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RESEARCH ARTICLE
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A Rapid and Simple Method for Screening Mixed Antigens as Candidates for a Multi-antigen DNA Vaccine against the Poultry Red Mite (*Dermanyssus gallinae*)

Gabriella Chandrakirana Krisnamurti [1], Saengchai Akeprathumchai [1,2], Surawat Chansuwan [1], Najmeh Khanefard [1], Yaowaluck Maprang Roshom [1,2] and Kanokwan Poomputsa [1,2,*]

[1]Biotechnology Program, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi (Bangkhunthian), Bangkok, Thailand 10150
[2]Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi (Bangkhunthian), Bangkok, Thailand 10150
*Corresponding author: kanokwan.poo@kmutt.ac.th

ABSTRACT

The poultry red mite (PRM), *Dermanyssus gallinae*, is a major blood-feeding parasite in chickens. These parasites have developed resistance to certain commercial acaricides. Vaccination, which induces antibodies in chicken blood to interfere with the PRM's biological functions upon feeding, could be a promising alternative for controlling PRM, particularly a multi-antigen vaccine. However, simultaneously evaluating multiple antigen candidates requires many chickens, making the process costly and time-consuming. To address this, we proposed a rapid, simple, and animal-friendly method. This approach involved the rapid production of antigens as a DNA vaccine, followed by administration to egg-laying chickens for antibody production. The antibodies, immunoglobulin Y (IgY), were conveniently obtained from egg yolks. Since vaccine efficacy depends on antibody function, the IgYs were systematically combined into various formulations using an experimental design method, namely fractional factorial design. These combined IgYs were then fed to PRMs via *in vitro* feeding assays, enabling the assessment of a wide range of IgY formulations. Five potential PRM antigens, Cathepsin D-1, Protein of Unknown Function 1 (PUF-1), Akirin, Serpin (SRP-1), and Histamine Release Factor (HRF), were used as models for the method. Mite survival was monitored for 120 hours, and survival times were analyzed using Kaplan-Meier curves with log-rank tests and fractional factorial design statistical analysis. Key IgYs that significantly impacted PRM survival, such as PUF-1 and SRP-1, as well as all IgY interactions, were identified. This led to the selection of optimal antigen formulations for further testing in chickens. With this rapid screening method, fewer chickens are required, thereby reducing overall time, labor, and costs.

Keywords: *Dermanyssus gallinae*; Immunoglobulin Y (IgY); *In vitro* feeding assay; Multi-antigen DNA vaccine; Poultry red mite (PRM)

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INTRODUCTION

Dermanyssus (*D.*) *gallinae* (De Geer, 1778), commonly known as the poultry red mite (PRM), is one of the most harmful ectoparasites affecting poultry farms in Europe (Sparagano et al., 2014). PRM primarily resides off-host, hiding in cracks and crevices within poultry houses. They become active and feed at night (Bartley et al., 2009) using their specialized mouthparts to pierce the skin of avian hosts. Mite feeding causes both direct and indirect reduction in productivity of egg and meat production as well as increased animal mortality (Kilpinen et al., 2005; Hwang, 2023, Schreiter et al., 2022). *D. gallinae* is also involved in the transmission of several pathogens causing serious diseases in animals and humans (Schiavone et al., 2022, Xu et al., 2025). Currently, PRM control relies heavily on synthetic and semi-synthetic acaricides (Hwang, 2023). However, widespread use of acaricides has resulted in the

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development of genetic resistance in PRMs, leading to uncontrolled outbreaks (Marangi et al., 2012; Sparagano and Ho, 2020; Guerrini et al., 2022; Schiavone et al., 2023). Additionally, the use of acaricides raises significant concerns regarding their impact on human health and the environment. Vaccination represents a promising alternative, and several research groups are actively working to identify vaccine candidate PRM antigens and evaluate their efficacy (Murata et al., 2021; Fujisawa et al., 2022; Win et al., 2023a). Since blood meals are the primary nutrient source for blood-feeding PRMs, antibodies induced by PRM protein antigens in the vaccine could interfere with or inhibit the function of these proteins. This interference may negatively impact PRMs by impairing host attachment, reducing feeding efficiency, disrupting blood digestion, and limiting nutrient extraction. These effects could ultimately increase mite mortality, contributing to PRM population control (Murata et al., 2021; Fujisawa et al., 2022; Win et al., 2023a).

Multi-antigen vaccines, designed to induce multiple antibodies targeting different PRM proteins, are expected to have greater potency and more rapid lethal effects on PRMs. Developing a multi-antigen vaccine against PRM requires screening a large number of potential antigens and evaluating their combined effects. Antigens can be identified using genomics (Schicht et al., 2013), transcriptomics (Burgess et al., 2018) and proteomics data (Lima-Barbero et al., 2019) of *D. gallinae*. These antigens may be prepared as purified proteins from PRM extracts or as recombinant proteins and evaluated through chicken immunization. The efficacy of the antigens can be assessed by infesting vaccinated chickens with PRMs. However, this approach is labor-intensive, costly, and time-consuming, as it requires a large number of chickens (Price et al., 2019). To overcome these challenges, a rapid and simple method was developed to assess mixed antigens as candidates for a multi-antigen vaccine while minimizing the use of chickens. This method included rapid antigen production, immunization of egg-laying hens to produce antibodies, antibody collection and purification, and assessment of antibodies in various combinations via *in vitro* feeding assays (McDevitt et al., 2006; Win et al., 2023b, Win et al., 2025). Using egg-laying chickens offers the advantage of producing antibodies, specifically immunoglobulin Y (IgY), in egg yolks. IgY can be conveniently collected from egg yolks without the need to draw blood from immunized chickens and can be easily purified (Redwan et al., 2021; Akhmetzyanov et al., 2022). IgY combinations were systematically designed using Design of Experiments (DOE) methods for efficient planning and statistical analysis (Lamidi et al., 2024). In this study, a fractional factorial design was chosen to reduce the number of samples compared to a full factorial design while still generating sufficient data for statistical analysis. The efficacy of the IgY combinations against PRM was determined by *in vitro* feeding assay and compared to identify the most effective antigens. *In vitro* feeding assays do not require chickens, making this method animal-friendly (Win et al., 2024).

Cathepsin D-1 is a lysosomal endopeptidase that digests hemoglobin in mites (Pritchard et al., 2015; Price et al., 2019). Anti-CatD-1 IgY has been reported to affect mite feeding (Bartley et al., 2012; Price et al., 2019) and may induce gut damage through direct binding to membrane-bound proteins (Pritchard et al., 2015). A Protein of Unknown Function-1 (PUF-1) has an unclear function but was identified as one of the three most effective antigens *in vitro* (Bartley et al., 2015). Serpins function as protease inhibitors, and their inhibition leads to the failure of protein synthesis and tissue repair (Simone and Higgins, 2015). Akirin (Dg-Akirin) immunization in chickens resulted in a 42% reduction in mite oviposition following feeding on vaccinated hens (Lima-Barbero et al., 2019). Histamine Release Factor-1 (HRF-1) induces histamine release and has been identified in several tick species. A single blood meal containing anti-Dg-HRF IgY fed to the mites increased their mortalities suggesting HRF-1 as a strong candidate for vaccination (Bartley et al., 2009, Win et al., 2025). These five known protein antigens were tested using the proposed method, and potential antigen and vaccine formulations were investigated.

MATERIALS & METHODS

Construction of DNA Vaccine

This study used five antigens with distinct functions, Cathepsin D-1, PUF-1, Serpin, Akirin, and Histamine Release Factor (HRF), as model antigens to demonstrate the method. The *D. gallinae* genes, *Dg-CatD1 (*GenBank accession No. HE565350.1), *Dg-PUF-1* (GenBank accession No. KR697568), *Dg-AKR* (GenBank accession No. MN310557.1), *Dg-SRP-1* (GenBank accession No. KR697565), and *Dg-HRF-1* (GenBank accession No. FM179713), were codon-optimized for expression in chickens. These genes were chemically synthesized (Integrated DNA Technologies, USA) and amplified by PCR using gene-specific primers. The PCR fragments were digested with the respective restriction enzymes and cloned into the multicloning site of a mammalian cell expression plasmid, pQCXIN (Clontech, USA). Additionally, the red fluorescence protein (RFP) gene was cloned into the same pQCXIN plasmid to serve as a marker for monitoring gene transcription and translation in chicken cells. The resulting recombinant plasmids were verified by restriction endonuclease digestion and nucleotide sequencing. Large-scale plasmid DNA extraction was performed using the QIAGEN EndoFree Plasmid Mega Kit (USA) by following the manufacturer's protocol. The DNA pellet was resuspended in endotoxin-free PBS.

Cell Lines

Chicken fibroblast cells (DF-1) (ATCC, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New York), supplemented with 10% fetal bovine serum (FBS). Cells were sub-cultured when they reached approximately 70% confluency. These cells were used for verification of gene expression in chicken.

Mite Pre-conditioning

Mixed developmental stages and sexes of *D. gallinae* were collected from SAFETist egg-laying hens at a local farm in Bangkok, Thailand. The PRM were stored in vented 75 cm^2 tissue culture flasks (Corning, USA). Starved PRM

were pre-conditioned by incubating them at room temperature for four days, followed by an additional two days of incubation at 4°C, before being used in the *in vitro* feeding assay.

Gene Expression Analysis in Chicken Cells by Reverse Transcription PCR (RT-PCR)

DF-1 chicken cells (4×10^5 cells/well) were seeded into six-well plates (Costar Corning, China) one day prior to transfection. Recombinant pQCXIN plasmid containing *D. gallinae* gene (2µg) was mixed with 2µg of polyethyleneimine (PEI, Sigma Aldrich, USA) and the mixture transfected into the DF-1 cells incubated for 3 hours before replacing with the fresh medium and continued incubation at 37°C for 72 hours. *D. gallinae* gene expression in the DF-1 cells was analyzed using RT-PCR. Briefly, transfected cells were harvested and RNA was extracted using TRIzol™ reagent (Invitrogen, USA), as per the manufacturer's protocol. To eliminate contaminating DNA, the RNA was treated with DNase I (New England Biolabs, USA). The treated RNA was then used as a template for first-strand cDNA synthesis, catalyzed by reverse transcriptase (Thermo Scientific, USA) and initiated with oligo (dT) primers. Specific primers for *D. gallinae* genes were employed for detecting specific transcripts by PCR. Direct monitoring of mRNA translation was performed using recombinant pQCXIN-RFP plasmid-transfected DF-1 cells. The expression of red fluorescent protein in the transfected cells was observed under a fluorescence microscope (Olympus IX73, Japan) 72 hours post-transfection.

Chicken Immunization

Eighteen female Babcock Brown chickens (18-week-old) were divided into six groups: five groups for the five plasmids, each containing a target *D. gallinae* gene and one negative control group that received only PBS. Each chicken was intramuscularly injected into the breast tissue at both sites with 200µg of endotoxin-free plasmid DNA. Two booster injections were administered at two-week intervals. Eggs from the immunized hens were collected the day before each injection and seven days after the final (third) injection for antibody analysis.

PRM Protein Preparation

Protein extracts were prepared by homogenizing 0.5g of PRM in 1mL of extraction buffer (0.1% SDS in PBS) using a handheld ultrasonic homogenizer set at 60W. The sample was homogenized for 30s and repeated three times. The resulting homogenate proteins, namely PRM, were separated on 10% SDS/PAGE and subjected to Western blot for specific IgY detection.

Immunoglobulin Y Extraction from Egg Yolk

IgY was extracted from egg yolk using the method described by Choi et al. (2010). Briefly, 0.5 g of egg yolk was mixed vigorously with 500µL of PBS (pH 7.4). The mixture was incubated for 1 hour at room temperature to allow IgY to dissolve in PBS, followed by centrifugation at 3000g for 30min at 4°C. The supernatant containing soluble IgY was collected, and total protein content was determined using the DC protein assay (Bradford, USA). Specific IgY against *D. gallinae* proteins was detected by Western blot analysis and quantity of each IgY was determined by indirect ELISA. IgY activity against the poultry red mite (PRM) was evaluated through an *in vitro* feeding assay.

Detection of the PRM-specific Antibody

Proteins from mite homogenates were separated by electrophoresis on a 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, USA) for Western blot. IgY solution (diluted 1:2500 in PBST) extracted from egg yolk was used as a primary antibody and a rabbit anti-chicken IgY horseradish peroxidase-conjugated antibody (Sigma, USA) at a dilution of 1:5000 as a secondary antibody. Specific signals were developed by applying a chemiluminescent substrate (Cyanagen, Italy) to the membrane. To verify the binding of IgY to the PRM, recombinant CatD-1, PUF-1, AKR, SRP-1 and HRF-1 proteins, produced by gene expression in *E. coli* and purified by affinity chromatography as described in the pET System Manual (Novagen, USA), were also run on separate Western blots.

***In vitro* Feeding Assay**

This assay was conducted to evaluate the effect of antibody on the PRM survival, as described by Wright et al. (2009). Egg yolk solutions containing anti-CatD-1 IgY, anti-AKR IgY, anti-PUF-1 IgY, anti-SRP-1 IgY, or anti-HRF IgY, all at titer 1,000, were prepared at three levels of total protein concentrations in PBS: low (-1=50mg), medium (0=150mg), and high (+1=250mg). All five IgYs were combined in various formulations, according to a 2^{5-1} fractional factorial design (FFD) (Table 1) in 250µL PBS then added into 250µL of non-immunized, heparinized chicken blood (36 USP units/mL). Blood containing egg yolk from the PBS-injected chickens, without any PRM-specific IgY (250mg), was served as the negative control. Blood samples were added to the reservoirs of the feeding devices (Nunn et al., 2020). Six *in vitro* feeding devices, each containing 20 PRM, were prepared for each formula (T1-T20) and incubated at 37°C with 75% relative humidity for 24 hours during the feeding period (Marangi et al., 2012). PRM were observed under a stereomicroscope at 0, 4, 24, 48, 72 and 120-hours post-feeding. PRM mortality was assessed based on the absence of movement in response to stimuli.

Statistical Analysis

The time to death among treatment groups was compared using the Kaplan-Meier survival curve, with the log-rank test used to assess the probability of survival distribution among the groups. Significant differences were set at $P<0.05$, with a 5% alpha level and a 95% confidence interval. Statistical analyses for the fractional factorial design included regression analysis, ANOVA, and t-tests to determine whether individual IgY and their interactions significantly affected PRM survival. All analyses were performed using Minitab Statistical Software, version 17.

Table 1: 2^{5-1} Fractional factorial design for IgY supplemented to chicken blood meals for *in vitro* feeding assay

Treatment	IgY combinations in 250µL PBS				
	anti-CatD-1 IgY	anti-PUF-1 IgY	anti-AKR IgY	anti-SRP-1 IgY	anti-HRF-1 IgY
T1	-1	-1	-1	-1	1
T2	1	-1	-1	-1	-1
T3	-1	-1	1	-1	-1
T4	1	-1	1	-1	1
T5	-1	1	-1	-1	-1
T6	1	1	-1	-1	1
T7	-1	1	1	-1	1
T8	1	1	1	-1	-1
T9	-1	-1	-1	1	-1
T10	1	-1	-1	1	1
T11	-1	-1	1	1	1
T12	1	-1	1	1	-1
T13	-1	1	-1	1	1
T14	1	1	-1	1	-1
T15	-1	1	1	1	-1
T16	1	1	1	1	1
T17	0	0	0	0	0
T18	0	0	0	0	0
T19	0	0	0	0	0
T20	Heparinized Chicken blood with non-specific IgY from PBS-injected chickens				

Note: -1 = 50mg, 0 = 150mg and, 1 = 250mg of protein containing IgY

RESULTS

DNA Vaccine Preparation

Construction of Plasmids Expressing *D. gallinae* Genes

Antigens were prepared as a DNA vaccine in the form of plasmids designed to express the target *D. gallinae* genes. The plasmid, pQCXIN, was used as the vector to deliver and express the target gene(s) in chickens under the control of CMV promoter. Five *D. gallinae* genes (*Dg-CatD-1*, *Dg-PUF-1*, *Dg-AKR*, *Dg-SRP-1*, and *Dg-HRF-1*) were amplified by PCR and individually ligated into this plasmid. Additionally, a marker protein gene encoding red fluorescent protein (RFP) was constructed as a model to evaluate the performance of pQCXIN as a gene delivery and expression vector. Recombinant pQCXIN plasmids containing the target genes, pQCXIN-CatD-1, pQCXIN-PUF-1, pQCXIN-AKR, pQCXIN-SRP-1, pQCXIN-HRF-1, and pQCXIN-RFP, were successfully obtained. The presence of the *D. gallinae* genes within the pQCXIN plasmids was confirmed by restriction endonuclease analysis using double digestion with *Not*I/*Eco*RI or *Pac*I/*Eco*RI (Fig. 1) and nucleotide sequence analysis.

D. gallinae Gene Expression Analysis in Transfected Chicken Cells

Before immunizing chickens, all recombinant plasmids were evaluated for their efficiency in delivering and expressing target genes in chicken cells. Following plasmid transfection into DF-1 chicken cell lines, *D. gallinae* gene expression was detected using reverse-transcription PCR. mRNA from plasmid transfected DF-1 cells was first converted into cDNA and then amplified by PCR using gene-specific primers. The PCR products of all cDNAs are shown in Fig. 2a indicating that all genes were successfully expressed. Additionally, DF-1 cells transfected with pQCXIN-RFP exhibited red fluorescent protein expression, confirming that RFP mRNA was efficiently translated in chicken cells (Fig. 2b). These results demonstrate that all recombinant plasmids effectively delivered and controlled the PRM gene to express in chicken cells. All the expressed gene products were also effectively translated to protein antigen in chicken cells.

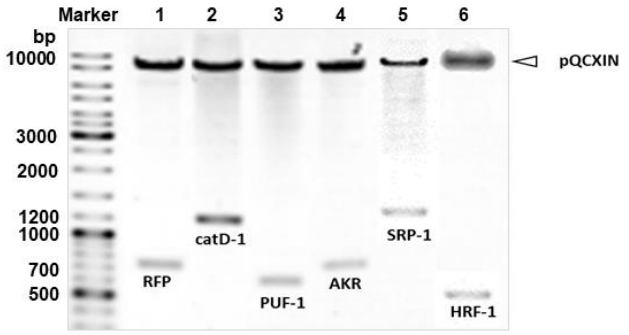

Fig. 1: Agarose gel electrophoresis (1%) of double enzyme digestion of recombinant pQCXIN plasmid containing *D. gallinae*-specific genes. Marker: 2-log DNA ladder. Lane 1-3: pQCXIN-RFP, pQCXIN-CatD-1 and pQCXIN-PUF-1 digested with *Pac*I/*Eco*RI, respectively, Lane 4: pQCXIN-AKR digested with *Not*I/*Eco*RI enzymes, Lane 5-6: pQCXIN-SRP-1 and pQCXIN-HRF-1digested with *Pac*I/*Eco*RI, respectively.

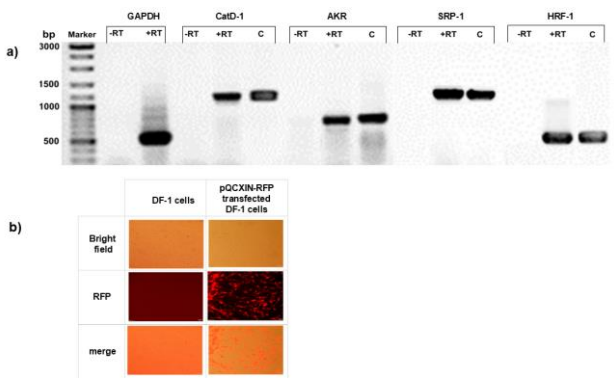

Fig. 2: a) Reverse transcription PCR for detection of the *D. gallinae* gene expression in DF-1 chicken cells. -RT: negative control, PCR of cDNA from reverse transcription reaction without reverse transcriptase. +RT: PCR of cDNA from reverse transcription reaction with reverse transcriptase. C: positive control, PCR using recombinant pQCXIN containing *D. gallinae* genes as a template. b) Fluorescence imaging analysis of pQCXIN-RFP transfected DF-1 cells, three-day post-transfection. Non-transfected DF-1 cells were used as negative control.

Chicken Immune Stimulation and PRM Specific IgY Detection

The efficacy of the PRM vaccine relies on the ability of the vaccine antigen to stimulate antibody production in chickens. After immunizing chickens with the recombinant plasmid, Western blots were performed to detect antibodies specific to each expressed protein. Since chicken antibodies (IgY) are present not only in the blood but also at high concentrations in egg yolks, they can be conveniently extracted from egg yolks. Diluted egg yolk samples were used as primary antibodies to bind specifically to PRM proteins on the Western blot. Fig. 3 shows multiple bands observed on the membrane,

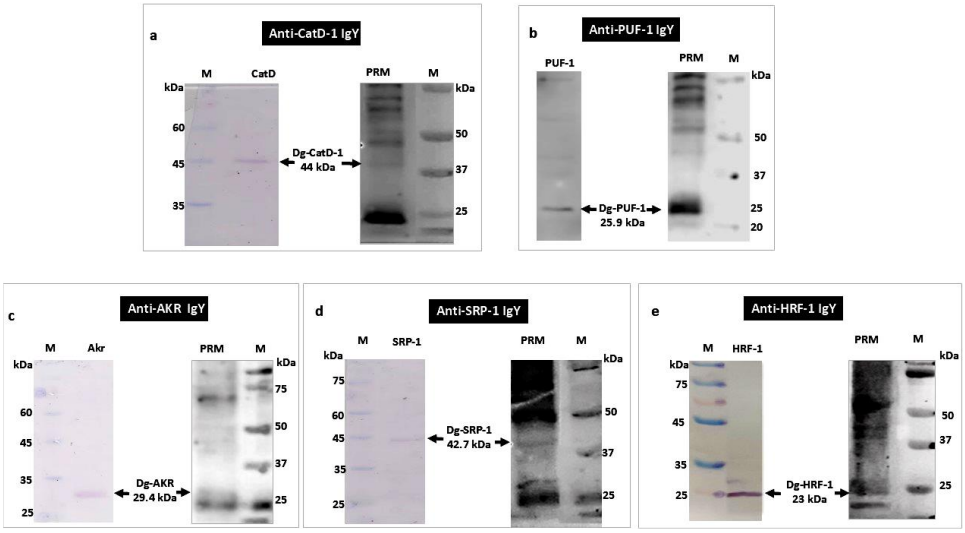

Fig. 3: Western blot for detection of antibody (IgY) specific to *D. gallinae* protein in the egg yolk of immunized chickens. Proteins used for Western blot were protein extracts from *D. gallinae* (PRM) and recombinant proteins which had been produced in *E.coli* and purified as controls. Primary antibodies were the diluted egg yolk solution containing IgY from a) pQCXIN-CatD-1 immunized chicken, b) pQCXIN-PUF-1 immunized chicken, c) pQCXIN-ARK immunized chicken, d) pQCXIN-SRP-1 immunized chicken, e) pQCXIN-HRF-1 immunized chicken. M: marker proteins; PRM: proteins extracted from poultry red mite; CatD-1, PUF-1, ARK, SRP-1and HRF-1 were purified recombinant proteins from *E. coli.*

including bands at the predicted sizes of each expressed protein. To confirm that these bands resulted from specific IgY binding to the expressed protein antigens, the same egg yolk sample was incubated with purified recombinant proteins expressed in *E. coli* as controls. As expected, single bands appeared at approximately 44kDa, 42.7kDa, 25.9kDa, 29.4kDa, and 23kDa, corresponding to recombinant CatD-1, SRP-1, AKR, PUF-1, and HRF-1 proteins, respectively. All egg yolks collected from the plasmid immunized chickens contained their specific IgYs (data not shown).

Assessment of DNA Vaccine Efficacy Against the PRM

The success of DNA vaccines depends on their ability to induce antibodies that interfere with PRM protein functions and subsequently eliminate the parasites. As previously demonstrated by Western blot analysis, anti-CatD-1 IgY, anti-PUF-1 IgY, anti-AKR IgY, anti-SRP-1 IgY, and anti-HRF-1 IgY were successfully produced. The effects of these IgYs on poultry red mite (PRM) survival were then investigated. These five IgYs were extracted from egg yolk, combined, and added to chicken blood, following the 2^{5-1}fractional factorial design (FFD). Three IgY concentration levels, 50mg, 150mg, and 250mg of total proteins, were prepared and designated as -1, 0, and 1, respectively. Nineteen formulations of mixed IgYs in chicken blood meals (T1–T19), including a negative control (T20), were prepared (Table 1) and subjected to *in vitro* feeding assays. Kaplan-Meier curves were generated, and log-rank tests were performed to analyze PRM survival probabilities when exposed to different IgY formulations.

The Kaplan-Meier curves along with the corresponding log-rank statistical analyses revealed that the presence of PRM-specific IgYs in blood meals across all treatments resulted in a significantly lower PRM survival percentage compared to the negative control (T20), $P<0.05$ (data not shown). Fig. 4a illustrates the effects of T1, T2, T3, T5, and T9, where blood meals were supplemented with only one IgY at the highest level (1 of +1 level), while the remaining IgYs were maintained at the lowest level (4 of -1 level). PRM survival curves for these treatments revealed that they shared the same median survival time, with survival dropping to 50% at 72 hours (Table 2). Notably, PRMs fed with a blood meal containing anti-AKR IgY at the highest level (T3) had the shortest 25% survival time (Q1) at just 24 hours.

Table 2: Survival time of PRM and PRM molarity at 96 hours after feeding with blood meals containing IgYs specific to the PRM protein

Treatment	Survival time			% Molarity at 96 hours
	Mean	Q1	Median	
T1	76.3	48	72	56.25
T2	74.9	48	72	58.75
T3	75.8	24	72	56.25
T4	73.3	24	72	57.5
T5	75.1	48	72	59.58
T6	76.6	48	72	56.25
T7	72.6	24	72	61.25
T8	73.5	24	72	58.75
T9	75.6	48	72	58.75
T10	73.7	48	72	60.41
T11	73.7	48	72	59.58
T12	74.7	48	72	57.91
T13	76.2	48	72	57.91
T14	73.8	48	72	60.41
T15	72.5	24	72	60.41
T16	67.9	24	48	66.25
T17	78.1	48	72	57.91
T18	78.2	48	72	56.25
T19	78.4	48	96	57.5
T20	101.6	96	-	-

Note: The mean survival time is estimated as the area under the survival curve in the interval 0 to t_{max}. Q1 survival time is the shortest time at which the PRM survival probability drop to 0.25 (25%). The median survival time is the shortest time at which the PRM survival probability drops to 0.5 (50%).

Fig. 4b presents the effects of T4, T7, T8 and T15, where blood meals contained three IgYs at the highest level (3 of +1 level), as well as T16, which contained all five IgYs at the highest level (5 of +1 level). Kaplan-Meier survival curves indicated that among all treatments, T16 had the shortest median survival time at 48 hours (Table 2). Log-rank test analysis revealed a significant difference in survival between T16 and T3 (P=0.003) but not between T16 and T4, T7, T8, or T15 (Table 3). However, a significant difference (P=0.004) was observed between T16 and T17, which contained all five IgYs at a medium level (5 of 0 level), Fig. 4c. This result further supports the impact of IgY concentration on PRM mortality.

Since the IgY formulations were systematically designed according to the 2^{5-1} fractional factorial design, further statistical analysis was performed to determine the

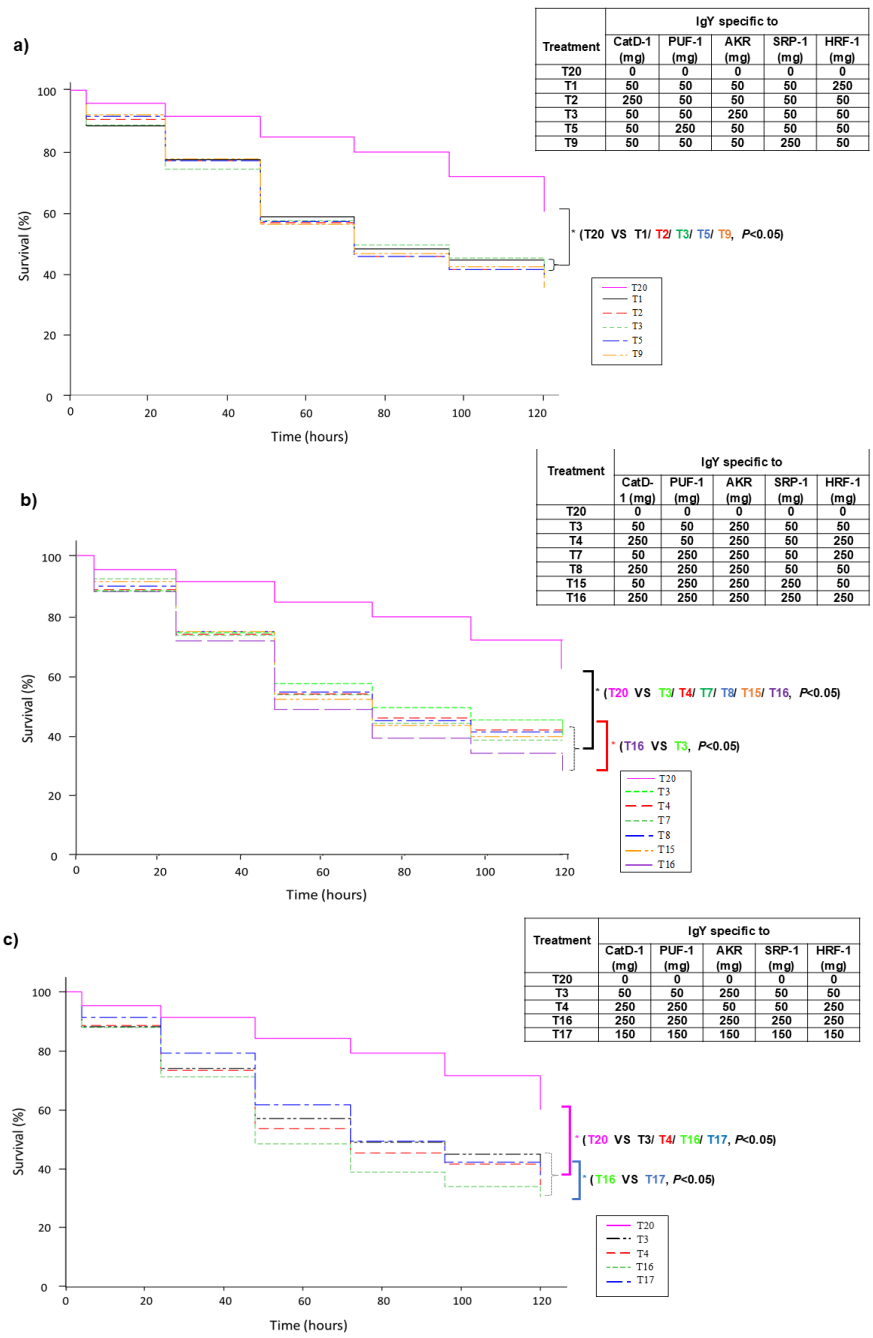

Fig. 4: Assessment of five IgY combinations supplemented in chicken blood meals on *D. gallinae* survival using an *in vitro* feeding assay. Kaplan-Meier plot of **a**) treatments with chicken blood containing one IgY at high concentration (1 at +1 level), **b**) treatments with chicken blood containing three IgYs at high concentration (3 at +1 level), and **c**) treatments with chicken blood containing one IgY at high concentration (1 at +1 level, T3), three IgYs at high concentration (3 at +1 level, T4), all five IgYs at high concentration (5 at +1 level, T16), and all five IgYs at medium concentration (5 at 0 level, T17), compared to the negative control (chicken blood, T20). * Indicates a statistically significant difference.

Table 3: Log rank tests of different T16 VS selected treatments of mite survival data from *in vitro* feeding assay

Treatments	Chi-square	*P*-value
T16 VS T1	7.87319	0.005
T16 VS T2	4.80460	0.028
T16 VS T3	7.64400	0.006
T16 VS T4	3.73226	0.053
T16 VS T5	4.914 21	0.027
T16 VS T7	1.95772	0.162
T16 VS T8	3.75742	0.053
T16 VS T9	5.87555	0.015
T16 VS T15	0.10310	0.748
T16 VS T17	8.2362	0.004
T16 VS T20	115.684	0.000

Note**:** Results with P<0.05 were considered significant. Degrees of Freedom (DF= 1) for all pairwise tests. The critical statistic is 3.841 for an alpha level of 0.05 and one degree of freedom

influence of each IgY and their interactions on PRM mortality. PRM mortality at 96 hours post-feeding (Table 2) was used for this analysis. The results showed that anti-PUF-1 IgY and anti-SRP-1 IgY were the main factors significantly affecting PRM mortality (P=0.047 and P=0.039, respectively) (Table 4). Additionally, significant interactions were observed between anti-AKR IgY and anti-PUF-1 IgY (AKR × PUF-1, P=0.047) and between anti-AKR IgY and anti-HRF-1 IgY (AKR × HRF-1, P=0.036). These findings suggest that the combined effects of these IgYs played crucial roles in PRM survival. Furthermore, log-rank test analysis, as previously described, showed that chicken blood meals containing either of these two pairs of IgYs, found in treatments T4, T7, T8, and T15, were as effective as T16 which contained all five IgYs at +1 level (5 at +1 level). Since a fractional factorial design of resolution V was used in this study, the main factors were theoretically aliased with four-factor interactions, while two-factor interactions were confounded with three-factor interactions. Anti-PUF-1 IgY and anti-SRP-1 IgY were

Table 4: Estimated Effects and Coefficients for PRM Mortality at 96-hour post-feeding

Term[a]	Effect	Coef	SE	Coef	T
Constant	59.141	0.216	272.74	0	-
CatD-1	0.781	0.391	0.2168	1.8	0.213
AKR	1.198	0.599	0.2168	2.76	0.11
PUF-1	1.927	0.964	0.2168	4.44	0.047*
SRP-1	2.135	1.068	0.2168	4.92	0.039*
HRF-1	0.573	0.286	0.2168	1.32	0.317
CatD-1×AKR	-0.052	-0.026	0.2168	-0.12	0.915
CatD-1×PUF-1	-0.156	-0.078	0.2168	-0.36	0.753
CatD-1×SRP-1	1.302	0.651	0.2168	3	0.095
CatD-1×HRF-1	0.573	0.286	0.2168	1.32	0.317
AKR×PUF-1	1.927	0.964	0.2168	4.44	0.047*
AKR×SRP-1	0.469	0.234	0.2168	1.08	0.393
AKR×HRF-1	2.24	1.12	0.2168	5.16	0.036*
PUF-1×SRP-1	0.156	0.078	0.2168	0.36	0.753
PUF-1×HRF-1	0.052	0.026	0.2168	0.12	0.915
SRP-1×HRF-1	1.094	0.547	0.2168	2.52	0.128

Note: [a] protein name referred to the IgY that is specific to that protein e.g. CatD-1 is anti-CatD-1 IgY. S = 0.867361, R-Sq = 98.53%, R-Sq(adj) = 86.76%
× represents interaction between IgY, * Statistical significance at 95% confidence interval

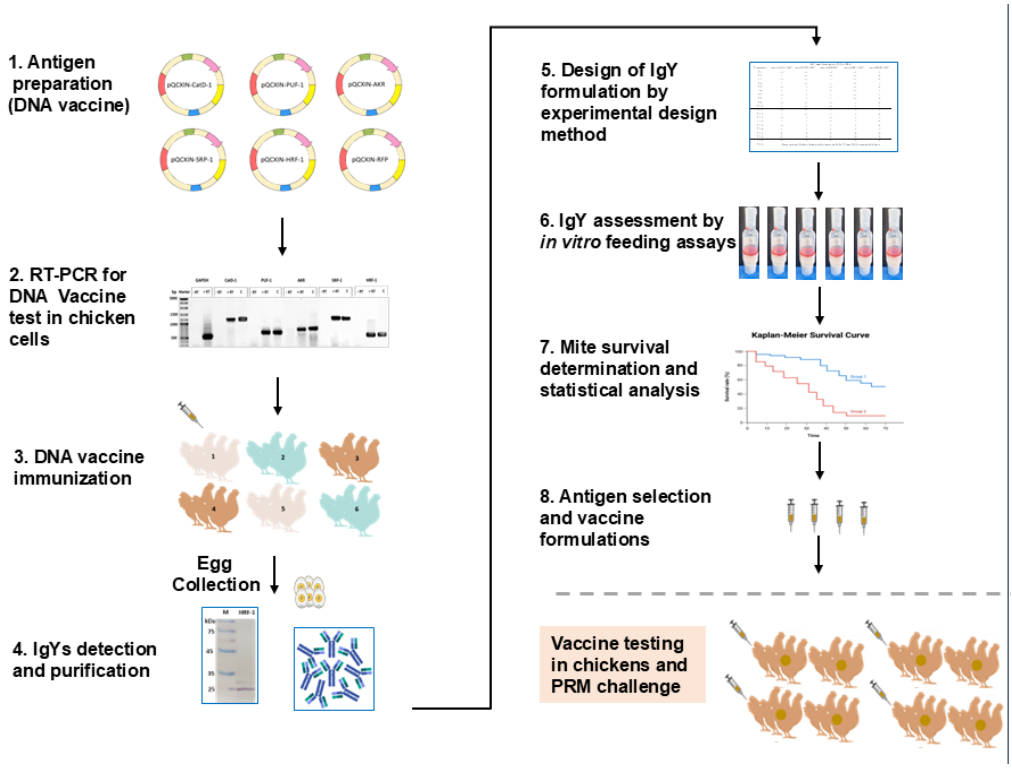

Fig. 5: A simple, rapid, and systematic method for assessing multiple PRM protein antigens simultaneously using *in vitro* feeding assays with an experimental design approach. With this screening method, fewer vaccine formulations need to be tested in chickens, thereby reducing the number of chickens used.

expected to be aliased with other four-factor combinations (CatD-1 × AKR × SRP-1 × HRF-1 and CatD-1 × AKR × PUF-1 × HRF-1, respectively). Therefore, it can be concluded that all five IgYs were significant. The information obtained from this screening method is valuable for selecting and designing candidate antigens for testing in chickens in the next step. A summary of this proposed screening method is illustrated in Fig. 5.

DISCUSSION

Five PRM proteins with different functions, previously confirmed as potential antigens for a single-antigen PRM vaccine, were selected as model antigens for method demonstration. These included Cathepsin D-1, a protein of unknown function (PUF-1), Akirin, Serpin-1, and Histamine Release Factor-1 (HRF-1). The genes encoding these proteins, Dg-catD-1, Dg-PUF-1, Dg-AKR, Dg-SRP-1, and Dg-HRF-1, were individually cloned into the pQCXIN plasmid. This plasmid was chosen because it contains a CMV promoter capable of driving gene expression in chickens (Yang et al., 2014). The recombinant plasmids were evaluated in DF-1 chicken cell lines, and all five genes were successfully expressed, as confirmed by the detection of all five mRNAs via RT-PCR. Additionally, red fluorescent protein (RFP) was readily detected in pQCXIN-RFP-transfected cells, indicating effective translation of the expressed mRNA. Therefore, the pQCXIN plasmid was deemed suitable as a vector for a DNA vaccine in chickens. Although this is the first report of using DNA vaccines against PRM, DNA vaccines have previously been used successfully in chickens (Liu et al., 2022; Valentin et al., 2024). They have been shown to elicit neutralizing antibodies as part of the humoral immune response and to stimulate T cells as part of the cellular immune response (Meunier et al., 2015). Furthermore, DNA vaccines can be administered to the target animal without the need for an adjuvant. Hence, a DNA vaccine antigen was chosen for this method.

The findings confirm that DNA vaccination using the pQCXIN vector successfully induced antigen-specific immune responses in chickens. The detection of anti-CatD-1 IgY, anti-AKR IgY, anti-SRP-1 IgY, and anti-HRF-1 IgY in egg yolks demonstrates that the encoded PRM antigens were effectively expressed and recognized by the avian immune system. This outcome highlights the potential of DNA vaccines to elicit strong humoral responses, even in the absence of adjuvants.

The presence of additional bands in the western blot, resulting from non-specific interactions between natural polyclonal IgYs and PRM proteins, is consistent with the natural immunological background of chickens. Importantly, the binding specificity of the induced IgYs was validated through the use of purified recombinant PRM proteins, confirming that the observed immune responses were directed against the intended target antigens rather than cross-reactive proteins. These results further support the suitability of pQCXIN as a DNA vaccine vector for poultry, aligning with previous studies showing that DNA vaccines can stimulate both humoral and cellular immune responses in chickens (Meunier et al., 2015; Liu et al., 2022; Valentin et al., 2024). The use of egg yolks as a non-invasive source of antibodies also underscores the practicality of this approach for evaluating antigenicity in poultry models.

The ability of antigen-specific IgYs to interfere with their target proteins and induce PRM mortality was evaluated to guide the selection of antigens for a multi-antigen vaccine. In this study, all treatments containing PRM-specific IgYs resulted in a significantly lower PRM survival rate compared to the negative control (T20), which lacked PRM-specific IgY. The mortality observed in some PRMs from T20 was primarily due to starvation and exposure to unfavorable conditions rather than immunological effects. Kaplan-Meier survival analysis showed no statistically significant differences among treatments with single IgYs at high concentrations (1 at +1 level). However, PRMs fed with blood meals containing anti-AKR IgY at the highest level (T3) exhibited the shortest 25% survival time (Q1), reaching just 24 hours. This highlights the critical role of Akirin, a regulatory protein that directly or indirectly interacts with transcription factors, chromatin remodelers, and RNA-associated proteins (Filimonova, 2013). The rapid killing effect observed is consistent with previous findings by Lima-Barbero et al. (2019), who reported a 42% reduction in mite oviposition following vaccination with Deg-AKR. Although oviposition was not assessed in this study, inhibition of Akirin by anti-AKR IgY likely disrupted multiple downstream pathways, leading to impaired biological processes and accelerated PRM mortality.

The log-rank test of survival curves indicated that treatment T16, which contained all five IgYs at the highest level (5 at +1 level), exhibited significantly greater efficacy ($P<0.05$) than treatments with only a single IgY at the highest level (1 at +1 level), specifically T1, T2, T3, T5, and T9. These results suggest that combining multiple antibodies exerts a stronger impact on PRM mortality than using a single antibody. Statistical analysis of the fractional factorial design further identified anti-PUF-1 IgY and anti-SRP-1 IgY as the main factors significantly affecting PRM survival. Although the function of PUF-1 remains unknown, this screening approach confirmed its potential as a vaccine antigen, supporting earlier findings by Bartley et al. (2017). Likewise, the role of Serpins as protease inhibitors (Simmone and Higgins, 2015) underlines their relevance as effective antigen targets. Both PUF-1 and Serpin are therefore recommended for inclusion in a next-generation multi-antigen PRM vaccine for testing in chickens.

Further analysis revealed significant interactions among all five IgYs, with particularly notable interactions between anti-AKR IgY and anti-PUF-1 IgY, as well as between anti-AKR IgY and anti-HRF-1 IgY ($P<0.05$). Given Akirin's regulatory role in conjunction with transcription factors and other proteins, it may influence pathways involving PUF-1 and HRF-1, making these combinations especially promising. Incorporating these antigen pairs into a multi-antigen PRM vaccine could therefore enhance efficacy. Among the four antigens evaluated using this screening method, Akirin, Serpin, PUF-1, and HRF-1, all showed significant contributions to PRM mortality, with the exception of Cathepsin D-1. Cathepsin D-1, a lysosomal endopeptidase responsible for hemoglobin digestion in mites (Pritchard et al., 2015; Price et al., 2019), may have had limited impact under the conditions of this *in vitro* assay, which could explain its lack of observable effect.

This study demonstrates the utility of an *in vitro* screening platform for evaluating multi-antigen combinations, providing valuable insights for rational vaccine antigen selection. The observed results align closely with the known biological functions of each antigen, supporting the reliability of this approach as an alternative to conventional antigen screening performed directly in chickens. By using *in vitro* pre-screening to narrow down high-potential antigen formulations, subsequent *in vivo* testing in chickens can be minimized, ultimately reducing animal use, costs, labor, and time.

Conclusion

This study establishes a simple, rapid, and animal-friendly method for assessing multiple protein antigens in the development of a poultry red mite (PRM) multi-antigen DNA vaccine. By utilizing plasmid-based antigen preparation and antibody collection from egg yolks of immunized laying hens, the approach minimizes animal use and stress. The use of artificial feeding devices and systematic experimental design methods, such as factorial design and response surface methodology, enables efficient evaluation of antibody combinations and their effects on PRM survival. Statistical tools, including Kaplan–Meier survival analysis, facilitate the identification of key antigen formulations with significant efficacy. As multi-antigen vaccines have demonstrated superior performance compared to single-antigen vaccines, and new candidate antigens continue to be discovered, this method provides a practical and effective framework for selecting optimal antigen combinations and accelerating the development of next-generation PRM vaccines.

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Author's Contribution: GCK: Performed the experiments and wrote the original draft. NK, SC: Performed protein expression and purification in *E. coli*. SA: Designed the statistical analysis and performed data analysis. YMS: Interpreted the results and experimental design. KP: Designed the research project, conceptualized the study, edited the original draft, and supervised the work.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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