

This is an original manuscript / preprint of an article published by Taylor & Francis in AVIAN PATHOLOGY on 29 April 2019, available online:

<http://www.tandfonline.com/10.1080/03079457.2019.1612514>.

The full details of the published version of the article are as follows:

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JOURNAL TITLE: Avian Pathology

PUBLISHER: Taylor & Francis

PUBLICATION DATE: 29 April 2019 (online)

DOI: 10.1080/03079457.2019.1612514

Publisher: Taylor & Francis & Houghton Trust Ltd

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Evaluation of vaccine delivery systems for inducing long-lived antibody responses to *Dermanyssus gallinae* antigen in laying hens

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Abstract

Dermanyssus gallinae, the poultry red mite, is a global threat to the commercial egg-laying industry. Control of *D. gallinae* is difficult, with only a limited number of effective pesticides and non-chemical treatments available. Here we characterise the candidate vaccine antigen *D. gallinae* cathepsin D-1 (Dg-CatD-1) and demonstrate that purified refolded recombinant Dg-CatD-1 (rDg-CatD-1) is an active aspartyl proteinase which digests haemoglobin with a pH optimum of pH 4. Soluble protein extracts from *D. gallinae* also have haemoglobinase activity, with a pH optimum comparable to the recombinant protein and both proteinase activities were inhibited by the aspartyl proteinase inhibitor Pepstatin A. Enzyme activity and the ubiquitous localization of Dg-CatD-1 protein in sections of adult female mites is consistent with Dg-CatD-1 being a lysosomal proteinase. Using Dg-CatD-1 as a model vaccine antigen, we compared vaccine delivery methods in laying hens *via* vaccination with: i) purified rDg-CatD-1 with Montanide™ ISA 71 VG adjuvant; ii) recombinant DNA vaccines for expression of rDg-CatD-1 and iii) transgenic coccidial parasite *Eimeria tenella* expressing rDg-CatD-1. In two independent trials, only birds vaccinated with rDg-CatD-1 with Montanide™ ISA 71 VG produced a strong and long-lasting serum anti-rDg-CatD-1 IgY response, which was significantly higher than control birds vaccinated with adjuvant only. Furthermore, we showed that egg laying rates of *D. gallinae* mites fed on birds vaccinated with rDg-CatD-1 in Montanide™ ISA 71 VG was reduced significantly compared with mites fed on unvaccinated birds.

Keywords. Sub-unit vaccine; DNA vaccine; live vaccine; vaccinology; ectoparasite; poultry red mite; aspartyl proteinase

Research Highlights

- *Dermanyssus gallinae* cathepsin D-1 (Dg-CatD-1) digests haemoglobin
- Vaccination of hens with rDg-CatD-1 in Montanide™ ISA 71 VG results in long-lasting IgY levels
- Serum anti-rDg-CatD-1 antibodies reduce egg laying in *D. gallinae* after a single blood meal

Introduction

The poultry red mite (PRM), *Dermanyssus gallinae* (De Geer) is a blood feeding ectoparasite that infests many avian species. Globally, from both economic and welfare perspectives, PRM is the most important parasite to the egg-laying industry. Across Europe, PRM prevalence is high, with up to 83% of commercial egg-laying facilities infested (George et al., 2015). In Europe alone, heavy infestations of poultry houses with PRM costs the industry an estimated 231 million Euro per annum (Sigognault Flochlay, Thomas, & Sparagano, 2017; Van Emous, 2017). PRM are difficult to control as they live off-host in inaccessible areas of the poultry house, and emerge during darkness to feed from birds for up to 1 hour every 2 – 4 days (George et al., 2015; Sparagano, George, Harrington, & Giangaspero, 2014). Heavy infestations of birds can reach up to 500,000 mites per bird and cause serious welfare issues, including anaemia, irritation and restlessness as well as significant reduction in egg production (Kilpinen et al., 2005; Sigognault Flochlay et al., 2017). Currently, PRM populations are controlled during periods when hen-houses are empty using synthetic acaricides, or during production cycles using an isoxazoline based drug (Miglianico et al., 2018; Sparagano et al., 2014). However, long-term repeated use of the older classes of acaricides has resulted in emergence of resistant PRM populations (Abbas, Colwell, Iqbal, & Khan, 2014; Beugnet, Chauve, Gauthey, & Beert, 1997). In addition, due to concerns about safety and environmental

contamination, many synthetic acaricides have been withdrawn from use, exacerbating the problem of PRM control (Sparagano et al., 2014).

Vaccination can provide high levels of protection against blood feeding ectoparasites, thereby reducing reliance on chemical control methods (de la Fuente & Kocan, 2003; Willadsen, 2004). For example, the commercial cattle vaccines, TickGARD™ and Gavac™, both based on administration of recombinant Bm86 protein, have been used to protect vaccinated cattle against the tick, *Rhipicephalus microplus* (Rand et al., 1989; Rodriguez et al., 1995). Moreover, studies show that vaccination has the potential to control PRM infestations of chickens: Experimental vaccination with protein extracts from *D. gallinae* mites (Bartley et al., 2015; Harrington, Din, Guy, Robinson, & Sparagano, 2009; Makert et al., 2016; Wright et al., 2009), or with recombinant-expressed *D. gallinae* antigens (Bartley, Huntley, Wright, Nath, & Nisbet, 2012; Bartley et al., 2009; Bartley et al., 2015; Harrington, Canales, et al., 2009; Wright, Bartley, Huntley, & Nisbet, 2016), induces antigen-specific serum IgY that reduces mite survival when fed to mites in *in vitro* feeding devices. Furthermore, we recently showed that vaccination of laying hens, housed in a commercial-style poultry facility, with a native PRM soluble protein extract results in protective immunity, reducing average mite counts in cages of vaccinated birds by 78% compared to cages of control, unvaccinated birds (Bartley et al., 2017).

Vaccine delivery methodology is particularly salient in the poultry layer sector. Vaccination programmes operate at large scales in hatchlings, or immature hens, and need to induce and sustain effective immunity through the full laying cycle, which commonly ranges between 60 and 80 weeks. This need for prolonged protection is one of the most challenging aspects of developing vaccines for laying flocks, particularly for ectoparasites where immunoprotective vaccine antigens (including Bm86) may be “hidden” because they are not naturally exposed to the host immune system during parasite feeding. This means there is no opportunity for natural immunological recall responses (boosting) against this type of antigen when hosts are exposed to the parasite.

In the current study we use a prototype vaccine antigen *D. gallinae* cathepsin D1 (Dg-CatD-1) (Bartley et al., 2012), and compare antigen delivery to laying hens by: i) immunization with purified recombinant Dg-CatD-1 in adjuvant; ii) recombinant DNA vaccination for expression of Dg-CatD-1 and iii) oral challenge with populations of the live transgenic coccidial parasite *Eimeria tenella* expressing Dg-CatD-1. These delivery platforms were chosen to compare antigen specific antibody titres in laying hens using tried and tested protein in adjuvant vaccination (Bartley et al., 2017), with new vaccine technologies which include DNA vaccination (Gupta, Dey, & Chellappa, 2016; Meunier, Chemaly, & Dory, 2016) and a novel experimental *Eimeria tenella* antigen delivery system (Clark et al., 2012; Marugan-Hernandez et al., 2016; Pastor-Fernandez et al., 2018), in order to identify the optimal system. We assess each delivery platform for the ability to induce specific anti-Dg-CatD-1 IgY in laying hens and monitor longevity of the response over a commercially relevant period. With the delivery method that gave the highest antibody responses and the longest duration of response, we went on to test vaccine efficacy of the prototype vaccine antigen using a recently-developed novel *in vivo* challenge system (Nunn et al., 2019).

Materials and Methods

Ethics statement. All experimental procedures were approved by the Moredun Research Institute Experiments and Ethics Committee and conducted under the legislation of UK Home Office Project License (reference P46F495BD), or by the Royal Veterinary College Animal Welfare & Ethical Review Body and conducted under the legislation of UK Home Office Project License (reference 70-7781).

Purification and refolding recombinant Dg-CatD-1. Expression of recombinant Dg-CatD-1 (rDg-CatD-1) was carried out in BL21-CodonPlus[®](DE3)-RIPL *E. coli* cells as previously described (Bartley et al., 2012). Following expression, cells were lysed by sonication and the insoluble inclusion bodies were isolated by centrifugation at 13,000 x g for 20 min at 4°C.

Inclusion bodies were solubilised in binding buffer (8M urea, 20 mM Tris-HCl, 0.5M NaCl, 45 mM imidazole, pH8.0) and rDg-CatD-1 was purified by nickel-affinity chromatography on HisTrap™ HP columns using an ÄKTA™ chromatography system (GE Healthcare Life Sciences). rDg-CatD-1 was eluted in binding buffer with a linear imidazole gradient (up to 500 mM imidazole), within 5 column volumes.

Fractions containing purified rDg-CatD-1 were pooled and refolded at 4°C by step-wise dialysis into refolding buffer (100 mM Tris-HCl, 400 mM L-arginine, 2 mM EDTA, 0.5 mM oxidised glutathione, 5mM reduced glutathione, pH8.0) with decreasing concentrations of urea. Briefly, rDg-CatD-1 was contained within cellulose membrane dialysis tubing (Sigma) and dialyzed for 3 h into refolding buffer with 6 M urea; 3 h into refolding buffer with 4 M urea; overnight into refolding buffer with 2 M urea; 3 h into refolding buffer with 1 M urea; and finally overnight into 10 mM Tris-HCl (pH 7.4), 0.5 M NaCl. After refolding, rDg-CatD-1 was concentrated using a Centriprep® centrifugal filter with Ultracel-10 membrane (Merck) followed by filtration through a 0.2 µm sterile filter (Sartorius). rDg-CatD-1 protein concentration was determined by BCA assay (Pierce) with BSA standards, and purity was confirmed by SDS-PAGE analysis. Purified, refolded rDg-CatD-1 was stored at 4°C prior to use.

Haemoglobin digest assay. Hydrolysis of haemoglobin by purified refolded rDg-CatD-1 was determined using a haemoglobin digest assay, according to (Houseman & Downe, 1982). As a positive control, the haemoglobin digest assay was also performed with a soluble mite extract, with protein extracts prepared as described previously (Wright et al., 2009). For each assay, equal volumes of 20 mg/ml bovine haemoglobin (Sigma) in 0.15 M NaCl, and of a 200 mM buffer of varying pH (pH 2 Glycine-HCl buffer; pH 3 - 6 citrate buffer; pH 7 HEPES buffer) were mixed and incubated at 37°C for 5 min. Digestion was initiated by adding refolded rDg-CatD-1, or soluble mite extract to the haemoglobin/buffer mixture and assays were incubated at 37°C for 20 h. Reactions were terminated by addition of 10% (w/v) trichloroacetic acid (final concentration) and precipitated polypeptides were removed by centrifugation at 13,000 x g for 10 min at 4°C. Absorbance values of trichloroacetic acid (TCA) soluble peptides in

supernatants were measured at 280 nm and used to calculate enzyme activity. For inhibition assays, a 1mg/ml pepstatin A (Sigma) stock was prepared in 10% acetic acid in methanol (9:1 methanol:acetic acid). Inhibition assays were performed as described above, in pH4 citrate buffer, with the addition of 2 μ M pepstatin A (final concentration; control reactions contained no pepstatin A, but an equivalent volume of 10% acetic acid in methanol). For all assays, controls lacking test samples were run in parallel and activity data were corrected for any spontaneous haemoglobin breakdown. One haemoglobin enzyme unit (Hb.E.U.) is defined as an increase of one absorbance unit at 280 nm, per mg protein, per hour.

SDS-PAGE and Western blot analysis. Proteins were analysed by SDS-PAGE using the NuPAGE[®] electrophoresis system (Invitrogen). Briefly, protein samples were prepared in NuPAGE LDS sample buffer with reducing agent and heated to 70°C for 10 min prior to loading on NuPAGE 4 – 12% Bis-Tris gels. Gels were run in MES SDS running buffer and either stained with SimplyBlue[™] SafeStain (Invitrogen) or prepared for Western blots.

For Western blot analysis, proteins were transferred to nitrocellulose membranes by electroblotting in 1X NuPAGE transfer buffer, according to the manufacturer's instructions (Invitrogen). After transfer, to prevent non-specific protein binding, membranes were blocked by incubation at room temperature for 2 hrs with 5% (w/v) milk powder in TBST (20 mM Tris-HCl, pH7.6, 150 mM NaCl, 0.1% (v/v) TWEEN[®] 20). Blots were washed three times with TBST, then incubated with antiserum raised in rabbits against rDg-Cat-D1. Antiserum was generated using a custom service provided by ImmunoTools and used at 1:3000 dilution in 5% milk powder in TBST. After washing three times in TBST, membranes were incubated with swine anti-rabbit IgG HRP (Dako) at 1:3000 dilution in TBST for 1 hour at room temperature. Membranes were washed three times with TBST and specifically bound antibodies were detected using SIGMAFAST[™] 3,3'-Diaminobenzidine (DAB) substrate.

Immunohistochemical localisation of Dg-CatD-1 in *D. gallinae* tissues. Mites were collected and stored at room temperature for 7 days to allow complete digestion of their blood

meal, and then fixed in Carnoy's fixative (60% absolute ethanol, 30% chloroform, 10% glacial acetic acid) for 5 days at 4°C. After fixation, mites were washed in 150 mM NaCl then orientated and pre-embedded in 2% agar:2.5% gelatin according to (Jones & Calabresi, 2007). Tissues were dehydrated using a graded ethanol series and then infiltrated with paraffin wax using a Tissue-Tek VIP 6 AI automated vacuum infiltration processor (Sakura). After processing tissues were embedded in paraffin wax blocks and sections of 5 µm thickness cut and placed on Superfrost® Plus glass slides (ThermoFisher Scientific). Tissue sections were deparaffinised in Histo-Clear (National Diagnostics) and then rehydrated in graded alcohols. Sections were washed in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) and endogenous peroxidase quenched with 3% (v/v) H₂O₂ at room temperature for 20 min. Tissue sections were blocked by incubation in 25% normal goat serum (v/v) (Vector Laboratories) in PBS with 0.5% (v/v) TWEEN® 80 (PBST80), then incubated overnight at 4°C with primary rabbit anti-Dg-CatD-1 antisera at 1:10,000 (final concentration) in 10% (v/v) normal goat serum in PBST80. Bound antibodies were detected using EnVision+ System-HRP/DAB (Dako), according to the manufacturer's instructions and then counterstained with haematoxylin. Sections incubated with pre-immune rabbit sera were processed in parallel and used as negative controls. Images of tissue sections were captured on an Olympus microscope equipped with a CCD camera.

Preparation of Dg-CatD-1 antigen for comparative vaccination experiments.

Formulation of rDg-CatD-1 in Montanide™ ISA 71 VG adjuvant

Purified refolded rDg-Cat-D1 protein was formulated in Montanide™ ISA 71 VG (SEPPIC) according to manufacturer's guidelines. Briefly, rDg-Cat-D1 in 10 mM Tris-HCl (pH7.4), 0.5 M NaCl was mixed with Montanide™ ISA 71 VG (adjuvant/antigen ratio of 70:30, based on weight) and a stable water-in-oil emulsion was produced using a IKA® T25 Ultra Turrax® high shear mixer with a S25N-8G head at 15,600 rpm for 3 min. For adjuvant-only control injections, 10 mM Tris-HCl (pH7.4), 0.5 M NaCl was mixed with Montanide™ ISA 71 VG and processed as described above.

Preparation of IL21::Dg-CatD-1::mCherry DNA Vaccine

Three synthetic *IL21::Dg-CatD-1::mCherry* fusion genes were designed for DNA vaccination and synthesized by GeneArt gene synthesis (Thermo Fisher Scientific). Each synthetic gene contained a continuous open reading frame, and encoded sequence variants of the following fusion partners: i) *Gallus gallus* IL-21 (NM_001024835); ii) Dg-CatD-1 (HE565350); and iii) fluorescent reporter mCherry (AY678264). In each of the synthetic genes, fusion partners were joined by an alanine (ala-ala-ala) linker region. Full details of each synthetic gene are shown in Table 1, and sequences of each of synthetic gene is shown in Supplementary File 1.

To produce DNA vaccines, each synthetic gene was PCR amplified using CloneAmp HiFi PCR premix (Takara) and directionally cloned into the XhoI site of pVAX1 (Thermo Fischer Scientific) using the In-Fusion HD cloning kit (Takara), according to the manufacturer's instructions. All synthetic *IL21::Dg-CatD-1::mCherry* fusion genes were sequenced after gene synthesis (Thermo Fisher Scientific), and re-sequenced using Sanger sequencing after cloning into pVAX1 to ensure correct orientation of each of the genes. Sequence data were edited and assembled into contiguous sequence using Contig Express module in Vector Nti (version 11) (Thermo Fisher Scientific). For both pilot and long-term vaccine trials, endotoxin-free plasmid DNA for pVAX1 DNA vaccines (Construct 1 - 3) was isolated using NucleoBond PC 10000 EF DNA purification kit (Macherey-Nagel, Düren, Germany) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Prior to DNA vaccination, purified pVAX1 vaccine DNA (construct 1 – 3) and negative control DNA (empty pVAX1 DNA) were validated by transfection of HEK-293 cells, and mCherry fluorescence monitored using a Nikon fluorescence microscope and images captured with a Nikon DXM1200F digital camera.

Generation of transgenic Eimeria tenella expressing Dg-CatD-1::mCherry

A synthetic *Dg-CatD-1::mCherry* reporter fusion gene was designed for expression in *E. tenella* to allow fluorescence detection of Dg-CatD-1::mCherry protein in transformed

parasites. The *Dg-CatD-1* coding sequence (HE565350) without the N-terminal signal sequence and stop codon (corresponding to 49 – 1150 bp of the *Dg-CatD-1* coding sequence) was fused to an in-frame C-terminal mCherry reporter (AY678264) via an alanine (ala-ala-ala) linker region, and included a C-terminal 6x-His-tag and glycosylphosphatidylinositol (GPI) signal from *E. tenella* surface antigen SAG D09 (AIR91965). The synthetic gene was codon optimized for *E. tenella* (<http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=5802&aa=1&style=GCG>), and included a 5' XbaI site and 3' BglII site to facilitate cloning into *Eimeria* transformation vectors. The sequence of the synthetic gene is shown in Supplementary File 1.

The synthetic *Dg-CatD-1::mCherry* reporter fusion gene was used to produce *E. tenella* transformation vectors for: i) cytoplasmic expression of *Dg-CatD-1::mCherry*; ii) secreted expression of *Dg-CatD-1::mCherry* and iii) GPI-anchored expression of *Dg-CatD-1::mCherry*. To produce transformation vectors for cytoplasmic and secreted expression of *Dg-CatD-1::mCherry* the synthetic *Dg-CatD-1::mCherry* reporter fusion gene was PCR amplified using MyTaq DNA polymerase (Bioline) with a forward primer with an XbaI site (5'-CATTCAAATGCTAGAGACCTG-3'), and a reverse primer with a BglII site and stop codon (5'-CGTAGATC**TTAGTGGTGGTGGTGGTGG**-3'); both restriction enzyme sites are underlined and the stop codon shown in bold. The PCR product was cloned using the pGEM-T Easy vector system (Promega), according to the manufacturer's instructions, fully sequenced (GATC Biotech) and sequence data assembled into a contiguous sequence using CLC Main Workbench (Qiagen). The sequenced coding region was digested with XbaI and BglII and cloned into the respective sites of either: p5M2-mChe (Marugan-Hernandez, Long, Blake, Crouch, & Tomley, 2017) to produce 'Cytoplasmic *Dg-CatD-1::mCherry*' *E. tenella* transformation vector; or p5M2-SP2-mChe (Marugan-Hernandez et al., 2017) to produce 'Secreted *Dg-CatD-1::mCherry*' *E. tenella* transformation vector. The GPI-anchored *Dg-CatD-1::mCherry* variant was produced by digesting the original (unamplified) Eurofins synthetic *Dg-CatD-1::mCherry* reporter fusion gene with XbaI and BglII and cloned into the respective sites

of p5M2-SP2-mChe-GPI to produce 'GPI-anchored Dg-CatD-1::mCherry'. All *Eimeria* p5M2 transformation vectors used here, were generated in a previous study (Marugan-Hernandez et al., 2017), and were a kind gift from Virginia Marugan-Hernandez (Royal Veterinary College, UK).

For transformation of *E. tenella* Wisconsin strain (McDougald & Jeffers, 1976), oocysts were propagated in 3 – 4 week old specific pathogen free (SPF) Light Sussex or Lohmann Selected Leghorn chickens (supplied by Millennium Hatchery) using established techniques (Long, Millard, Joyner, & Norton, 1976). Oocysts were recovered and sporulated, then sporozoites were purified through columns of nylon wool and DE-52, using standard methods (Schmatz, Crane, & Murray, 1984; Shirley, Bushell, Bushell, McDonald, & Roberts, 1995). Sporozoites transformed with 10 µg PstI linearized p5M2-Dg-CatD-1::mCherry transfection plasmids (Plasmids 1-6) by restriction enzyme-mediated integration (REMI) transfection using the Nucleofector 4D system (Lonza) according to (Marugan-Hernandez et al., 2017). Transformation vectors included: p5M2-Dg-CatD-1::mCherry, for cytoplasmic expression of Dg-CatD-1::mCherry; p5M2-SP2-Dg-CatD-1::mCherry, for secreted expression of Dg-CatD-1::mCherry; and p5M2-SP2-Dg-CatD-1::mCherry-GPI, for GPI-anchored expression of Dg-CatD-1::mCherry. For control *E. tenella* populations, sporozoites were excysted and transformed as described above using p5M2 transformation vectors without the Dg-CatD-1 coding sequence (control vectors included: p5M2-mChe; p5M2-SP2-mChe and p5M2-SP2-mChe-GPI). To obtain stable transgenic populations, transfected sporozoites were used for cloacal administration in birds ($12-24 \times 10^3$ sporozoites/bird). Oocysts were harvested 7 days after infection, sporulated and used for subsequent *in vivo* passages after population enrichment for fluorescent parasites by FACS (BD FACS Aria III) (Marugan-Hernandez et al., 2016). A full list of transgenic *E. tenella* strains generated in this study is shown in Table 1.

Dg-CatD-1 pilot (P) immunisation trial. A pilot vaccination trial was designed to compare circulating serum anti-Dg-CatD-1 IgY antibody levels in Hy-Line Brown hens after Dg-CatD-1

antigen delivery by: i) vaccination with purified rDg-CatD-1 formulated in Montanide™ ISA 71 VG; ii) vaccination with DNA for transient expression of Dg-CatD-1::mCherry; or iii) oral vaccination with transgenic *Eimeria tenella* expressing Dg-CatD-1::mCherry. The pilot trial was performed under specific pathogen free-conditions (SPF) and ran for 8 weeks. Prior to the start of the trial, 72 x one day old chicks (supplied by Millennium Hatchery, UK) were co-housed for 8 days in a loose litter floor pen (diameter 2.4 m) with layer mash and water provided *ad libitum*. At the beginning of the trial (experimental day 0, eight days of age) 6 groups of 6 chicks were randomly selected, assigned to groups P7-P12, and housed in loose litter floor pens of 1.0 m diameter. The remaining chicks, representing groups P1-P6, were co-housed in the original 2.4m diameter pen. All chicks were wing tagged to facilitate identification. All groups received a standard layer mash and water *ad libitum* throughout the trial. Blood samples were taken from two birds per group prior to each treatment and at intervals throughout the trial. Groups received the following treatments:

Groups P1 & P2. Recombinant protein vaccination. Each bird from the protein vaccination group (Group P1), received three doses of 50 µg rDg-CatD-1 in Montanide™ ISA 71 VG via intramuscular (IM) injection into the thigh at days 2, 12, and 29 of the trial. The adjuvant-only negative control group (Group P2) received equivalent injections with 10 mM Tris-HCl (pH7.4), 0.5 M NaCl in Montanide™ ISA 71 VG.

Groups P3 - P6. DNA vaccination. Each bird was vaccinated three times with 56 µg DNA vaccine with 1 mg/kg bupivacaine via IM injection into the thigh at days 2, 12, and 29 of the trial. Group P3 birds were vaccinated with DNA construct 1; Group P4 birds were vaccinated with DNA construct 2; Group P5 birds were vaccinated with DNA construct 3. Details of all DNA constructs are shown in Table 1. The empty vector negative control group (Group P6) received equivalent injections with 56µg pVAX1 (empty vector) with 1mg/kg bupivacaine.

Groups P7 - P12. *E. tenella* – Dg-CatD-1::mCherry oral challenge. Each bird was orally infected three times with *E. tenella* Dg-CatD-1::mCherry or the appropriate control *E. tenella* population, with each bird receiving 1000, 3000 and 5000 oocysts at days 2, 12, and 22 of the trial, respectively. Groups P7, P9 and P11 were infected with the experimental *E. tenella* Populations 1, 3, or 5, respectively. Group P8, P10 and P12 were infected with the control *E. tenella* Populations 2, 4, or 6, respectively. Details of all transgenic *Eimeria* Populations are shown in Table 1.

Dg-CatD-1 long-term immunisation trial. To investigate longevity of the immune response in immunized laying hens, a longer-term trial was performed under specific pathogen free-conditions (SPF) for 28 weeks. At the beginning of the trial (experimental day 0, eight days of age), 80 x one day old chicks (supplied by Millennium Hatchery, UK) were randomly assigned to 8 groups (Group L1- L8) of 10 and housed in a loose litter floor pen (diameter 1.6m) with layer mash and water provided *ad libitum*. Blood samples were taken from 2 birds per group prior to each treatment and throughout the trial. Groups received the following treatments:

Group L1 & L2. Recombinant protein vaccination.

Each bird from the protein vaccination group (Group L1), received four injections with 50 µg rDg-CatD-1 in Montanide™ ISA 71 VG via IM injection into the thigh at day 3, 13, 30 and 177 of the trial. The adjuvant-only negative control group (Group L2) received equivalent injections with 10 mM Tris-HCl (pH7.4), 0.5 M NaCl in Montanide™ ISA 71 VG.

Group L3 & L4. DNA vaccination.

Each bird from the DNA vaccination group (Group L3), received four injections with 120µg DNA vaccine Construct 3 with 1mg/kg bupivacaine via IM injection into the thigh at days 3, 13, 30 and 177 of the trial. Details of all DNA constructs are shown in Table 1. The empty vector negative control group (Group L4) received equivalent injections with 120 µg pVAX1 (empty vector) with 1mg/kg bupivacaine.

Group L5 & L6. DNA vaccination prime/recombinant protein vaccination boost.

Each bird from the DNA vaccination prime/recombinant protein vaccination boost (Group L5), received two injections of 120µg DNA vaccine Construct 3 with 1mg/kg bupivacaine via IM injection into the thigh at day 3 and 13 of the trial and injections of 50 µg rDg-CatD-1 in Montanide™ ISA 71 VG via IM injection into the thigh at day 30 and 177 of the trial. The control group (Group L6) received two injections with 120 µg pVAX1 (empty vector) with 1mg/kg bupivacaine via IM injection into the thigh at day 3 and 13 of the trial and two injections of 50 µg rDg-CatD-1 in Montanide™ ISA 71 VG via IM injection into the thigh at day 30 and 177 of the trial.

Group L7 & L8. Eimeria tenella – Dg-CatD-1::mCherry oral challenge.

Each bird from the *Eimeria* challenge group (Group L7), received four oral administrations of *E. tenella* Population 1, with each bird receiving 1000, 3000, 5000 and 10000 oocysts at day 3, 13, 23 and 72 of the trial. Control birds (Group L8) each received equivalent doses of *E. tenella* Population 2. For all *Eimeria* challenged birds, cage litter was replaced at day 59 of the trial.

Evaluation of immune responses to Dg-CatD-1. Levels of serum anti-Dg-CatD-1 IgY were monitored throughout the pilot and long-term trials by ELISA. Microtitre plates were coated overnight at 4°C with 50 µl rDg-CatD-1 (10 µg/ml in 50 mM bicarbonate/carbonate coating buffer, pH 9.6). Plates were washed six times with phosphate-buffered saline with TWEEN20 (PBST; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.1% v/v TWEEN20), and then blocked with 10% soya milk powder in Tris-buffered saline with TWEEN20 (TBST; 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% v/v TWEEN20) for 2 h at room temperature. After washing, plates were incubated with 50 µl sera (diluted 1/1600 in TBST) and incubated for 1 h at room temperature. Plates were washed again and incubated with 50 µl rabbit anti-chicken IgY-peroxidase conjugate (Sigma-Aldrich) at 1/30,000 in TBST for 1 h at room

temperature. Plates were washed and specifically bound antibodies were detected by addition of 50 µl SIGMAFAST™ OPD (Sigma-Aldrich) to each well. After 20 min in the dark, the colour reaction was stopped by addition of 25 µl of 2.5 M H₂SO₄ to each well and the OD values read at 490 nm. Each serum sample from individual birds was assayed in duplicate.

On-bird Dg-CatD-1 vaccine trial. The Dg-CatD-1 vaccine trial was performed under specific pathogen free-conditions (SPF) and ran for 37 days. Prior to the start of the trial, 10 x nineteen week old Hy-Line Brown pullets were randomly assigned to 2 groups (Group V1 and Group V2) of five. Throughout the trial birds were housed in an enclosed, loose litter floor pen of 2.1m x 0.9m with natural lighting. All groups received layer mash and water *ad libitum* throughout the trial, perches and nest boxes were provided. At the beginning of the trial (experimental day 0, 17 weeks of age) and throughout the trial, blood samples were taken from birds in each group. Each bird from the Dg-CatD-1 protein vaccination group (Group V1), received two injections of 50 µg rDg-CatD-1 in Montanide™ ISA 71 VG via IM injection into the breast at day 2 and day 16 of the trial. The adjuvant-only negative control group (Group V2) received equivalent injections with 10 mM Tris-HCl (pH7.4), 0.5 M NaCl in Montanide™ ISA 71 VG.

For on-bird *D. gallinae* feeding trials, mixed stage and mixed sex mites were collected from commercial egg-laying facilities and stored in vented 75 cm² tissue culture flasks (Corning, NY, USA) at room temperature (RT) for seven days, after which they were stored at 4 °C for 3 weeks without access to food. On-bird feeding trials were performed, according to methods previously described (Nunn et al., 2019). Briefly, sealed mesh feeding devices were constructed from 105µm aperture width, 63 µm depth polyester mesh, and each mesh feeding device was filled with 100 conditioned adult female *D. gallinae* mites. Prior to feeding assays, feathers were plucked from the outer thigh of each hen, and a single feeding pouch containing mites was placed on the plucked thigh and held in place using Leucoplast tape and further secured using a cohesive bandage (Central Medical Supplies Ltd., Pontefract, UK), according to methods described by (Nunn et al., 2019). Feeding devices were left on the birds for 3 hours, after which devices were removed and engorged fed mites were recovered and

transferred to individual wells of a 96 well tissue culture plate (Costar, Corning, NY, USA), sealed with AeraSeal tape (Sigma-Aldrich Co Ltd, Dorset, UK). Plates were incubated at 25°C with 85% relative humidity and mites were monitored daily over 8 days and scored for survival and cumulative egg production. The experiment was repeated three times at day 28, day 30 and day 32 of the experiment (at peak levels of serum anti-Dg-CatD-1 IgY), with each experiment using fresh batches of conditioned adult female *D. gallinae* mites and the same Dg-CatD-1 vaccinated birds (Group V1) and adjuvant only vaccinated birds (Group V2).

Statistical analyses. Analysis of antibody levels induced by different modes of immunisation in the pilot and long term vaccine trials; analysis of anti-Dg-CatD-1 IgY antibody titres in egg yolks; and analysis of mite feeding rates on vaccinated and unvaccinated chickens were analysed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Datasets were analysed using either: Student's *t*-test; one-way ANOVA with Dunnett's multiple comparison test; or Mann-Whitney U test (as indicated). *P* values of <0.05 were considered significant. For the analysis of numbers of eggs per mite in the on-hen vaccine trial study, data were analysed with a Poisson generalised linear mixed model (GLMM) including an offset to account for the number of fed mites.

Results

Expression and validation of recombinant Dg-CatD-1 vaccine antigen. To investigate antigen delivery platforms in laying hens, recombinant vaccine antigen Dg-CatD-1 was expressed in *E. coli* and purified as an insoluble protein (Fig. 1A); encoded as a Dg-CatD-1::mCherry fusion in DNA vaccines (Fig. 1B); or expressed as a Dg-CatD-1::mCherry fusion in transgenic *E. tenella* parasites (Fig. 1C).

In *E. coli*, rDg-CatD-1 was expressed as an insoluble protein at approx. 60 mg/litre cell culture. Purified refolded rDg-CatD-1 migrated as a 42 kDa protein when analysed by reducing SDS-PAGE, in agreement with its calculated molecular weight (Fig. 1A). To aid detection of the expressed antigen in DNA vaccines and transformed *Eimeria* parasites, Dg-CatD-1 was

fused at the C-terminus to an in-frame mCherry reporter tag. All vaccine constructs are detailed in Table 1. Prior to DNA vaccination of hens, expression of the encoded transgenes in each DNA vaccine construct was validated by transfection and expression in HEK 293 cells (Fig. 1B). For DNA vaccine Constructs 1 - 3, production of rDg-CatD-1::mCherry was confirmed by the presence of the fused mCherry reporter by fluorescence microscopy (Fig. 1B). *E. tenella* transformants were selected by FACS enrichment of mCherry expressing parasites (background transfection control). After 5 passages the number of mCherry positive transformants was increased between 10- and 20-fold. Positive transformants were selected by FACS sorting before each trial ensuring >95% of inoculated oocysts were expressing the transgene. All transgenic *Eimeria* parasites generated in this study are detailed in Table 1. Expression of Dg-CatD-1 protein was confirmed by detection of the fused mCherry reporter by fluorescence microscopy (Fig. 1C).

Characterisation of Dg-CatD-1 haemolytic activity. Purified refolded rDg-CatD-1 is an enzymatically active aspartyl proteinase, with detectable haemoglobin digestion activity. High levels of protease activity were detected in acidic conditions (pH2 – pH5), with a pH optimum at pH 4. In comparison, *D. gallinae* soluble protein extracts also had high levels of haemoglobin digestion protease activity in acidic conditions, with a comparable pH profile and pH optimum to rDg-CatD-1 (Fig. 2A). Protease activity of both rDg-CatD-1 and *D. gallinae* soluble protein extracts were inhibited by 2 μ M Pepstatin A (Fig. 2B).

Localisation of Dg-CatD-1 in *D. gallinae* whole mite sections. Antisera raised against rDg-CatD-1 was first validated in Western blot experiments. Rabbit anti-rDg-CatD-1 antisera was found to specifically bind purified rDg-CatD-1 (Supplementary Fig. 1, Lanes 1 and 2) as well as native Dg-CatD-1 in soluble protein extracts derived from both fed and starved *D. gallinae* mites (Supplementary Fig. 1, Lanes 3, 4 and 5). Control Western blots probed with pre-immune sera from the same rabbit showed no non-specific cross reactivity (Supplementary Fig. 1). For localisation experiments, consecutive sections of fixed, wax-embedded mites,

were probed with either rabbit anti-rDg-CatD-1 sera or rabbit pre-immune sera (Fig. 3). Mite tissue sections probed with anti-rDg-CatD-1 antisera showed ubiquitous staining, with the exception of ovary tissue in female mites (Fig. 3A). As a control, a consecutive section of the same mite probed with pre-immune sera showed no non-specific antibody staining (Fig. 3B).

Comparative analysis of anti-Dg-CatD-1 IgY levels – Pilot Study

Recombinant protein vaccination. Group P1 birds immunized three times (V1, V2, V3) with 50 µg rDg-CatD-1 in Montanide™ ISA 71 VG generated a specific serum anti-Dg-CatD-1 IgY response, with a peak serum IgY level 10 days after V2 (Fig. 4A). Anti-Dg-Cat-1 IgY levels remained above levels observed in adjuvant-only immunized birds (Group P2) for at least 22 days post V3 (Fig. 4A). Adjuvant-only control birds (Group P2) did not generate anti-Dg-CatD-1 IgY antibodies (Fig. 4A).

DNA Vaccination. Group P3, P4 and P5 birds were vaccinated three times with 56 µg DNA Constructs 1, 2 and 3, respectively. All DNA vaccine constructs (Constructs 1 – 3) are detailed in Table 1. Birds vaccinated with DNA vaccine Construct 1 did not generate an anti-Dg-CatD-1 IgY response and were comparable to control birds vaccinated with empty pVAX1 DNA vector. Birds vaccinated with DNA vaccine Construct 2 and DNA Construct 3 had elevated sera anti-Dg-CatD-1 IgY level 7 – 22 days after V3 (Fig. 4B and Fig. 4C). However, anti-Dg-CatD-1 IgY levels at 22 days post V3 were not significantly different to control birds immunized with empty pVAX1 DNA vector (One-way ANOVA with Dunnett's multiple comparison test, $n = 4$).

Eimeria tenella – Dg-CatD-1::mCherry oral challenge. Group P7, P9 and P11 birds were immunised orally with Dg-CatD-1 expressing transgenic *E. tenella* populations 1, 3 or 5, respectively. All transgenic *E. tenella* parasites (populations 1 – 6) generated in this study are detailed in Table 1. Chickens challenged with transgenic *E. tenella* expressing three different variants of Dg-CatD-1::mCherry, did not generate a specific anti-Dg-CatD-1 IgY response

(Fig. 4D), and showed no difference from control birds immunised orally with transgenic *E. tenella* populations expressing only mCherry (Fig. 4D).

Comparative analysis of anti-Dg-CatD-1 IgY levels – Long term Study

Recombinant protein vaccination. Group L1 birds immunized three times (V1, V2, V3) with 50 µg rDg-CatD-1 in Montanide™ ISA 71 VG generated a specific anti-Dg-CatD-1 IgY response, with a peak serum IgY level 7 days after V2 (Fig. 5A). Serum anti-Dg-Cat-1 IgY levels remained above those observed in adjuvant-only control birds (Group L2) for at least 100 days post V3 (Fig. 5A). Adjuvant-only control birds (Group L2) did not generate anti-Dg-CatD-1 IgY antibodies (Fig. 5A). Finally, a fourth rDg-CatD-1 immunization (V4) at day 177 of the experiment elevated the circulating anti-Dg-CatD-1 IgY level in immunized birds (Group L1) relative to adjuvant only control birds (Group L2). Specific anti-Dg-CatD-1 antibodies were also detectable in egg yolks from Group L1 birds at day 64 and day 65 (relative to first bleed), and were significantly higher than in control adjuvant-only vaccinated birds (t-test $P < 0.0001$) (Fig. 6).

DNA Vaccination. Group L3 birds vaccinated four times (V1, V2, V3 and V4) with 120 µg DNA Construct 3 did not generate an anti-Dg-CatD-1 IgY response and were comparable to negative control birds (Group L4) vaccinated with control DNA Construct 4 (Fig. 5B).

DNA vaccination prime/recombinant protein vaccination boost. Group L5 birds vaccinated twice (V1, V2) with 56 µg DNA Construct 3 and then once (V3) with rDg-CatD-1 in Montanide™ ISA 71 VG generated a specific anti-Dg-CatD-1 IgY response after the protein boost immunization (Fig. 5C). However, vaccination with DNA Construct 3 did not prime a response, and comparable anti-Dg-CatD-1 IgY levels were observed in negative control birds vaccinated with pVAX1 followed by a rDg-CatD-1 protein boost immunization (Fig. 5C).

Eimeria tenella – Dg-CatD-1::mCherry oral challenge. The pilot study demonstrated that birds challenged with *E. tenella* expressing three different variants of Dg-CatD-1::mCherry did not generate a specific anti-Dg-CadD-1 IgY response. As an extension of this preliminary trial, we performed an additional long-term trial to test whether prolonged exposure to *E. tenella* expressing Dg-CatD-1::mCherry was sufficient to generate a detectable anti-Dg-CatD-1 IgY response. For the long term study Group L7 and L8 birds administered with transgenic *E. tenella* Population 1 or 2, respectively, generated an anti-*Eimeria* IgY response, confirming establishment of *E. tenella* infection (Supplementary Fig. 2). However, birds infected with *E. tenella* test Population 1 did not generate an anti-Dg-CatD-1 IgY response and were comparable to negative control birds infected with control *E. tenella* Population 2 expressing only mCherry (Fig. 5D).

Mite feeding on-bird Dg-CatD-1 vaccine trial. Birds immunized twice (V1 and V2) with 50 µg rDg-CatD-1 in Montanide™ ISA 71 VG generated a specific serum anti-Dg-CatD-1 IgY response, with similar dynamics to those in the pilot vaccine trial (Fig. 4) and the long-term vaccine trial (Fig. 5). Peak serum anti-Dg-CatD-1 IgY levels were observed 13 days after V2 (Fig. 7A), while birds immunized with only adjuvant did not generate anti-Dg-CatD-1 IgY antibodies. On-bird *D. gallinae* feeding trials, where conditioned adult female mites in mesh pouches were fed on either Dg-Cat-D-1 vaccinated or control birds, were conducted between day 28 – 32 (relative to the first bleed), at peak levels of serum anti-Dg-CatD-1 IgY antibodies (Fig. 7B). Feeding rates of female *D. gallinae* mites on chickens vaccinated with either rDg-CatD-1 or adjuvant only control were 62% ± 5.4 ($n=12$) and 70% ± 7.1 ($n=10$), respectively, with no statistically significant difference between groups (Mann–Whitney U test, $P=0.2276$). The total number of fed female mites recovered varied within group and experiment and the total number of recovered fed female mites for the 3 repetitions of the experiment were as follows: rDg-CatD-1 $n = 186, 337$ and 151 ; Control $n = 232, 258$ and 146 . (Fig. 7B). Cumulative mite egg production per fed mite over 7 days was analysed using a Poisson generalised linear mixed model (GLMM) including an offset to account for the varied number of fed mites. The

results show statistically significant differences between treatment groups, with fed mites from the rDg-CatD-1 vaccinated group (Group V1) producing fewer eggs than mites which had fed on control hens (Group V2) ($p=0.0033$, LRT $p=0.01456$).

Discussion

We have characterised the activity and localisation of the aspartyl proteinase Dg-CatD-1 in the poultry red mite, demonstrating that Dg-CatD-1 digests haemoglobin, and has a ubiquitous localisation, with the exception of the ovary, in female mite tissues. In blood-feeding mites, haemoglobin digestion takes place in intracellular vesicles through the combined activities of lysosomal endopeptidases Cathepsin D (Dg-CatD-1) and Cathepsin L (Filimonova, 2013; Nisbet & Billingsley, 2000; Pritchard, Kuster, Sparagano, & Tomley, 2015). While, Dg-CatD-1 is predicted to function in the gut as a digestive proteinase, based on its localisation, it is also likely to function in lysosomes throughout mite tissues. Using Dg-CatD-1 as a model vaccine antigen, we analysed different methods of delivering this protein as a vaccine to chickens in order to induce high and sustained serum antibody levels, and demonstrated its potential for inclusion in a vaccine to control red mite infestations.

Almost every licensed vaccine available on the market for use against infectious agents confers protection by inducing pathogen/antigen-specific antibody production in the host (reviewed by (Rappuoli, Bottomley, D'Oro, Finco, & De Gregorio, 2016)). The efficacy of vaccine-induced antibody responses against eukaryotic haematophagous parasites is established for some ecto- and endo-parasites (e.g. see (Nisbet & Huntley, 2006; Nisbet, Meeusen, Gonzalez, & Piedrafita, 2016)). However, the requirement for long-lasting (>1year) immunity in commercial laying hens is a particular challenge, given the scale of these operations and associated difficulties in administering booster vaccinations by injection during the laying cycle. For this reason, we investigated three different methods of vaccine administration to attempt to gain high, sustained levels of antigen-specific circulating antibody.

Montanide™ ISA 71 VG has been used previously to immunise hens with *Eimeria* recombinant profilin protein because of the enhanced immune repertoire generated by this adjuvant (Jang et al., 2013). In the current study, this method of antigen administration proved to be the most superior for inducing antigen-specific serum IgY. A series of 3 immunisations of hens administered before 6 weeks of age led to high antigen-specific serum IgY levels which were sustained for >100 days.

Vaccination of chickens with DNA vaccines encoding Dg-CatD-1 fused with chicken IL-21 and mCherry reporter did not induce a significant level of antigen-specific antibodies, unless boosted by immunisation with the rDg-CatD-1 protein, though antigen-specific antibody levels were short-lived following the booster. The *Gallus gallus* IL-21 coding sequence was included in the construct of the DNA vaccines, as it has been shown to facilitate specific humoral responses in DNA vaccines against *Toxoplasma* (Chen et al., 2014). Although a number of interleukins had shown some promise, IL-21 was one of the few *Gallus gallus* interleukin genes available in a public database at the time of design of DNA-constructs in this project. Interleukin IL-21 had shown some promise to induce B-cell differentiation into antibody-secreting plasma cells (Ettinger et al., 2005) and to increase levels of humoral responses after DNA-vaccination in synergy with other interleukins after being included into DNA-constructs (Li et al., 2014). Since higher levels of antibody production towards the mite protein Dg-CatD-1 was the purpose of this study, we hoped these additional gene fragments could facilitate humoral responses in our experiments. For IL-21, there is now conflicting evidence suggesting that although it may facilitate humoral responses in some cases, the opposite may also occur (Leonard and Wan, 2016). The failure to provoke an anti-Dg-CatD-1 humoral response in these studies, may therefore be explained by this double edged effect of IL-21.

Oral inoculation of birds with transgenic populations of *E. tenella* expressing a selected exogenous antigen has been shown to confer partial protection against challenge with *E. maxima*, with levels of efficacy comparable to those obtained using recombinant protein or DNA vaccines (Pastor-Fernandez et al., 2018). However, this method of antigen delivery did

not generate a specific humoral immune response against rDg-CatD-1 in the present study, possibly due to low levels of antigen expression or misfolding of the expressed protein, among other recurrent issues when ectopically expressing proteins in evolutionarily unrelated organisms. Serial *E. tenella* infection induces a robust immune response including elevated serum antibody levels specific for soluble *E. tenella* oocyst antigen (Gilbert, Bhanushali, & McDougald, 1988). However, while recent studies using transgenic *E. tenella* to deliver vaccine antigens targeting *Campylobacter jejuni*, infectious laryngotracheitis virus or infectious bursal disease virus demonstrated capacity to induce specific antibody responses, all were generally low titre and variable between individuals (Clark et al., 2012; Marugan-Hernandez et al., 2016). Comparison of these results suggest that current transfection protocols for *E. tenella* promote more effective induction of cell-mediated than humoral transgene-specific immune responses.

Immunisation of hens with rDg-CatD-1 in Montanide™ ISA 71 VG resulted in antigen-specific serum IgY which, when consumed by adult female *D. gallinae* feeding on the immunized hens in *in vivo* feeding devices, induced an ~50% reduction in egg production by the mites (Fig. 7B). Dg-CatD-1 was selected as a model antigen for our work because its expression is induced by blood feeding (Bartley et al., 2012), it is ubiquitously expressed in tissues accessible to ingested IgY (Fig. 3 and (Nisbet, Huntley, Mackellar, Sparks, & McDevitt, 2006)) and antibodies to Dg-CatD-1 increased mortality of *D. gallinae* in *in vitro* assays (Bartley et al., 2012). In other haematophagous parasites, aspartyl proteinases, including orthologues of Dg-Cat-D1, initiate haemoglobin digestion and are vital for parasite nutrition (Williamson et al., 2004). A similar role has been proposed for aspartyl proteinases in *D. gallinae* marking them out as important potential vaccine candidates (Hamilton, Nisbet, Lehane, Taylor, & Billingsley, 2003; Nisbet & Billingsley, 2000).

In this study, we have made substantial progress towards identifying a method to administer prototype vaccines for *D. gallinae* in hens to stimulate long-lasting circulating antibody responses. With the recent publication of the *D. gallinae* genome (Burgess et al., 2018), we expect an increase in activity in development of prototype vaccines for this species.

However, for any vaccine which relies on induction of protective antibodies there is a threshold level of antigen-specific antibody below which efficacy is compromised. This threshold needs to be determined empirically, along with detailed analysis of the population dynamics of *D. gallinae* (see (Mul et al., 2017)), to establish the required levels of both antibody and vaccine efficacy to impact on PRM numbers and to keep parasite numbers below the economic threshold of infestation.

Acknowledgements

This work was supported by the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 635408 ('PARAGONE: vaccines for animal parasites'). We are very grateful to Javier Palarea-Albaladejo (Biomathematics & Statistics Scotland) for assistance in analysis of the data and to the staff of Bioservices Division, Moredun Research Institute for expert care of the experimental animals.

Disclosure Statement

All authors declare that they have no competing interests

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Figures

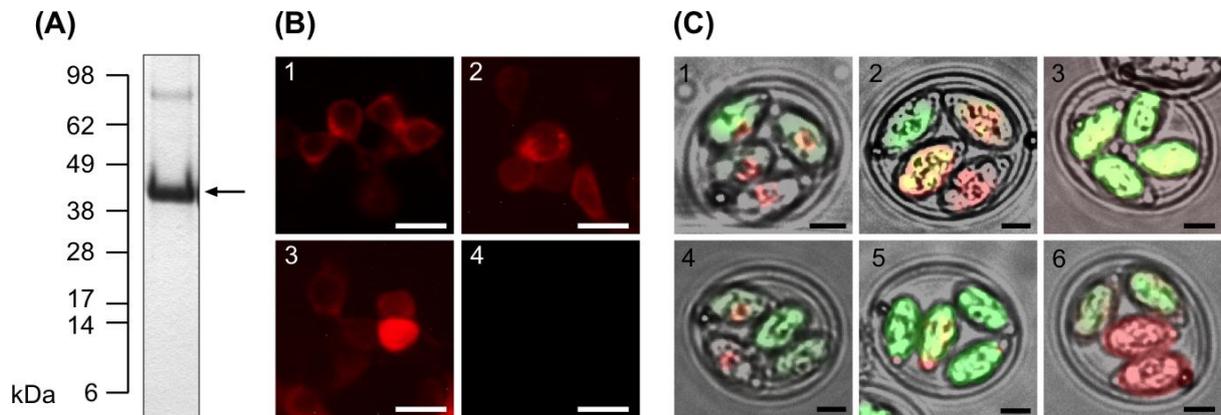


Figure 1. Expression of rDg-CatD-1 and rDg-CatD-1::mCherry. (A) SDS-PAGE analysis of purified refolded rDg-CatD-1 with Coomassie staining. Arrow indicates purified rDg-CatD-1 at 42 kDa. (B) Validation of Dg-CatD-1::mCherry DNA vaccine constructs in HEK 293 cells transfected with Construct 1 (panel 1), Construct 2 (panel 2) and Construct 3 (panel 3). Negative control cells (panel 4) were transfected with empty pVAX1. In each panel localisation of mCherry reporter (red) is shown. Scale bar is 50 μm . (C) Expression of Dg-CatD-1::mCherry in transgenic *E. tenella* oocysts. Panels 1 – 6 represent *Eimeria tenella* Populations 1 - 6, respectively. In each panel localisation of mCherry (red) and mCitrine (green) reporters is shown. Scale bar is 10 μm .

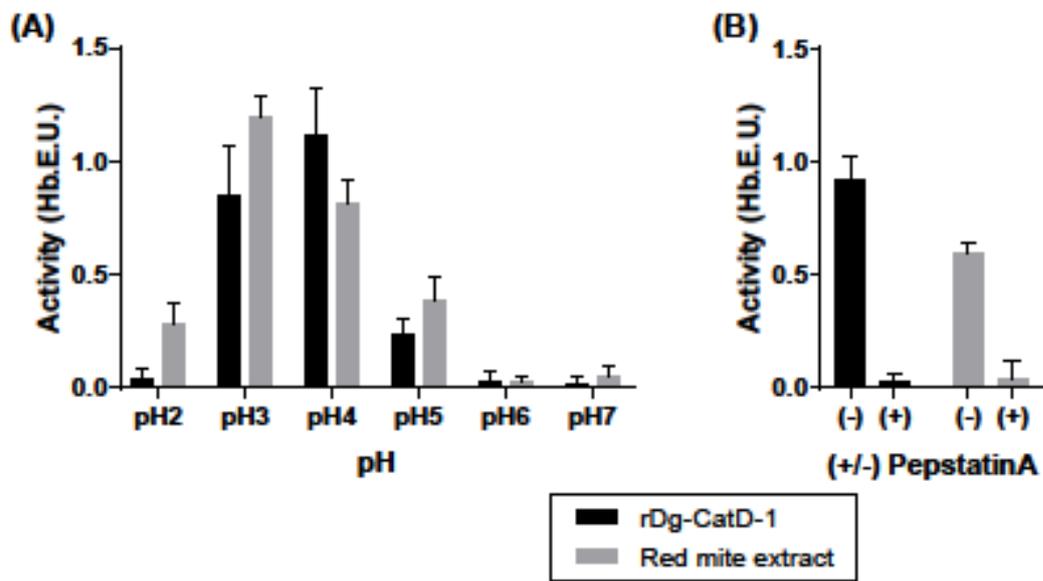


Figure 2. Haemoglobinolytic protease activity analysis of Dg-CatD-1. (A) Activity of refolded rDg-CatD-1 and soluble *D. gallinae* protein extract at pH 2 – pH 7 (as indicated in the figure). (B) Activity of refolded rDg-CatD-1 and soluble *D. gallinae* protein extract at pH 4 in the presence (+) or absence (-) of pepstatin A (2 μ M final concentration). Each value is the mean \pm SEM, $n = 4$.

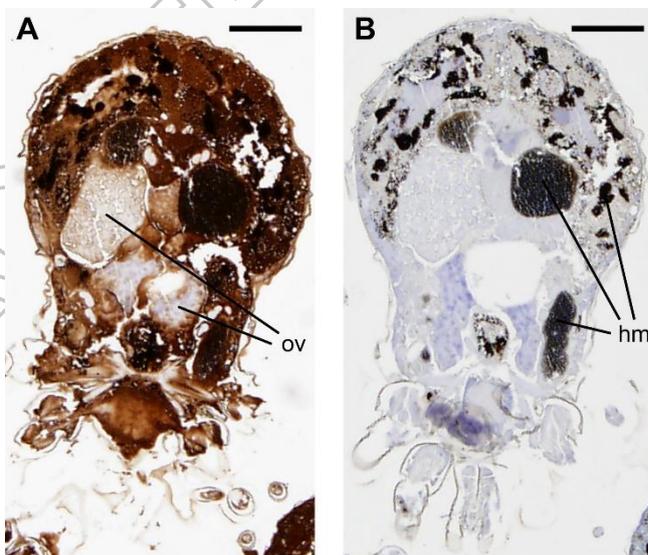


Figure 3. Immunohistochemical localisation of Dg-CatD-1 in *D. gallinae*. Sequential *D. gallinae* tissue sections (5 μ m thickness) were probed with either: (A) rabbit polyclonal anti-Dg-CatD-1 sera; or (B) pre-immune rabbit sera. Specifically bound antibodies were detected using EnVision+ System-HRP (DAB) (Dako) and counterstained with haematoxylin. Ov, ovary; hm, haematin granules. Scale bar is 125 μ m.

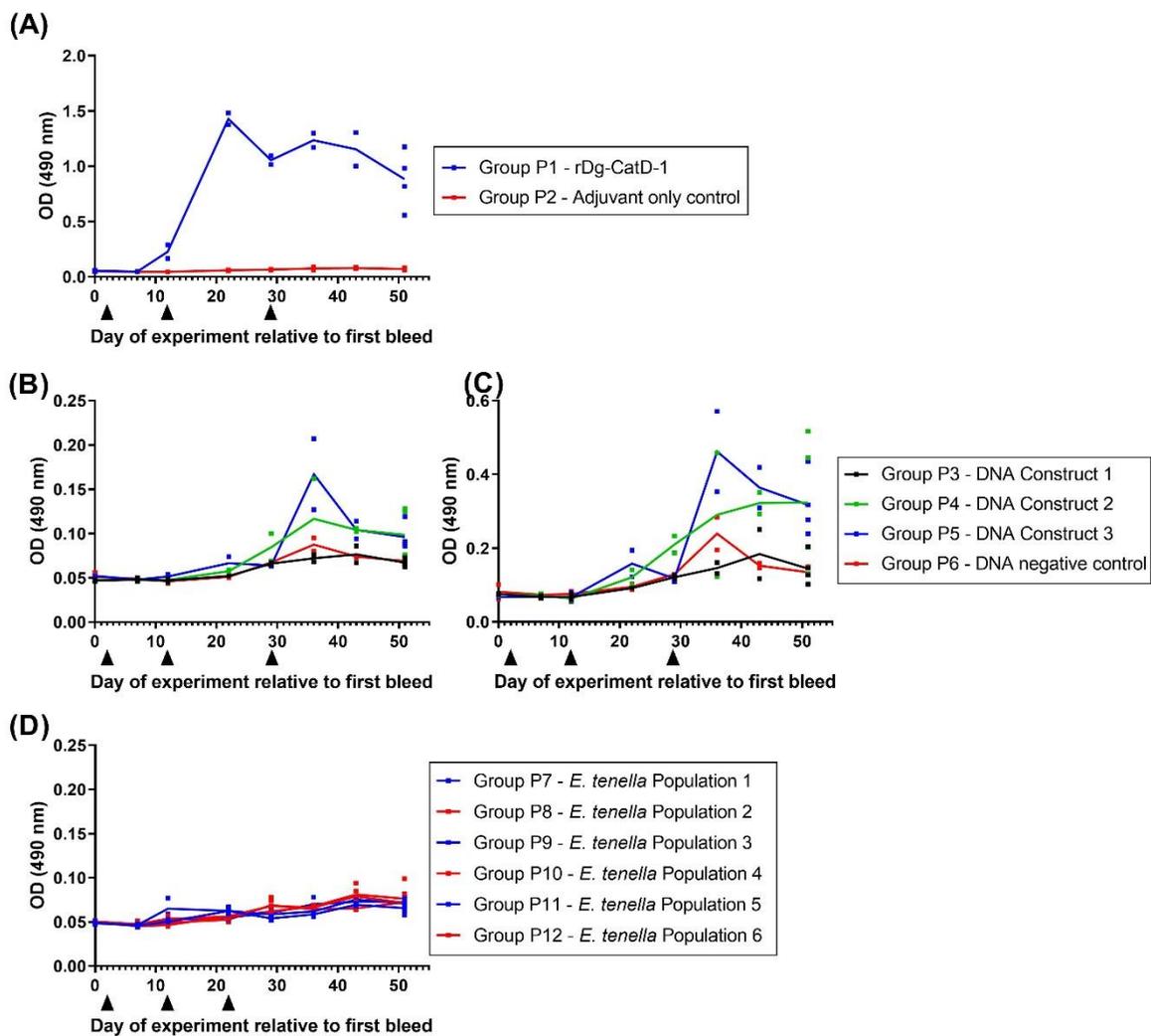


Figure 4. Comparative analysis of serum anti-Dg-CatD-1 IgY levels during the pilot vaccine trial. Comparative ELISA showing levels of circulating anti-Dg-CatD-1 IgY antibodies

in chicken sera after delivery of Dg-CatD-1 antigen by: (A) recombinant protein vaccination; (B) and (C) DNA vaccination; and (D) transgenic *Eimeria*. Black triangles indicate time points for either rDg-CatD-1 protein vaccination, Dg-CatD-1 DNA vaccination, or transgenic Dg-CatD-1 *Eimeria* challenge. Sera in panels (A), (B) and (D) were used at 1/1600; sera in panel (C) was used at 1/200. Individual data points are shown for each replicate sample. Each time point $n = 2$, except day 51 were $n = 4$. Connecting line represents mean value at each time point.

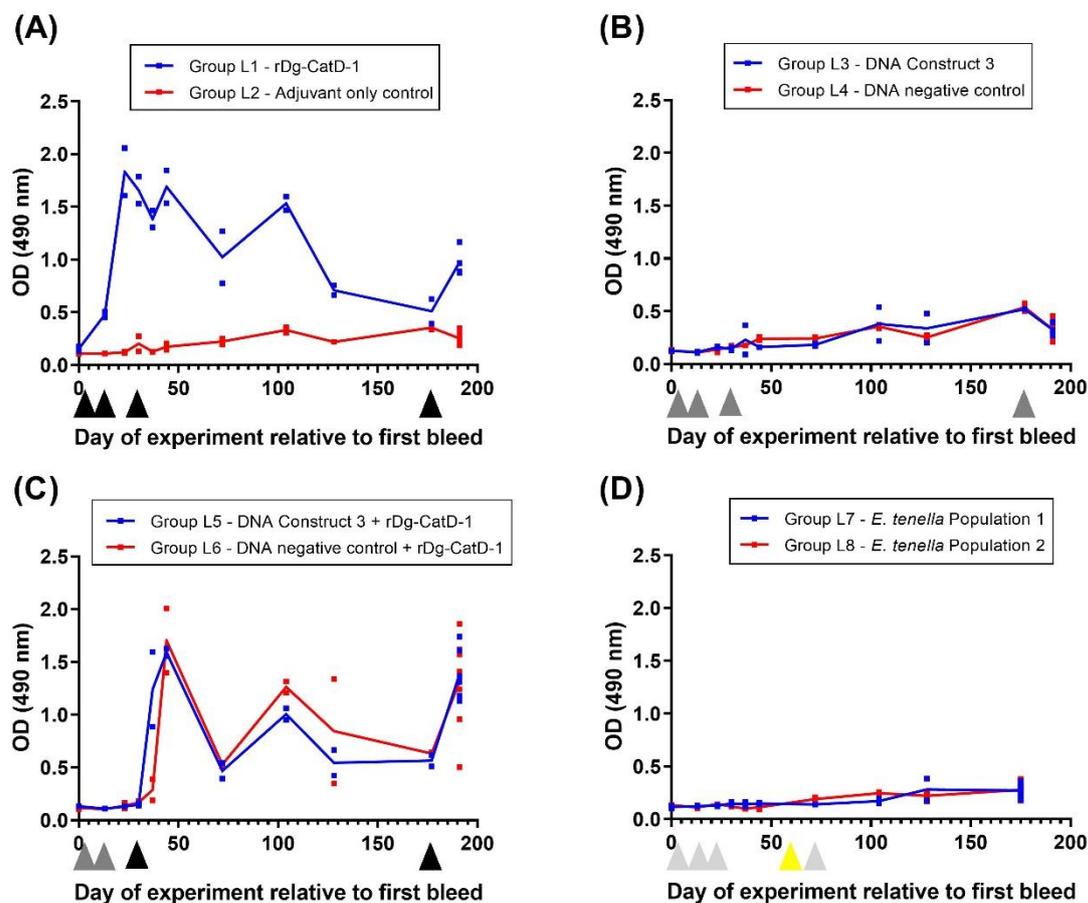


Figure 5. Comparative analysis of serum anti-Dg-CatD-1 IgY levels during the long-term vaccine trial. Comparative ELISA showing levels of circulating anti-Dg-CatD-1 IgY antibodies in chicken sera after delivery of Dg-CatD-1 antigen by: (A) recombinant protein vaccination; (B) DNA vaccination (C) DNA vaccination prime followed by a recombinant protein boost; and (D) transgenic *Eimeria*. Black triangles indicate rDg-CatD-1 protein vaccination; dark grey triangles indicate Dg-CatD-1 DNA vaccination; light grey triangles indicate transgenic Dg-

CatD-1 *Eimeria* inoculation; yellow triangle indicates litter change for chickens receiving transgenic *Eimeria*. All sera were tested at 1/1600. Individual data points are shown for each replicate sample. Each time point $n = 2$, except day 191 were $n = 4 - 8$. Connecting line represents mean value at each time point.

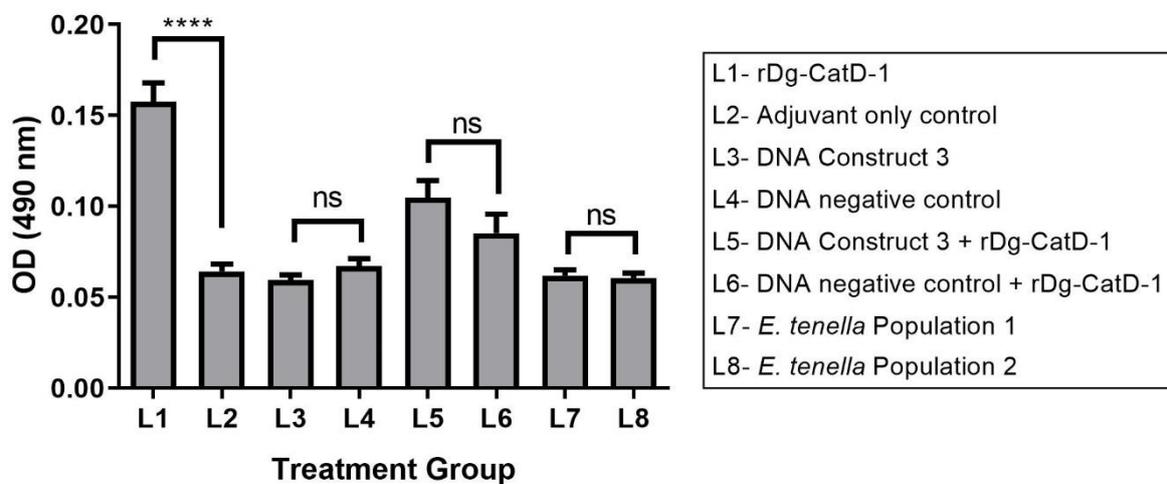


Figure 6. Comparative analysis of egg yolk anti-Dg-CatD-1 IgY antibodies

from the long-term vaccine trial. Comparative ELISA showing levels of anti-Dg-CatD-1 IgY antibodies in egg yolk from birds in treatment Groups L1 – L8 (as indicated in the figure). Eggs were collected from each group at days 64 and 65 relative to the first bleed, and diluted yolks from individual eggs were used at 1/1600. Each bar represents mean \pm SEM, $n = 3 - 10$. Non-significant (ns) and significant differences (****) between treatment groups are shown (t-test, $P < 0.0001$).

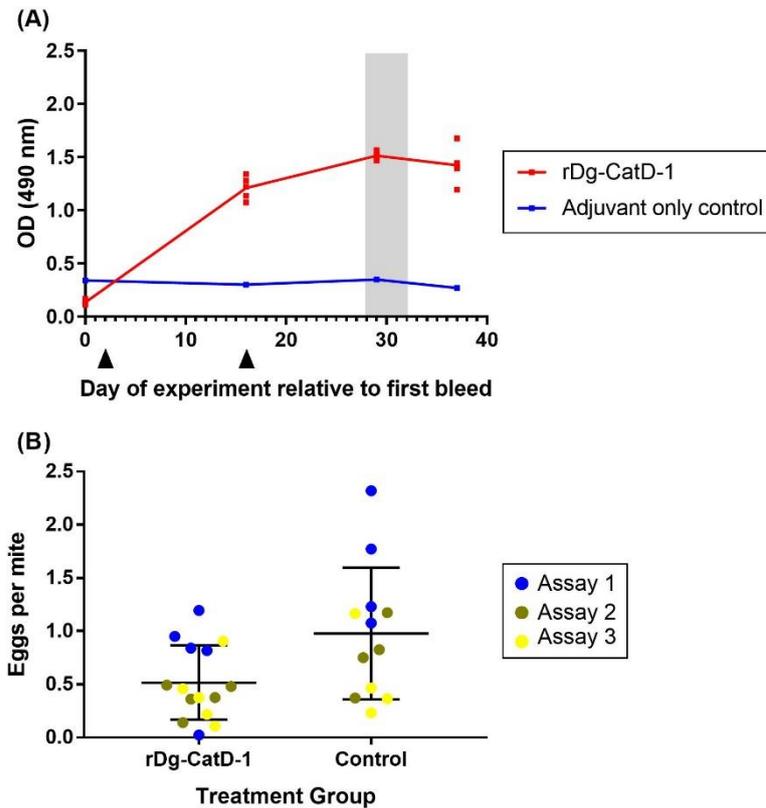


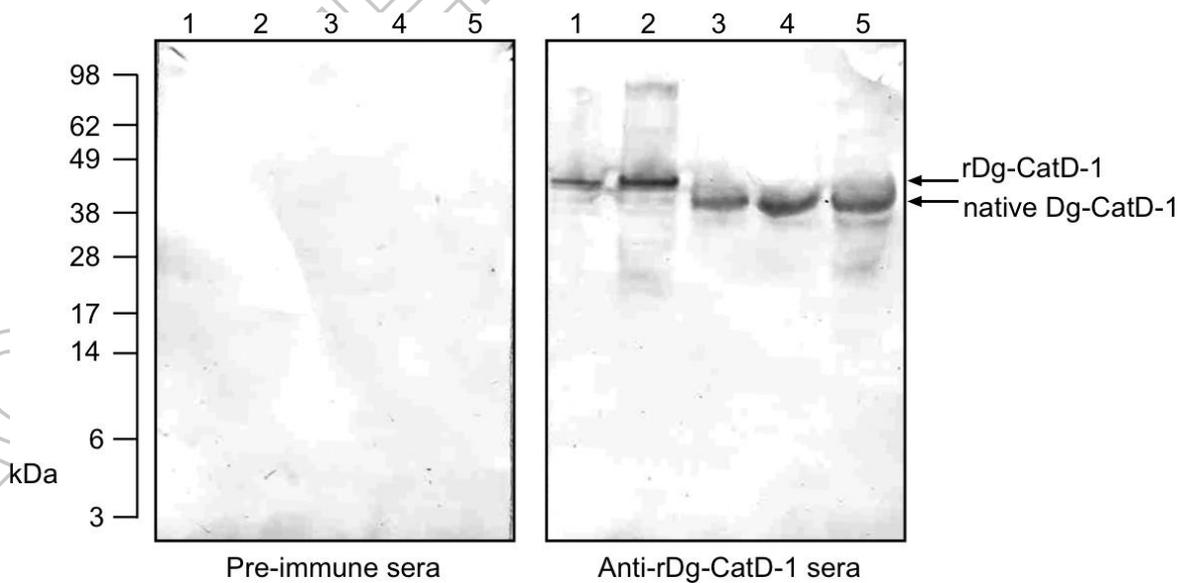
Figure 7. *D. gallinae* feeding trial on hens vaccinated with rDg-CatD-1. (A) Comparative ELISA showing levels of circulating anti-Dg-CatD-1 IgY antibodies in chicken sera after vaccination with rDg-CatD-1 in Montanide ISA 71 VG, or adjuvant only control. Black triangles indicate vaccination time points. All sera were tested at 1/1600. Individual data points are shown for each replicate sample ($n=5$), with exception of control group ($n=4$) as a bird was removed from the trial due to health issues unrelated to the study. Following vaccination, a *D. gallinae* on-bird feeding trial was conducted on days 28, day 30 and day 32 (shaded grey). (B) Egg production of *D. gallinae* mites after feeding on birds vaccinated with either rDg-CatD-1 or adjuvant only (control). Eggs per fed mite in assay is shown at 196 hour post-feeding time point. The feeding assay was repeated 3 times (assay 1 – 3) using the same vaccinated birds. Individual data points are shown for each assay with mean \pm SEM ($n=15$, rDg-CatD-1 group; $n=12$, control treatment group).

Tables

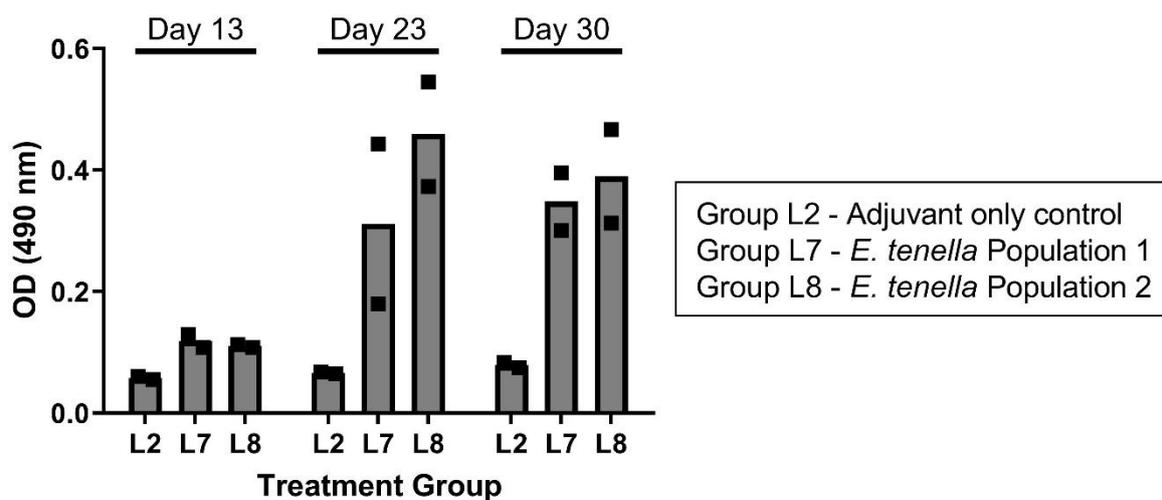
Vaccine Delivery	Identifier	Description	*Native Dg-CatD-1 SP removed?	Codon optimised [species]
Recombinant protein	rDg-CatD-1	Dg-CatD-1	Yes	No
DNA vaccine	Construct 1	IL21::Dg-CatD-1::mCherry	No	Yes [<i>Gallus gallus</i>]
	Construct 2	IL21::Dg-CatD-1::mCherry	Yes	Yes [<i>Gallus gallus</i>]
	Construct 3	IL21::Dg-CatD-1::mCherry	Yes	No
<i>Eimeria tenella</i>	Population 1	Cytoplasmic Dg-CatD-1::mCherry	Yes	Yes [<i>Eimeria tenella</i>]
	Population 2	Cytoplasmic mCherry		Yes [<i>Eimeria tenella</i>]
	Population 3	Secreted Dg-CatD-1::mCherry	Yes	Yes [<i>Eimeria tenella</i>]
	Population 4	Secreted mCherry		Yes [<i>Eimeria tenella</i>]
	Population 5	GPI-anchored Dg-CatD-1::mCherry	Yes	Yes [<i>Eimeria tenella</i>]
	Population 6	GPI-anchored mCherry		Yes [<i>Eimeria tenella</i>]

Table 1. Summary of Dg-CatD-1 delivery platforms. *Dg-CatD-1 is a predicted secreted protein, with native signal peptide (SP) predicted at amino acids 1 – 16.

Supplementary Figures



Supplementary Figure 1. Western blot validation of rabbit anti-rDg-CatD-1 sera. Purified recombinant Dg-CatD-1 (50 ng lane 1; 200 ng lane 2) and soluble red mite extract from fed mites (10 µg lane 3; 5 µg lane 4) and starved mites (10 µg lane 5) were resolved by SDS-PAGE and proteins transferred to nitrocellulose membranes. Blots were probed with either rabbit preimmune sera (negative control) or rabbit anti-rDgCatD-1 immune sera, with sera both used at 1:3000 dilution. Specifically bound antibodies were detected using swine anti-rabbit IgG HRP (Dako) at 1:3000 dilution and visualised using SIGMAFAST™ 3,3'-Diaminobenzidine (DAB) substrate. Arrows indicate the presence of an rDg-CatD-1 immune reactive band (42 kDa) and a native rDg-CatD-1 immune reactive band (40 kDa).



Supplementary Figure 2. Anti-*E. tenella* IgY response of birds challenged with *E. tenella* from the long-term vaccine trial. Comparative ELISA showing levels of anti-*E. tenella* IgY antibodies in sera from birds in treatment Group L2 (control, no *E. tenella* challenge) and birds challenged with transgenic *E. tenella* Population 1 and Population 2 (Groups L7 and Group L8) at days 13, 23 and 30 of the long-term vaccination trial. ELISA

performed with sera diluted 1/200. Each bar represents mean and individual data points are shown for each replicate sample ($n = 2$).

Supplementary File 1. Nucleotide and translated amino acid sequences for recombinant Dg-CatD-1 protein expression; IL21::Dg-CatD-1::mCherry DNA vaccination; and Dg-CatD-1::mCherry *Eimeria* transformation.

Dg-CatD-1 coding sequence for *E. coli* expression

```
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```

ATG - Start codon
Sequence from pET22b plasmid (poly-His region underlined)
Dg-CatD-1 coding sequence
TGA - Stop codon

```
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Amino acid change - Dg-CatD-1 sequence in NCBI is FQVIF

IL21::Dg-CatD-1::mCherry synthetic genes for DNA vaccination

>Construct_1

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ATG - Start codon

IL21 coding sequence

Dg-CatD-1 signal peptide

Linker region

Dg-CatD-1 coding sequence

mCherry coding sequence

>Construct_1

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Amino acid change - Dg-CatD-1 sequence in NCBI is FQVIF

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ATG - Start codon

IL21 coding sequence

Linker region

Dg-CatD-1 coding sequence

mCherry coding sequence

>Construct_2

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Amino acid change - Dg-CatD-1 sequence in NCBI is FQVIF

>Construct_3

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ATG - Start codon
IL21 coding sequence
Linker region
Dg-CatD-1 coding sequence
mCherry coding sequence

>Construct_3

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Amino acid change - Dg-CatD-1 sequence in NCBI is FQVIF

Dg-CatD-1::mCherry synthetic gene for *Eimeria* transformation

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>Dg_CatD_1::mCherry_synthetic_gene
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GCAGCGGCAGCGTGTGAGCGCGTGTGAGCACAGACACAGTGTGAGCGTGTGAGCGGCATTACAG
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XbaI restriction enzyme site (TCTAGA)
ATG - Start codon
Dg-CatD-1 coding sequence
Linker region
mCherry coding sequence (poly-His region underlined)
TAA- Stop codon
BglII restriction enzyme site (AGATCT)

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Amino acid change - Dg-CatD-1 sequence in NCBI is FQVIF