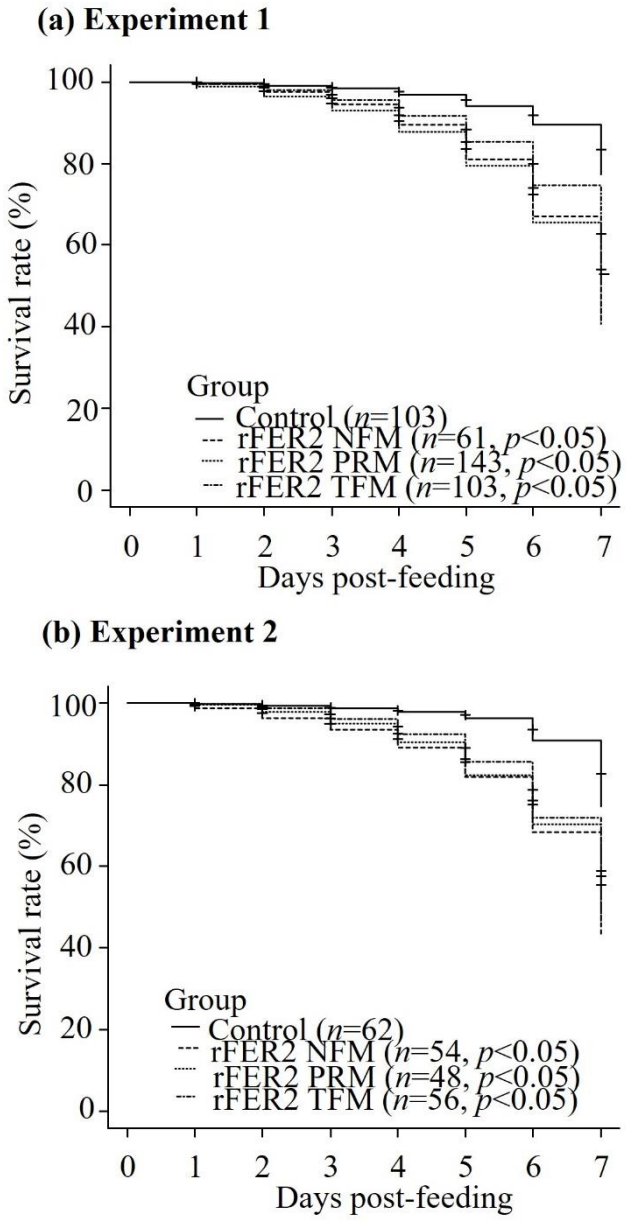

Figure IV-5. Evaluation of the acaricidal potential of plasmas from chickens immunized with rFER2 proteins.

PRMs were artificially fed with fresh blood containing immune plasmas by the *in vitro* feeding assays. The mortality rates of PRMs fed with immune or control plasma were monitored daily for a week. The *in vitro* feeding assays were performed twice. The total number of PRMs used in these assays was as follows: (a) Experiment 1: rFER2 PRM (n=143), rFER2 TFM (n=103), rFER2 NFM (n=61) and control (n=103); (b) Experiment 2: rFER2 PRM (n=48), rFER2 TFM (n=56), and rFER2 NFM (n=54) and control (n=62). The number of dead PRMs was recorded. A log-rank test with Bonferroni multiple comparisons was performed, and Kaplan–Meier survival curves were generated. p<0.05 was considered statistically significant between each immunized and control groups.

Table IV-5. Mortality of PRMs fed plasma from chickens immunized with recombinant ferritin 2 (rFER2) from different species of mites (experiment 1)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=103)							
No. of dead PRMs post-feeding	2	4	4	6	9	10	14
Mortality (%)	1.94	3.88	3.88	5.82	8.73	9.71	13.59
Immunized group (rFER2 PRM, *n*=143)							
No. of dead PRMs post-feeding	11	22	25	33	40	50	58
Mortality (%)	7.69	15.38	17.48	23.07	27.97	34.96	40.55
p value (Fisher's exact)	0.079	0.003*	1.06E-03*	1.77E-04*	1.67E-04*	4.3E-06*	4.12E-06*
Odds ratio	4.19	4.48	5.21	4.82	4.04	4.97	4.31
95% confidence interval	0.88–39.69	1.45–18.45	1.72–21.31	1.89–14.69	1.80–9.98	2.32–11.67	2.18–9.02
Immunized group (rFER2 TFM, *n*=103)							
No. of dead PRMs post-feeding	4	9	13	17	20	26	33
Mortality (%)	3.88	8.73	12.62	16.50	19.41	25.24	32.03
p value (Fisher's exact)	0.683	0.251	0.040*	0.025*	0.044*	5.39E-03*	2.56E-03*
Odds ratio	2.03	2.36	3.55	3.18	2.51	3.12	2.98
95% confidence interval	0.28–22.96	0.63–10.85	1.05–15.51	1.13–10.31	1.02–6.61	1.36–7.73	1.42–6.52
Immunized group (rFER2 NFM, *n*=61)							
No. of dead PRMs post-feeding	2	7	10	13	17	21	24
Mortality (%)	3.27	11.47	16.39	21.31	27.87	34.42	39.34
p value (Fisher's exact)	0.629	0.102	8.35E-03*	4.56 E-03*	1.77 E-03*	1.56E-04*	6.18E-10*
Odds ratio	1.71	3.18	4.80	4.34	3.99	4.83	9.63
95% confidence interval	0.12–24.11	0.77–15.51	1.31–22.03	1.43–14.81	1.54–11.04	1.97–12.60	4.30–22.70

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

Table IV-6. Mortality of PRMs fed plasma from chickens immunized with recombinant ferritin 2 (rFER2) from different species of mites (experiment 2)

	Days post-feeding						
	1	2	3	4	5	6	7
Control group (*n*=62)							
No. of dead PRMs post-feeding	1	2	2	2	3	7	11
Mortality (%)	1.61	3.23	3.23	3.23	4.84	11.29	17.74
Immunized group (rFER2 PRM, *n*=48)							
No. of dead PRMs post-feeding	2	5	7	9	13	14	17
Mortality (%)	4.17	10.42	14.58	18.75	27.08	29.17	35.47
p value (Fisher's exact)	0.579	0.236	0.039*	0.010*	0.002*	0.027*	0.047*
Odds ratio	0.63	3.45	5.05	6.81	7.18	3.19	2.52
95% confidence interval	0.13–158.83	0.53–37.84	0.9–52.18	1.31–68.15	1.8–41.94	1.08–10.37	0.97–6.81
Immunized group (rFER2 TFM, *n*=56)							
No. of dead PRMs post-feeding	1	4	7	9	12	18	20
Mortality (%)	1.79	7.14	12.5	16.07	21.43	32.14	35.71
p value (Fisher's exact)	1	0.421	0.083	0.024*	0.011*	0.007*	0.036*
Odds ratio	1.11	2.29	4.24	5.67	5.29	3.68	2.56
95% confidence interval	0.01–88.41	0.31–26.31	0.76–43.61	1.09–56.31	1.32–30.97	1.31–11.49	1.02–6.69
Immunized group (rFER2 NFM, *n*=54)							
No. of dead PRMs post-feeding	3	5	8	10	13	18	20
Mortality (%)	5.56	9.26	14.81	18.52	24.07	33.33	37.04
p value (Fisher's exact)	0.337	0.248	0.043*	0.012*	0.006*	0.006*	0.022*
Odds ratio	3.55	3.03	5.15	6.72	6.14	3.88	2.70
95% confidence interval	0.28–191.19	0.47–33.16	0.96–52.02	1.33–66.14	1.55–35.7	1.38–12.16	1.07–7.11

*$p<0.05$ was considered statistically significant.
PRM, poultry red mite; TFM, tropical fowl mite; NFM, northern fowl mite

DISCUSSION

Genetic characterization revealed that the *FER2* genes of PRMs, TFMs, and NFMs were classified into the cluster of secretory FERs and were different from the cluster of intracellular FERs. FER2 of avian mites included signal peptides at the N terminus. FERs of vertebrates and insects are composed of H subunits containing ferroxidase centers (iron-binding sites) and L subunits containing amino acid residues with ferrihydrite nucleation centers [Pham *et al*., 2010]. FER2 of avian mites was entirely conserved in the ferroxidase centers, and in addition, rFER2 of PRMs, TFMs, and NFMs exhibited iron-binding ability, suggesting that these FER2 were H-type subunits with catalytic function in Fe(II) oxidation. In ticks, two types of FERs have been reported [Kopácek *et al*., 2003; Galay *et al*., 2013], and FER2 was characterized as a secretory protein in the hemolymph, in contrast to FER1 [Hajdusek *et al*., 2009]. Similar to those of ticks, FER1 and FER2 were identified in PRMs [Xu *et al*., 2021]. The mRNA of the *FER2* gene expressed throughout the developmental stages of PRMs, and RNAi studies indicated the essential roles of FER2 for survival and reproduction [Xu *et al*., 2021]. Unfortunately, owing to a lack of TFMs and NFMs samples in Japan, the expression patterns of the *FER2* genes throughout the developmental stages of TFMs and NFMs and physiological roles were not characterized. However, the immune plasma against rFER2 TFMs or NFMs was verified to cross-react with the rFER2 PRM and to exhibit acaricidal effects on PRMs. Collectively, these findings suggest that the FER2 identified in the present study is a secretory molecule that plays a critical role in the physiology of avian mites.

Hematophagous ectoparasites acquire nutrients from the host blood for development and reproduction. A PRM can suck up to 0.2 μL of the host blood in a single feeding [Mul *et al*., 2009] and is exposed to a large amount of iron. Excessive exposure to non-heme iron following hemoglobin digestion in the midguts could be hazardous to mites. FERs are supposed to have a function in iron homeostasis in ticks [Kopácek *et al*., 2018], while iron metabolism in avian mites is poorly known. A previous study reported that silencing *FER1* and *FER2* genes negatively impacts tick feeding and oviposition; moreover, *FER2* depletion is associated with *FER1* expression and impaired iron homeostasis in ticks [Galay *et al*., 2013]. Therefore, FER2 has been considered an effective vaccine antigen candidate against ticks [Hajdusek *et al*., 2010; Knorr *et al*., 2018; Githaka *et al*., 2020; Oleaga *et al*., 2022]. Moreover, the cross-immunoprotection efficacy of the FER2 vaccine against different tick species has been demonstrated [Xavier *et al*., 2021; Zeb *et al*., 2024]. In PRMs, knocking down *FER1* and *FER2* genes resulted in a negative impact on blood digestion and oviposition, in addition to the increased mortality; moreover, vaccination of chickens with rFER2 (rDg-FER1 in the original work) resulted in a significant increase in the mortality of PRMs [Xu *et al*., 2021]. In this

study, immune plasmas against each rFER2 showed cross-reactivities with FER2 proteins from different avian mites; moreover, the immune plasma against rFER2 TFM and NFM showed anti-PRM effects. Therefore, these data suggest the utility of FER2 as a vaccine antigen against TFMs and NFMs, in addition to PRMs, and its potential as an antigen candidate for the development of a universal vaccine against hematophagous avian mites.

From the knowledge of tick control, the application of common antigens with broad protective efficiency against several tick species is valuable because ticks are nonuniformly distributed throughout the world [Hajdusek *et al*., 2010; Ndawula *et al*., 2019; Githaka *et al*., 2020; Xaiver *et al*., 2021; Oleaga *et al*., 2022; Zeb *et al*., 2024]. Similar strategies should be applied to control avian mites on poultry farms because the distribution of avian mites is different in each region. Therefore, the development of an anti-PRM vaccine that is effective against other avian mites, such as TFMs and NFMs, would further enhance its applications. In this study, the immune plasma against each rFER2 cross-reacted with rFER2 proteins from different mites, and, immune plasma against rFER2 TFM and NFM had acaricidal effects on PRMs. Thus, FER2 could be a common antigen candidate for the development of a vaccine with broad-spectrum protection across multiple avian mites. Moreover, based on the current knowledge, FER2 is a unique molecule in blood-feeding arthropods to transport iron from the midguts to other tissues; therefore, it may be selected as a preferred vaccine antigen for formulation of cocktail vaccine.

SUMMARY

Since the current control approaches against avian mites are insufficient, alternative strategies are preferable. Vaccine approaches have gained attention as a novel method for controlling PRMs. In addition, the development of a vaccine with broad-spectrum effects on other avian mites, such as TFMs, and NFMs, could provide advantages in terms of cost-effectiveness.

In this chapter, therefore, the feasibility of FER2, a vaccine antigen candidate against PRMs as a common antigen, was examined. FER2 identified in TFMs and NFMs possessed the amino acid residues essential for the oxidization of Fe(II) and were classified into the cluster of secretory FER, similar to that of PRMs. In addition, rFER2 proteins of PRMs, TFMs, and NFMs exhibited iron-binding abilities. These data support that the molecules identified in this study serve as FER2 in each avian mite. Importantly, the immune plasmas against rFER2 of PRMs, TFMs, and NFMs cross-reacted with rFER2 proteins from different avian mites. Furthermore, the immune plasmas containing antibodies against each rFER2 exhibited acaricidal effects on PRMs, even in the immune plasmas against rFER2 of TFMs and NFMs. Thus, FER2 could be a vaccine antigen candidate with broad effects on hematophagous avian mites. Further studies are required to determine the effectiveness of FER2 as a common vaccine antigen against avian mites.

GENERAL DISCUSSION

The present study evaluated the efficacy of HRF, GST, CP, and FER2 as vaccine antigens for the utilization of universal vaccines in Chapters I, II, III, and IV, respectively. The results of *in vitro* feeding assays could be affected by differences in PRM lots. Although it is difficult to simply compare the data of each antigen, the total mortality at 7 days post-feeding in *in vitro* feeding assays for each antigen was summed to calculate the increased rate of deaths in PRMs compared to the death rate of PRMs in the control group in the assays for each antigen (Table 2). Among four antigens, FER2 showed the highest increased rate of deaths, followed by GST, HRF, and CP. Therefore, FER2 can be assumed as the most effective antigen. In addition, the current knowledge of FER2 with its unique function strongly suggests FER2 as a suitable antigen for vaccine production. GST and CP can be considered as second and third candidates for universal vaccine antigens, respectively. Based on the current understanding of the physiology of mites, mites have several GSTs and CPs, and some of them may have the substitutive functions of GST or CP. Therefore, the addition of antigens, which have similar functions and can interact with GST or CP, in vaccine formulation may enhance the protective efficacy. In the present study, HRF showed its acaricidal effects mostly on nymphs. Therefore, HRF could be applied for an antigen of a cocktail vaccine while the use of HRF as a single antigen may be less effective. Collectively, these findings suggest that FER2 is a more effective antigen for the universal vaccine than other antigens evaluated in this study. To further determine the protective efficacy, studies are required to compare the reduction rate of avian mites infested on vaccinated and unvaccinated chickens *in vivo*.

Table 2. Increased mortality of PRMs fed with immune plasma against each candidate vaccine antigen

	Total mortality rate (%)[a]				Increased rate of deaths (%, vs. control)[b]			
	HRF	GST	CP	FER2	HRF	GST	CP	FER2
Control	15.17	10.27	25.37	15.15	-	-	-	-
PRM	27.24	22.31	37.77	39.27	79.61	117.15	48.89	159.16
TFM	33.64	29.09	46.40	33.33	121.85	183.15	82.92	120.0
NFM	26.86	19.94	39.60	38.26	77.08	94.11	56.12	152.52

[a]The total mortality rate of PRMs was calculated based on the summed results of *in vitro* feeing assays at 7 days post-feeding.
[b]Increased rate of deaths in PRMs fed with each immune plasma was calculated based on the total mortality rate of PRMs in each immunized group compared to that of control group.

CONCLUSION

Avian mites, including poultry red mites (PRMs, *Dermanyssus galline*), tropical fowl mites (TFMs, *Ornithonyssus bursa*), and northern fowl mites (NFMs, *Ornithonyssus sylviarum*), are blood-feeding parasites that infest poultry and other birds, and infestation with these mites causes several negative impacts on the health and welfare of poultry, resulting in severe economic losses to the poultry industry. The emergence of drug-resistant mites has reduced the effectiveness of chemical acaricides commonly used, making it challenging to control avian mites in the field. Thus, the establishment of alternative control strategies against avian mites is urgently needed. In recent years, approaches using vaccines have been proposed as promising alternative methods against PRMs. Nevertheless, the efficacy of anti-PRM vaccines needs to be improved for field application; therefore, the development of cocktail vaccines that combine multiple antigens is required. Moreover, although vaccines against TFMs and NFMs that cause similar problems to PRMs have not been studied, the development of anti-PRM universal vaccines that are effective against other avian mites including TFMs and NFMs would greatly enhance their utility. Therefore, multiple vaccine antigens with broad efficacy against avian mites are required to be determined, and these antigens should have different physiological functions for future application as cocktail vaccines to enhance their efficacy. In the present study, histamine release factor (HRF), glutathione S-transferase (GST), cysteine protease (CP), and ferritin 2 (FER2), which have been reported as candidate vaccine antigens against PRMs, were identified from TFMs and NFMs, and the potentials as universal vaccine antigens with broad-spectrum efficacy across avian mites were assessed.

In Chapter I, the potential of HRF, a multifunctional protein involved in biological activities and allergic responses, as a vaccine antigen against multiple avian mites was evaluated. The *HRF* genes identified from TFMs and NFMs were 12 amino acid sequences shorter than that of PRMs and belonged to a cluster of mite HRFs, including PRMs. Increased antibody production was observed in chickens immunized with recombinant HRF (rHRF) of PRMs, TFMs, and NFMs. The immune plasma containing antigen-specific antibodies cross-reacted with rHRFs from different avian mites. In addition, the mortality of nymph PRMs artificially fed immune plasmas was increased. This study demonstrates the effects of HRF immunization on nymphs across mite species, suggesting the utility of HRF as a candidate antigen for universal vaccine development.

In Chapter II, the potential of GST, which is a multifunctional protein involved in acaricide detoxification, as a vaccine antigen was assessed. The *GST* genes identified from TFMs and NFMs were highly conserved with that of PRMs and clustered in the cytosolic mu class GSTs. The recombinant GST (rGST) proteins generated from PRMs, TFMs, and NFMs exhibited catalytic activity to conjugate glutathione with a substrate.

Immunization with each rGST induced antigen-specific antibodies in chickens, and each immune plasma was cross-reacted with rGSTs of different mites. Moreover, the immune plasma against rGSTs of TFMs and NFMs exerted acaricidal effects on PRMs *in vitro*. These results indicate the potential use of GSTs as vaccine antigens against avian mites, assuming that specific antibodies against GSTs can disrupt the physiology of mites by interacting with naïve GSTs in the midgut cells.

In Chapter III, the feasibility of CP, a proteolytic enzyme vital for hemoglobin digestion in blood sucking arthropods, as a universal vaccine antigen, was investigated. The *CP* genes identified from TFMs and NFMs were characterized as digestive *CP* genes by the phylogenetic analysis, and the peptidase domains (PDs), which are required for the enzyme activities of CPs, were highly conserved to that of PRMs. The recombinant PDs of CPs (rCP-PDs) generated from PRMs, TFMs, and NFMs exhibited cathepsin L-like enzymatic activities. In addition, plasmas from chickens immunized with rCP-PDs from PRMs, TFMs, and NFMs exhibited cross-reactivities with rCP-PDs of different mites and exerted acaricidal effects on PRMs *in vitro*. Therefore, CP could be an effective antigen for developing a vaccine with broad-spectrum efficacy across avian mite species.

In Chapter IV, the feasibility of FER2, an iron-binding protein important for iron homeostasis, as a vaccine antigen against multiple avian mite species, was assessed. Phylogenetic analysis indicated that *FER2* genes of avian mites belonged to a cluster of secretory ferritins. The deduced amino acid sequences of TFMs and NFMs included amino acid residues, which serve as the ferroxidase diiron binding center critical for the oxidation of Fe (II), and they are conserved with those of PRMs. In addition, the recombinant FER2 (rFER2) generated from PRMs, TFMs, and NFMs exhibited iron-binding activities. The immune plasmas against each rFER2 recognized the rFER2 from different avian mites and exhibited the acaricidal effects on PRMs *in vitro*. These data suggest that antigens associated with biological features conserved among avian mites, such as FER2, are suitable for application as universal vaccine antigens.

In conclusion, the present study identified four candidates as universal vaccine antigens with broad protective efficacy against avian mite species. The development of universal vaccines could be advantageous for commercial manufacturing, and their application on poultry farms may minimize economic losses in production. However, the observations obtained in the present study are limited based on *in vitro* findings using PRMs. Therefore, to accurately assess cross-protective efficacy against avian mites, the effects of each antigen on TFMs and NFMs are necessary to be examined *in vitro* and *in vivo*. In addition, it is necessary to analyze the effects of these vaccines on the fecundity of adult females fed with immune plasma. Analysis of the expression levels of each antigen gene at different developmental stages will provide a more accurate picture of their effects on each mite. Moreover, to enhance the protective efficacy, a combination of antigens analyzed in this study as a cocktail vaccine needs to be investigated. According

to the findings of this study, FER2 should be used as a base antigen, in combination with others such as HRF or GST or CP, in the formulation of cocktail vaccines. Thus, although there are still some limitations, the findings of this study could encourage the future application of universal vaccines against avian mites, thereby improving the economic losses in the poultry industry.

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SUMMARY IN JAPANESE

和文要旨

ワクモ（*Dermanyssus gallinae*）は鳥類に寄生する吸血性のダニで、鶏に貧血や産卵数や産卵率の低下を引き起こし、国内外の多くの養鶏場で深刻な生産性の低下を引き起こしているほか、動物福祉の観点からも問題視されている。ワクモの防除は、殺虫剤の使用により行われているが、薬剤耐性ダニの出現により、現行の対策に変わる新規防除法の確立が望まれている。近年、新規防除法としてワクチンによる防除法の開発が注目されている。しかし、野外への応用に向けて、さらなる効果の改善が求められおり、複数抗原を組み合わせたカクテルワクチンの開発が必要である。また、その他の家禽の吸血性のダニであるトリサシダニ (*Ornithonyssus sylviarum*) やミナミトリサシダニ (*Ornithonyssus bursa*) においてもワクモと同様な被害や問題点が報告されているが、これらの吸血性ダニの防除に向けたワクチンの開発研究は行われていない。そのため、トリサシダニやミナミトリサシダニにも幅広く効果を示す抗ワクモユニバーサルワクチンの開発は、その有用性を大きく高めると考えられる。また、ユニバーザルワクチンとしての効果を高めるためには、生理機能の異なる複数の抗原を組み合わせたカクテルワクチンの開発が望ましい。本稿では、家禽の吸血性ダニに幅広く応用可能なユニバーサルワクチンの開発に向けて、既に抗ワクモワクチンの抗原候補として報告されている histamine release factor (HRF)、glutathione S-transferase (GST)、cysteine protease (CP)、ferritin 2 (FER2) をトリサシダニおよびミナミトリサシダニより同定し、ユニバーサルワクチン用抗原としての可能性について検討した。

第 1 章では、HRF のユニバーサルワクチン抗原としての応用の可能性を評価した。HRF は様々な生物種に保存されている分子であり、免疫応答の調節や細胞増殖などの複数の機能を有する。トリサシダニおよびミナミトリサシダニより同定された *HRF* 遺伝子の予想アミノ酸配列は、ワクモに比べて 12 アミノ酸を欠損していたが、系統樹解析では mite の *HRF* 遺伝子が属するクラスターに分類された。また、ワクモ、トリサシダニおよびミナミトリサシダニの組換え HRF をニワトリに免疫したところ、抗体価の上昇と HRF 特異的抗体の産生が認められた。各組換え GST に対する免疫血漿は、異なるダニに由来する組換え HRF に対して交差反応性を示し、免疫血漿を人工的にワクモに吸血させたところ、幼ダニの死亡率増加が認められた。

第 2 章では、GST の有用性を評価した。GST も第 1 章で解析した HRF と同様に様々な生物種に保存される分子で、代謝や解毒作用に関与し、特に節足動物では薬剤の代謝に関わることが知られている。ワクモ、トリサシダニおよびミナミトリサシダニの *GST* 遺伝子は高度に保存されており、系統樹解析では Mu クラス GST に分類され、各ダニの組換え GST はいずれも触媒活性を示した。各組換え GST をニワトリに免疫したところ、抗体価の上昇と特異的抗体の産生が認められ、それぞれの免疫血漿は異なるダニに由来する組換え GST に交差反応性を示した。さらに、トリサシダニおよびミナミト

リサシダニの GST に対する免疫血漿ワクモに人工的吸血させたところ、死亡率の増加が認められた。

第 3 章では、CP のユニバーサルワクチン用抗原としての有用性を評価した。第 1 章および第 2 章において、様々な生物種に高度の保存されている HRF と GST に有用性が確認された。本章では、吸血性節足動物において重要となる血液消化に関与するプロテアーゼに着目した。系統樹解析の結果、トリサシダニおよびミナミトリサシダニより同定された *CP* 遺伝子は、mite のシステインプロテアーゼが属するクラスターに分類され、酵素活性に重要なペプチダーゼドメインは家禽のダニ間において高い相同性を示した。さらにその組換えタンパク質はいずれもカテプシン L 様の酵素活性を有した。各ダニに由来する CP ペプチダーゼドメインの組換えタンパク質に対する免疫血漿は、異なる種のダニにも交差反応性を示し、免疫血漿を人工的に吸血されたワクモの死亡率を上昇させた。

第 4 章では、FER2 の家禽のダニに対するワクチン抗原としての有用性を評価した。FER2 は吸血性節足動物において、各組織への鉄の輸送に関わる分泌タンパク質であり、恒常性維持や繁殖のために重要な機能を持つ。トリサシダニおよびミナミトリサシダニの *FER2* 遺伝子はワクモと同様に分泌型 ferritin のクラスターに分類され、鉄(II)イオンの酸化に重要な ferroxidase diiron binding center となるアミノ酸残基が保存されていた。また、各組換え FER2 は鉄結合活性を示した。組換え FER2 に対する免疫血漿は、異なるダニに由来する組換え FER2 に交差反応性を示し、全ての免疫血漿にワクモの死亡率を上昇させる効果が認められた。

以上より、HRF、GST、CP、FER2 は家禽の吸血性ダニに対して幅広く効果を示すユニバーサルワクチン抗原としての効果が示唆された。ユニバーサルワクチンの開発は、様々な地域での利用を可能とし、養鶏場における生産性向上に貢献する可能性を持つ。しかし、本研究におけるワクチン抗原としての評価は、*in vitro* において、ワクモのみを用いて実施している。そのため、より正確にユニバーサルワクチン用抗原としての有効性を評価するためには、トリサシダニやミナミトリサシダニを用いて、*in vitro* および *in vivo* における試験を行う必要がある。また、ワクチン抗原の効果範囲をより詳細に検証するためには、メス成ダニの繁殖効率や各発育ステージにおけるワクチン抗原遺伝子の発現解析が求められる。さらに、効果を高めるためには、本研究で同定された抗原を組み合わせたカクテルワクチンとしての応用も検討する必要がある。このように、家禽の吸血性ダニに対するユニバーサルワクチンの開発には複数の検討課題が残されている。しかし本研究では、4 つのユニバーサルワクチン抗原候補の同定に成功しており、本成果をもとにした、養鶏産業における吸血性ダニによる経済損失の改善に向けたワクチン開発への展開が期待される。