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Research paper

Eimeria tenella oocysts attenuated by low energy electron irradiation (LEEI) induce protection against challenge infection in chickens

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ABSTRACT

In vitro and *in vivo* studies were performed to assess whether *Eimeria tenella* (*E. tenella*) oocysts, exposed to low energy electron irradiation (LEEI), might be considered potential vaccine candidates against cecal coccidiosis. Sporulated oocysts were exposed to LEEI of 0.1 kGy to 10.0 kGy. Reproduction inhibition assays (RIA) were performed in MDBK cells to assess infectivity of sporozoites excysted from irradiated and non-irradiated oocysts. LEEI of 0.1 kGy or 0.5 kGy resulted in 73.2% and 86.5% inhibition of *in vitro* reproduction (%I_{RIA}), respectively. Groups of 12 one day old (D1) chicken were orally inoculated with Paracox®-8 (G1), 2.0×10^3 non-irradiated oocysts (G2) or 1.0×10^4 irradiated oocysts exposed to LEEI of 0.1 kGy (G3, G4) or 0.5 kGy (G5). Chicken of groups G1, G2, G4 and G5 were challenged 3 weeks later (D21) by a single inoculation of 7.5×10^4 non-attenuated oocysts of the same strain while G3 remained unchallenged. All chickens were subject to necropsy 7 days after challenge (D28) to estimate lesion scores (LS) and oocyst index (OI). A positive control (PC, non-vaccinated, challenged) and a negative control (NC, non-vaccinated, non-challenged) were kept in parallel. Chicken of group G5 had similar weight gain as the Paracox®-8 group (G1) after challenge and higher weight gains as compared to the other vaccinated groups. Feed conversion ratio (FCR) did not differ between chickens inoculated with oocysts irradiated with 0.5 kGy (G5) and negative control (NC) before challenge (1.25–1.52). After challenge FCR was 1.99 (G5) to 2.23 (G4) in the vaccinated chicken compared to 1.76 in group NC. LS and OI were significantly lower in all vaccinated groups as compared to group PC. Progeny oocysts collected from the feces of chickens following vaccination with irradiated oocysts exhibited lower *in vitro* infectivity/reproduction in MDBK cells with %I_{RIA} of 89.7% and 82.4% for progeny of oocysts irradiated with 0.5 kGy and 0.1 kGy, respectively, suggesting hereditary attenuation by LEEI treatment. Seroconversion was demonstrated by ELISA before challenge (D21) in all vaccinated groups, however, chicken inoculated with irradiated oocysts displayed higher antibody levels than those inoculated with precocious oocysts (G1). In Western blot analysis chicken vaccinated with virulent (G2) or 0.1 kGy-irradiated *E. tenella* oocysts (G3, G4) showed more protein bands compared to G5 (0.5 kGy). We conclude that LEEI could be a promising technology for production of attenuated oocyst vaccines.

1. Introduction

Eimeria tenella is an intracellular apicomplexan protozoon that causes relevant losses in poultry industry (McDougald and Fitz-Coy, 2013). Legal restrictions on the use of pharmacologically active feed additives, *e.g.* in the European Union (ANON, 2003), resistance development and increasing consumer concern reinforce attempts to control coccidiosis through applying new vaccine candidates (Ahmad et al., 2016) or using extracts from plants (Muthamilselvan et al., 2016).

Vaccination is a long-known measure to protect chicken against

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coccidiosis (Cox, 1998). Various vaccination regimes have been proposed including passive immunization (Wallach, 2010), or active immunisation with either virulent or attenuated *Eimeria* spp. strains (Williams, 2002). Attenuated precocious strains of *Eimeria* spp. induce a satisfactory degree of immunity against coccidiosis challenge (Price, 2012). Another option to induce attenuation is to expose the pathogen to ionizing radiation. For instance, Fetterer et al. (2014) reported on a vaccine based on oocysts exposed to gamma ray. *E. tenella* oocysts attenuated by X-ray exposure also induced a protective immune response (Jenkins et al., 1991). However, inactivation by gamma ray or X-ray requires suitable shielding to avoid health risks and has technical limitations (Chalise et al., 2004). High-energy electron beam irradiation was suggested as an alternative technique to attenuate pathogens (Brahmakshatriya et al., 2009; Grasso et al., 2011; Nims and Plavsic, 2015), but this procedure also requires adequate and expensive shielding. In contrast, low energy electron irradiation (LEEI) requires less stringent measures to protect staff working in such facilities even when high dose rates are applied. Inactivation of pathogens in liquid solution within a short period of time and maintenance of antigenic structures are important advantages of LEEI (Fertey et al., 2016; Bayer et al., 2018). In this study, we tested whether LEEI is suitable to attenuate *E. tenella* oocysts and whether such oocysts induce protection to the pathogen in chicken.

2. Materials and methods

2.1. E. tenella (Houghton strain)

E. tenella (Houghton strain) oocysts were kindly provided by Prof. D. P. Blake, Royal Veterinary College, University of London, UK. The strain was passaged every 6 months as described before (Shirley, 1997). Oocysts were collected from feces, incubated for sporulation and subsequently stored at 4 °C in 2% potassium dichromate solution (Raether et al., 1995).

2.2. Irradiation of oocysts

Sporulated oocysts were stored for less than 4 weeks at a density of ˜ $1.0–2.0 \times 10^5$ oocysts/ml. To purify and concentrate oocysts, 25 ml of stock oocyst suspension were washed in phosphate-buffered saline (PBS, pH = 7.2) through repeated centrifugation (three times, 400 x g, 10 min). Oocysts were sterilized by exposure to 12% sodium hypochlorite for 10 min. To remove sodium hypochlorite, sterilized oocysts were centrifuged (1085 x *g*, 5 min) and supernatant was removed. Oocyst pellets were then resuspended in PBS (1:10) and centrifuged (1085 x *g*) and again resuspended in PBS. This washing procedure was repeated three times. Finally, the oocyst suspension was adjusted to approximately $1.5–3.0 \times 10^6$ oocysts/ml in PBS corresponding to optical density (OD600) of 2.75 (BioPhotometer Plus, Eppendorf AG, Hamburg, Germany). The adjusted oocyst suspension was stored at 4 °C in PBS until use.

Irradiation was carried out as described (Fertey et al., 2016). Briefly, 230 µl of oocyst suspension were centrally applied into a sterile 100 mm petri dish (Primaria™, Corning). The suspension was overlaid with a round OPP-foil (diameter 55 mm), to avoid potential shadowing, resulting in a liquid film of even thickness (˜ 100 µm). The petri dishes were placed on a cooled sample holder and covered with a layer of PET/PE-foil. The samples were then irradiated with the intended dose at 8 °C using a 200 keV electron emitter (KeVac system, Linac Technologies, Orsay, France 200 kV/ 5 mA). The applied doses were calculated based on measurements performed before by applying 5.5 kGy to a radiochromic dosimeter film (Risø B3 dosimeter, Risø High Dose Reference Laboratory, Denmark). The irradiated oocysts were stored at 4 °C until further use.

2.3. In vitro reproduction inhibition assay (RIA)

Reproduction inhibition assay was used to determine $\%I_{RIA}$ to identify the lowest irradiation dose sufficient to distinctly reduce but not prevent sporozoite replication within 96 h p.i. of *in vitro* incubation as previously described (Thabet et al., 2015). Sporozoites were artificially excysted from oocysts (Raether et al., 1995) that were previously exposed to LEEI (0.1, 0.5, 1.0, 2.5, 5.0, and 10.0 kGy). In short, excystation was achieved by vigorous shaking of oocysts with 0.5 mm glass beads (BioSpec Products, Bartlesville, OK, USA) followed by incubation with 0.25% trypsin (w/v) (Carl Roth, Karlsruhe, Germany) and 4% sodium taurocholic acid (w/v) (Sigma, Taufkirchen, Germany) at 41 °C for 90 min. Sporozoite suspensions were filtered (Whatman 595.5 folded filters, GE Healthcare Life Science, UK) as described previously (Mattig et al., 1993), and centrifuged (2778 x *g*). Sporozoite pellets were washed with PBS (1.0 ml) and again centrifuged (2778 x *g*) three times. Sporozoites were microscopically counted using a hemocytometer.

Madin-Darby bovine kidney (MDBK) cells (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were used to provide host cell monolayers. They were grown in 24-well plates with supplemented Dulbecco's modified Eagle's medium (DMEM, with high glucose 4.5 g/l and L-Glutamine, 5% newborn calf serum; Gibco, Germany) to obtain semi-confluent monolayers. During infection, antibiotics were added (penicillin, 100 U/ml; streptomycin, 100 g/ml; and amphotericin-B, 0.25 g/ml; GE Healthcare, Germany) to the medium. MDBK cells were inoculated with 5.0×10^4 sporozoites/well and further incubated at 41 °C and 5% CO_2. Extracellular (non-invasive) sporozoites were removed by washing cultures three times with PBS after 24 h p.i and incubation was continued until 96 h p.i. (Thabet et al., 2015). MDBK cells infected with sporozoites from non-irradiated oocysts (0 kGy) cultured without (positive control, PC) or in the presence of monensin (0.25 µg/ml, MON) were included in each experiment. MON is a strong inhibitor of sporozoite reproduction *in vitro* and served as a respective control. Non-infected MDBK cells were included as negative controls (NC). All *in vitro* trials included four biological replicates.

Trypsin-versene (Lonza, Thermo Scientific, Germany) was used to detach adherent MDBK cells at 96 h p.i. For DNA extraction, QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions (blood and body fluid spin protocol). DNA was eluted in 50 µl nuclease-free water and concentrations were measured using a spectrophotometer (Nanodrop 2000, Thermo Scientific, Germany) at 260 nm wavelength. DNA was diluted to a concentration of 20 ng/l and stored at −20 °C until use for qPCR.

ITS-1 gene copy numbers for *E. tenella* were measured in all DNA preparations. A pSCA-147 plasmid standard dilution series was generated using the primers ET-F and ET-R (Kawahara et al., 2008) as described before (Thabet et al., 2015). Stratagene MX3000 P (La Jolla, USA) was applied for qPCR using the following reaction volumes: 10 µl of SYBR Green® master mix (Thermo Scientific, Germany), 0.4 µl of a 25 µM stock of primer ET-F (0.5 µM), 0.4 µl of a 25 µM stock of primer ET-R (0.5 µM), and 7.2 µl of nuclease-free water. DNA template volume was 2 µl, yielding a final 20 µl volume in the reaction capillary. The cycling reaction was as follows: 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 20 s at 62 °C, and 20 s at 72 °C A melting curve program involving heating from 60 to 95 °C at a rate of 0.1 °C/s was applied to create the dissociation curve. Data represent the mean of three replicates with an acceptable standard deviation of less than 0.5 Ct values were used to calculate value of individual biological replicate.

In vitro inhibition percentage ($\%I_{RIA}$) was calculated as follows:

$$\%IRIA = 100 * (1 - \frac{gene\ copies\ LEEI\ exposed}{gene\ copies\ PC})$$

Table 1
Experimental design to study the protective potential of LEEI attenuated *E. tenella* oocysts against challenge infection.

Group	category	inoculation dose per chicken (D1)	challenge dose per chicken (D21)	n =
NC	**negative control**	–	–	12
PC	**positive control**	–	7.5×10^4 oocysts	12
G1	**vaccination control**	Paracox®-8*	7.5×10^4 oocysts	12
G2	**non-irradiated**	2.0×10^3 oocysts	7.5×10^4 oocysts	12
G3	**irradiated (0.1 kGy)**	1.0×10^4 oocysts	–	12
G4	**irradiated (0.1 kGy)**	1.0×10^4 oocysts	7.5×10^4 oocysts	12
G5	**irradiated (0.5 kGy)**	1.0×10^4 oocysts	7.5×10^4 oocysts	12

*100 µl.

2.4. In vivo vaccination experiment

2.4.1. Experimental design

A total of 84 Cobb-500 chickens, one day of age, were purchased (Cobb Germany Avimex GmbH, Wiedemar, Germany) and used to study immunity induced by inoculation of LEEI exposed oocysts. Two different doses were chosen, based on results of the *in vitro* studies. Chickens were allocated to seven experimental groups of 12 birds each and kept under floor pen conditions (NC, PC, and 5 vaccinated groups G1-G5; see Table 1). Feed and water were provided *ad libitum*.

Vaccination (G1, G2, G3, G4, G5) was performed at 1 day of age (D1). G1 was vaccinated with Paracox®-8 (Intervet GmbH, Germany) following the manufacturer's instructions (one dose/chicken contains: *E. acervuline* HP: 500 oocysts, *E. brunetti* HP: 100 oocysts, *E. maxima* CP: 200 oocysts, *E. maxima* MFP: 100 oocysts, *E. mitis* HP: 1000 oocysts, *E. necatrix* HP: 500 oocysts, *E. praecox* HP: 100 oocysts, and *E. tenella* HP: 500 oocyts). Chicken of G2 were applied non-irradiated oocysts (2.0×10^3 oocysts/chicken) into the crop while those of groups G3, G4, and G5 were inoculated each with 1.0×10^4 oocysts exposed to LEEI at 0.1 kGy (G3 and G4) or 0.5 kGy (G5). Positive controls (PC, non-vaccinated) and vaccinated groups, except for G3, were challenged on D21 by inoculation of 7.5×10^4 non-irradiated oocysts/chicken into the crop. Negative controls (NC) were neither vaccinated nor challenged. All chickens were euthanized and necropsied on D28.

2.4.2. Assessment of efficacy

Feed consumption, clinical observations and mortality were recorded daily. All birds were individually weighed at D1, D7, D14, D21, and D28. Feed conversion ratio (FCR) per group was calculated as follows:

$$FCR = \left(\frac{weekly\ feed\ intake}{number\ of\ chickens}\right) \div average\ weight$$

2.4.3. Oocyst excretion

Fecal samples were taken from each chicken by pressing prudently against the cloaca at D1, D7, D14, D20, and D27. Each sample was weighed, the same weight of tap water was added, and the sample homogenized. Saturated NaCl (density: 1.2 g/l) was added to the sample at 10-fold amount and the suspension thoroughly mixed for 2 min using a magnetic stirrer.

Litter samples were taken on days D6, D9, D12, D15, D18, D20, D24, and D27. 12 g of each individual litter sample were dissolved in 180 ml of saturated NaCl and homogenized using a household blender.

Oocysts per gram (opg) of feces or litter were determined using a McMaster slide. Four chambers per sample were evaluated for oocysts and the mean count was calculated according to the following formula:

$$opg = average\ oocyst\ counts * 100$$

2.4.4. Progeny of LEEI exposed oocysts

Oocysts from litters of G2, G4, and G5 were collected on D12 to D14. Oocysts were isolated from the litter of the respective groups separately and stored in 2% potassium dichromate. To support sporulation, oocyst suspensions were gently shaken for about 96 h at 25 °C. RIA was performed as described in Section 2.3.

2.4.5. Dissection

All birds were dissected on D28 after euthanizing using carbon dioxide for 10 min following the guided euthanasia protocol. Individual weight, mean lesion score (LS = 0–4, according to Johnson and Reid, 1970), and mean oocyst index (OI = 0–5, according to Hilbrich, 1978) were calculated.

2.4.6. Humoral immune response

Blood samples were taken from the wing vein of each chicken on D21 and centrifuged at 2000 x *g* for 10 min. Sera were collected and stored at −20 °C until use. Antibodies against *E. tenella* were detected by an enzyme-linked immunosorbent assay (ELISA; Constantinoiu et al., 2007; Alnassan et al., 2013). In short, *E. tenella* Houghton strain sporozoites were obtained as described in section 2.3. Purified sporozoites were disrupted by freezing and thawing five times followed by sonication on ice (Bandelin Sono Plus Sonicator; 70% amplitude) until no sporozoites were visible under light microscope (Liu et al., 2014). The suspension was centrifuged at 16,000 × *g* for 10 min and protein concentration was determined by Pierce BCA protein assay kit (Thermo Scientific, USA) using bovine serum albumin as standard (Sigma-Aldrich, Germany). A 96-well plate was coated with 100 mg/ml *E. tenella* protein in 0.05 M carbonate buffer (pH 9.6) overnight (10 µg/well) and free binding sites were blocked with 3% bovine serum albumin (BSA) in PBS with 0.05% Tween 20 (PBST) for 2 h at room temperature. Serum samples were diluted 1:100 in PBST and applied to the plate and incubated for 1 h at room temperature. After washing (five times) with PBST, the sera were further incubated for 1 h with the secondary antibody (horseradish peroxidase-labelled goat anti-chicken IgG; 1:8000 in PBS, Southern Biotech, Birmingham, AL, USA) followed by washing five times with PBST. Substrate (3,3′,5,5′-tetramethylbenzidine) was added and the reaction stopped after 20 min by adding 200 µl of 1 M H_2SO_4. The optical density (OD) was determined in a plate reader at 450 nm (Anthos htll, Anthos Labtec Instruments GmbH, Wals, Austria). A standard curve using a twofold dilution series (625-0.3 ng/ml) of unlabelled chicken IgY (Southern Biotech, Birmingham, Al, USA) as antigen were used to calculate the concentration (ng/ml) of IgY through determining the OD after incubation with horseradish peroxidase-labelled goat anti-chicken IgG (Alnassan et al., 2013). All measurements were performed in technical triplicate. The cut-off value was defined as the mean value calculated for all negative sera plus the threefold standard deviation.

2.4.7. Western blot

Serum samples collected on D21 from NC, G1, G2, G4, and G5 were pooled per group. Sporozoite antigen (50 µg per lane, according to Pierce BCA protein assay kit (Thermo Fisher Scientific, USA)) was loaded on two different denaturing SDS gels (gradient gel 4%–20% acrylamide and 10% acrylamide). Blotting was performed over night at 4 °C at 40 mA in Tris-Glycine buffer with 20% methanol. The membrane was blocked with 5% skim milk powder in PBS (w/v) and cut into lanes. The lanes were probed with chicken serum diluted 1:500 for 2 h at room temperature. The membrane was washed 3 times (5 min) with PBS-Tween (0.1%) and incubated with anti-chicken HRP diluted 1:1000 with PBS-Tween (0.1%) containing 5% skim milk powder for 1.5 h at room temperature. The membranes were washed 3 times (5 min) with PBST (0.1%) and detection was performed using ECL-substrate Pierce™32,209 (Thermo Scientific, Rockford, IL). Film was developed after 15 s exposure time. Molecular weight in kDa was indicated using Page Ruler™ Plus 26,620 (Thermo Scientific, Vilnius, Lithuania).

2.5. Data analysis

GraphPad Prism® 5.0 (Version 5.01, GraphPad Software, Inc., USA) was used to generate the standard curve for ELISA applyinga non-linear regression model to determine serum antibody concentration (ng/ml) in chicken serum samples. Statistical analyses were performed with the software package SPSS statistic 22® (IBM, New York, USA, 2014). Kolmogorov-Smirnov test was used to evaluate data for normal distribution. Normally distributed data were compared using ANOVA and Bonferroni test. Kruskal-Wallis and Mann-Whitney U-tests were applied for non-normally distributed data. Pearson product-moment correlation coefficient was calculated to assess correlation between *in vivo* parameters (LS, OI) and gene copy numbers of irradiated oocysts or their progeny, respectively. Wilcoxon signed-rank test was used to compare $\%I_{RIA}$ for sporozoites extracted from irradiated parental oocysts with those of their progeny after *in vivo* passage.

3. Results

3.1. In vitro reproduction inhibition assay (RIA)

5.1×10^4 gene copies/well were detected in MDBK cell monolayers infected with sporozoites originating from parental stock oocysts, while 4.6×10^4 gene copies/well were measured in cultures infected with the same number of sporozoites excysted from oocysts that were, except for irradiation, subjected to all other procedural steps of the irradiation protocol. This insignificant difference ($p > 0.05$) proofs that handling of oocysts during the irradiation procedure has no negative impact on parasite viability *in vitro*.

LEEI inactivated oocysts in a dose dependent manner. Oocysts exposed to LEEI of 1 kGy or more were almost completely inhibited ($\%I_{RIA} > 98.0\%$), whereas a dose of 0.5 kGy had a lower but still clear inhibitory impact on replication ($\%I_{RIA} = 86.5\%$, $p < 0.05$). Even oocysts exposed to only 0.1 kGy displayed distinct inactivation with $\%I_{RIA} = 73.2\%$, $p < 0.05$. Consequently, LEEI doses correlated significantly ($r_s = 0.812$, $p < 0.01$) with $\%I_{RIA}$ values. As expected, MON caused almost complete inactivation ($\%I_{RIA} > 99.8\%$).

3.2. Immunisation experiment

3.2.1. Clinical signs, weight gain, and feed conversion

Based on the *in vitro* data, LEEI doses of 0.1 kGy and 0.5 kGy were chosen for subsequent testing of immunisation of chicken by LEEI attenuated oocysts.

Average weight gain per week was calculated for each group separately. During D1-D7, all groups gained similar weight except for G2 that displayed impaired growth after vaccination with 2.0×10^3 non-attenuated oocysts (G2 *vs*. PC and G3: $p < 0.001$; G2 *vs*. NC, G1, and G5: $p < 0.05$). In the week after challenge (D22-D28), the lowest average weight gain per week was observed in PC (not vaccinated, challenged; Table 2), and weight gain was significantly lower in PC than in NC (not vaccinated, not challenged; $p < 0.05$).

G5 (0.5 kGy) exhibited the lowest FCR values in period D1-D20 (before challenge) compared with other vaccinated groups. Performance (FCR) was on the same level in G5 as in NC during periods D1-D7 and D15-D21. After challenge at D21, G5 and G1 (Paracox®-8) presented similar weight gain, however, G5 showed the best FCR (1.99) of all challenged groups and FCR was only slightly above those of NC chicken (1.76), whereas in all other groups FCR was higher than 2 (Table 2).

Table 2
Average weight gain (WG) per week and feed conversion ratio (FCR) per week calculated for each group separately.

	D1-D7		D8-D14		D15-D21		D22-D28	
Group*	WG (g)	FCR	WG (g)	FCR	WG (g)	FCR	WG (g)	FCR
NC	98.29 ± 14.66	1.44	247.89 ± 24.95	1.51	464.59 ± 42.92	1.54	541.67 ± 182.46	1.76
PC¶	102.97 ± 12.65	1.39	258.01 ± 30.40	1.54	429.94 ± 46.18	1.61	416.08[d] ± 107.79	2.22
G1¶	96.53 ± 10.38	1.45	229.93 ± 26.79	1.64	440.55 ± 98.29	1.61	491.49 ± 153.10	2.11
G2¶	77.45[a, b] ± 14.82	1.48	196.62[c] ± 57.15	1.75	370.28[c] ± 90.41	1.72	419.87[e, f] ± 60.89	2.21
G3	112.01 ± 16.73	1.29	248.82 ± 39.28	1.62	441.79 ± 59.98	1.64	469.51 ± 64.58	2.06
G4¶	92.13 ± 11.47	1.32	227.42 ± 28.04	1.55	416.83 ± 60.98	1.63	426.24[e] ± 68.82	2.23
G5¶	99.51 ± 18.95	1.25	236.39 ± 50.76	1.52	447.91 ± 84.34	1.52	490.20 ± 65.05	1.99

*Refer to Table 1 for detailed description of groups. ¶Challenge using 7.5×10^4 oocysts/bird was performed in these groups at D21.
[a, b]Average weight gain in period (D1-D7) was significantly lower in G2 compared with PC and G3 ($p < 0.01$) [a], and NC, G1, G5 ($p < 0.05$) [b]. [c] Average weight gain in period (D8-D14) was significantly lower in G2 compared with NC, PC, and G3 ($p < 0.05$). [d] Average weight gain in period (D22-D28) was significantly lower in PC compared with NC ($p < 0.05$). [e] at the same period of time, average weight gain was significantly lower in both G2 and G4 compared with NC ($p < 0.01$). [f] Average weight gain in G2 was also lower than G3 and G5 ($p < 0.05$).

In general, vaccination at D1 induced no clinical signs or mortalities in the respective groups. However, bloody feces were observed in litter of groups G3 and G4 (vaccinated with 0.1 kGy irradiated oocysts) and one chicken of G3 died due to cecal coccidiosis at D7. In PC, challenge of chicken with non-attenuated parental oocysts resulted in severe clinical coccidiosis with hemorrhagic diarrhea, retarded growth, decrease in feed intake, and death of 2 out of 12 (16.7%) chicken (Table 2). Following challenge, clinical coccidiosis was generally mild in all vaccinated groups, although hemorrhagic diarrhea was seen in floor of G2 and G3.

3.2.2. Oocyst counts

No oocysts were detected in the feces of chicken of group NC during the whole experiment and in group PC before D27 (Fig. 1).

At D7, oocysts were excreted in all vaccinated groups (G1-G5) with no significant difference in opg observed between groups G1 (Paracox®-8) and G5 (0.5 kGy). Compared with the other vaccinated groups (G2, G3, G4) oocyst excretion was significantly lower in G5 ($p < 0.05$), and G2 and G3 showed significantly higher opg than G1 ($p < 0.05$; Fig. 1a).

At D14, excretion of oocysts by chickens of G3 and G5 was significantly lower than recorded for G1 ($p < 0.05$), while G2 showed the highest average opg of all groups (Fig. 1b).

All vaccinated groups shed oocysts at D20. Interestingly, opg recorded for group G5 were on a rather low level at D20 and did not differ significantly ($p > 0.05$) from the zero values of NC and PC (Fig. 1c).

Subsequent challenge resulted in considerable oocyst excretion one week later (D27), particularly in the non-vaccinated control (PC). The opg values were significantly ($p < 0.05$) higher in PC chicken compared to all vaccinated groups except for G2 ($p > 0.05$). Amongst all challenged vaccinated chickens lowest average opg were recorded for G4 and G5 (Fig. 1d).

Cumulative opg for the whole investigation period were

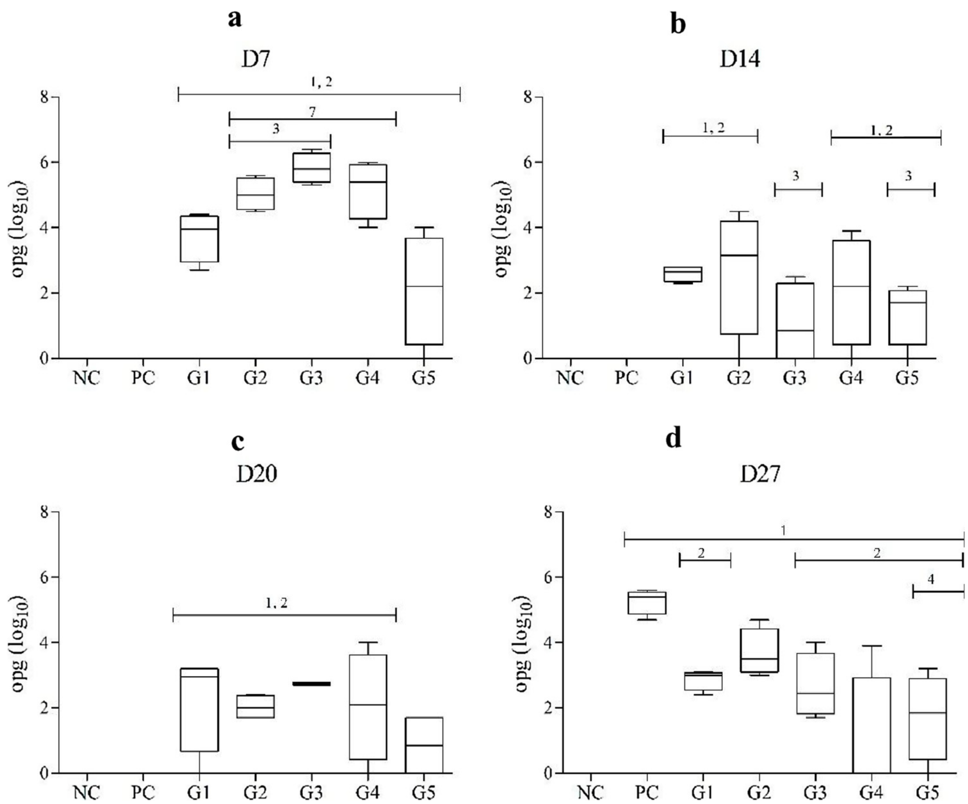

Fig. 1. Fecal oocyst counts (log_{10} opg). **1a**: D7; **1b:** D14 ($p < 0.05$). **1c**: D20; **1d**: D28 (=one week after challenge). Superscripts indicate statistically significant differences ($p < 0.05$) by comparison with [1] NC, [2] PC, [3] G1, [4] G2, and [7] G5. Refer to Table 1 for detailed description of groups.

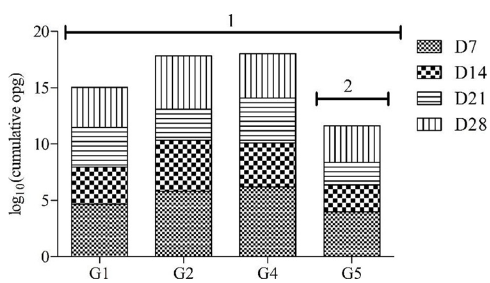

Fig. 2. Cumulative fecal oocyst counts (log_{10} opg). Stacked column figures represent fecal oocyst count values collected from chickens directly on a weekly basis. [1] Cumulative opg values significantly different from PC ($p < 0.01$). [2] Opg values significantly different from those of G1, G2, and G4 ($p < 0.05$). Refer to Table 1 for detailed description of groups.

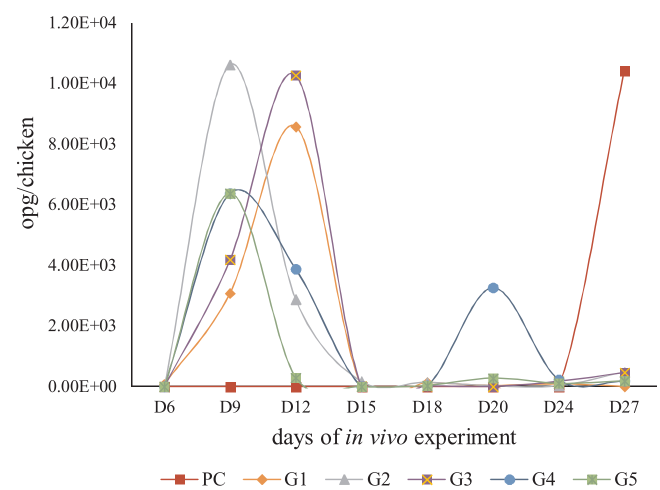

Fig. 3. Litter oocyst counts (expressed as average opg/chicken). Refer to Table 1 for detailed description of groups.

significantly ($p < 0.05$) lower in group G5 as compared to all of the other vaccinated and challenged groups (Fig. 2).

Oocysts were first detected in litter collected at D6 after vaccination from the pens of G1 and G2. Litter collected at D9 from pens of groups G1 and G3 contained particularly large amounts of oocysts. Litter opg were highest in G3 at D12 (average litter opg/chicken = 1.03×10^4). From D12 onwards, litter opg distinctly decreased and remained low until the end of the experiment, except for a moderate and temporary opg increase in G4 on D20. In contrast, challenge of the not vaccinated controls (PC) induced a steep increase of litter opg on D27 (Fig. 3).

3.2.3. Lesion score (LS) and oocyst index (OI)

As expected, LS and OI were zero in the uninfected and unchallenged NC chicken. Chicken vaccinated with non-attenuated oocysts (G2) or oocysts exposed to 0.1 kGy (G4) showed lowest post challenge LS of 0.17 and 0.50. Average LS in groups G1 (Paracox®-8) and G5 (0.5 kGy) were moderately higher with values of 0.75 and 0.83, respectively, but still distinctly lower than in group PC (2.73). Some variation was observed between vaccinated groups (G4 *vs.* G1: $p = 0.061$; G5 *vs.* G2: $p < 0.05$; Fig. 4a), however, in all immunized groups average LS were significantly lower than in PC chicken ($p < 0.01$).

Similarly, the highest average OI of 5.0 was found in the PC, whereas OI was significantly lower in each of the vaccinated challenged groups (G1, G2, G4, G5; $p < 0.01$). No difference in OI was observed between the vaccinated challenged groups (Fig. 4b) confirming protection from challenge by LEEI treated oocysts (G4, G5).

3.2.4. Sustainability of attenuation

Oocysts were collected from feces of groups G2, G4, and G5 on D12 to D14 and allowed to sporulate. $\%I_{RIA}$ of these progeny oocysts were

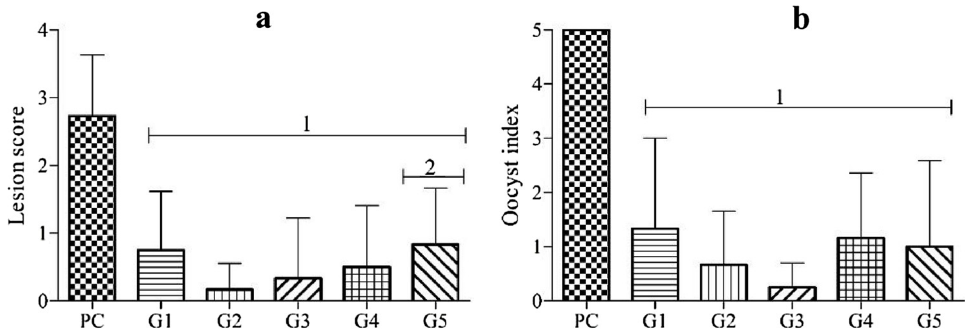

Fig. 4. Mean lesion score (LS) and mean oocyst index (OI) of vaccinated and challenged groups after dissection at D28. [1] LS and OI significantly different from PC ($p < 0.01$). [2] LS significantly different from G2 ($p < 0.05$). Refer to Table 1 for detailed description of groups.

compared to the values obtained for LEEI treated parental oocysts. Sporozoites excysted from oocysts exposed to 0.1 kGy LEEI exhibited $\%I_{RIA}$ of 73.2%, while their progeny was slightly less reproductive with $\%I_{RIA}$ of 82.4%. Sporozoites originating from LEEI treated parental oocysts at a higher dose of 0.5 kGy had almost the same $\%I_{RIA}$ as their progeny (86.5% and 89.7%, respectively).

LS and OI recorded for chicken of group PC and for chicken vaccinated with LEEI treated oocysts (G4, G5) were analyzed for correlation and for correlation with $\%I_{RIA}$ values. OI showed a significant correlation with LS ($r_s = 0.800$, $p < 0.01$) and with $\%I_{RIA}$ of the LEEI exposed oocysts ($r_s = -0.921$, $p < 0.01$). OI induced by infection of chicken with parental LEEI exposed oocysts also negatively correlated with $\%I_{RIA}$ calculated for the respective offspring oocysts ($r_s = -0.903$, $p < 0.01$). LS also negatively correlated with $\%I_{RIA}$ of LEEI exposed oocysts ($r_s = -0.622$, $p < 0.01$) and with respective offspring oocysts ($r_s = -0.654$, $p < 0.01$). $\%I_{RIA}$ measured for parental irradiated oocysts and for their progeny also exhibited correlation ($r_s = 0.967$, $p < 0.01$) (Fig. 5). No significant difference ($p > 0.05$) of $\%I_{RIA}$ values between parental and offspring sporozoites was observed irrespective of the applied irradiation dose (Fig. 5b).

3.2.5. Humoral immune response

Standard curve generated from nonlinear regression analysis, through measurement of OD values resulted from binding known concentrations of horseradish peroxidase-labelled goat anti-chicken IgG to coated unlabelled chicken IgY was suitable to determine concentrations of serum antibodies in chickens ($R^2 > 0.996$, $p < 0.05$). For assessment of humoral immune response at D21 (before challenge) NC and PC were both considered as negative control (not infected) and were used to calculate the cut-off value of 9.42 ng/ml. Seroconversion was detected in all vaccinated groups. G2 (vaccinated with virulent *E. tenella* oocysts) had the highest concentration of *E. tenella* specific serum antibodies ($p < 0.01$ in relation to G1 and G5; $p < 0.05$ in relation to G4). Amongst all vaccinated groups G1 (Paracox®-8) displayed the lowest serum antibody concentrations, and the difference was significant ($p < 0.01$) in comparison with G2, G3, and G4 (Fig. 6).

3.2.6. Western blot

Different antibody binding patterns were observed amongst the vaccinated groups. Only few bands were visible with sera collected from G1 chicken while G5, G4, and G2 chicken revealed differences in numbers and intensities of bands (Fig. 7).

4. Discussion

Malpractice and vast use of anticoccidial agents resulted in the emergence of drug-resistant *Eimeria* strains (Quiroz-Castañeda and Dantán-González, 2015)) and critical appraisement of extensive medication may increase. Nonetheless, banning of anticoccidials would be a serious challenge for the poultry industry and thus such products are still regarded and widely used as poultry feed additives, *e.g.* in the European Union (Anon, 2003). Vaccination could offer a solution for this problem. In general, live vaccines are superior to inactivated vaccines in inducing protective immunity due to their ability to complete their life cycle in enterocytes (Yun et al., 2000). *Eimeria* strains selected for precociousness are particularly suited for vaccination since they replicate in the host intestine, thus produce offspring oocysts that booster primary immunisation, but are of reduced virulence (Williams, 1998). In the EU, only attenuated live vaccines are licensed and various products based on this principle have been commercialized. However, their production requires large numbers of chickens and causes high production costs. This is a major hindrance for extensive routine use of precocious attenuated vaccine strains (Shivaramaiah et al., 2013).

Ionizing radiation is a suitable option for production of attenuated vaccines (Nims and Plavsic, 2015) and has been previously used for oocysts (Jenkins et al., 1991; Fetterer et al., 2014). Recent studies on

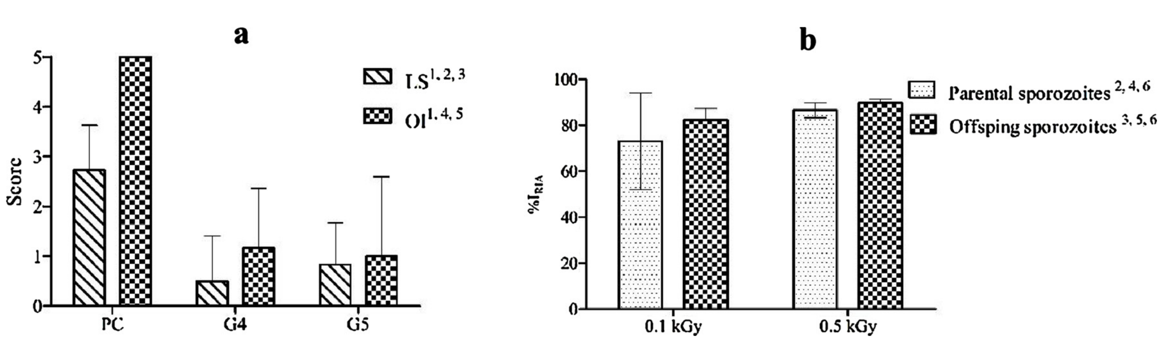

Fig. 5. Comparison of *in vivo* and *in vitro* parameters between G4 (0.1 kGy) and G5 (0.5 kGy) in reference to their corresponded PC. Spearman's rank correlation coefficient (r_s; Mukaka, 2012) was calculated. Interpretation of coefficient values (+/- values indicate positive or negative correlation, respectively): very high (0.90–1.0), high (0.70-0.90), moderate (0.50-0.70), low (0.3-0.5), negligible (0.00-0.30). [1] LS *vs.* OI ($r_s = 0.800$, $p < 0.01$), [2] LS *vs.* parental $\%I_{RIA}$ ($r_s = -0.622$, $p < 0.01$), [3] LS *vs.* offspring $\%I_{RIA}$ ($r_s = -0.654$, $p < 0.05$); [4] OI *vs.* parental $\%I_{RIA}$ ($r_s = -0.921$, $p < 0.01$), [5] OI *vs.* offspring $\%I_{RIA}$ ($r_s = -0.903$, $p < 0.01$), [6] parental $\%I_{RIA}$ *vs.* offspring $\%I_{RIA}$ ($r_s = 0.967$, $p < 0.01$).

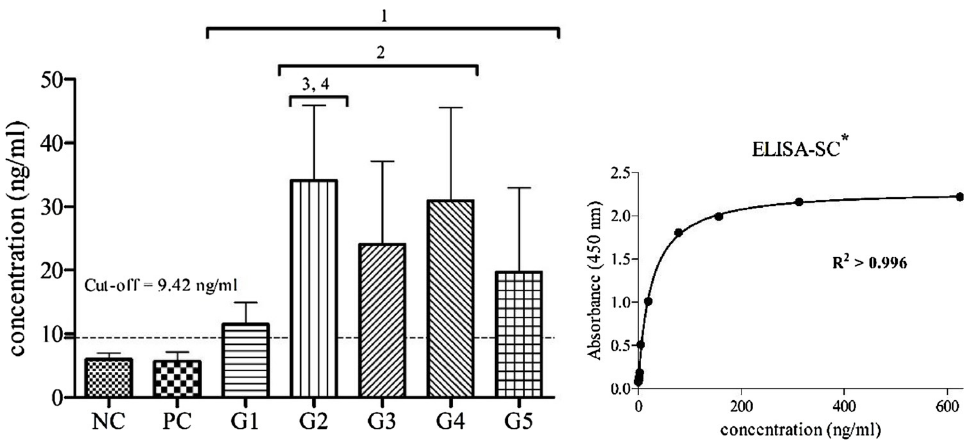

Fig. 6. *E. tenella* specific humoral immune response at D20 (before challenge infection). Significantly higher serum antibody concentrations are marked by superscripts as follows: [1] groups G1 – G5 as compared to NC or PC ($p < 0.01$); [2] G2-G4 as compared to G1 ($p < 0.01$); [3] G2 as compared to G1 and G5 ($p < 0.01$); [4] G2 as compared to G4 ($p < 0.05$). Refer to Table 1 for detailed description of groups. * Goodness-of-fitness value ($R^2 > 0.996$; $p < 0.050$) was determined for standard curve generated from measurement the OD values of known concentrations of labelled goat anti-chicken IgG after binding to unlabeled chicken IgY using nonlinear regression analysis.

the effects of gamma-irradiated sporozoites of another member of the apicomplexa, *Plasmodium falciparum*, demonstrated alteration of many parasite proteins, *e.g.* cell surface proteins, membrane transporters, and stress-induced proteins (Oakley et al., 2016). LEEI is a versatile technology for vaccine manufacturing, due to its safety and exact definition of application parameters compared with other forms of irradiation. LEEI was used successfully to inactivate viruses and bacteria that were demonstrated to elicit protective immune responses (Fertey et al., 2016; Bayer et al., 2018). The major effect of LEEI is to damage nucleic acids rather than proteins of the pathogen, thus maintaining antigenicity (Nims and Plavsic, 2015; Seo, 2015).

LEEI does neither require complex shielding nor a radioactive source (Rögner et al., 2009; Nims and Plavsic, 2015; Gotzmann et al., 2018). Therefore, the technology is well suited to be incorporated in standard biological laboratories or production facilities, and probably suited for vaccine production at reasonable costs.

RIA was applied successfully before to assess anticoccidial sensitivity profiles (*i.e.* ionophores, Thabet et al., 2015) and natural products supposed to inhibit parasite replication (Alnassan et al., 2015). RIA appeared reliable to determine inhibition of *in vitro* parasite reproduction and the calculated $\%I_{RIA}$ values correlated well with *in vivo* parasitological (OI) and pathological (LS) indicators of coccidiosis (Thabet et al., 2017). Generally, *in vivo* trials are necessary to proof immunogenicity of attenuated oocysts and to determine the most suitable vaccination dose (Fetterer et al., 2014). Such trials are time consuming and large numbers of chickens may have to be sacrificed. Alternatively, RIA appears to be a suitable *in vitro* method that allows rapid and sensitive investigation of viability of LEEI attenuated oocysts.

Sporozoites extracted from oocysts irradiated with various doses of LEEI were motile, under microscopic examination, before applying *in vitro* infection. LEEI at doses of 1.0 kGy or higher completely inactivated sporozoites' ability to replicate as did the ionophorous anticoccidial MON, while sporozoites extracted from oocysts exposed to lower doses of LEEI of 0.5 kGy and 0.1 kGy displayed reduced *in vitro* replication of approximately 15% or 30%, respectively. Consequently, we decided to use oocysts treated with doses of 0.1 kGy or 0.5 kGy LEEI to immunise chicken. Appropriate dose of irradiated oocysts to be used as vaccination during *in vivo* infection was unknown. A vaccination dose using irradiated oocysts, 1.0×10^4 oocysts/bird, was consistent with other experimental vaccine trials in chickens (Fetterer et al., 2014). In other hand, vaccination dose using virulent strain (1.0×10^2 oocysts/bird) was determined following routinely guidelines used during passage of *Eimeria* spp. (Raether et al., 1995). Interestingly, progeny oocysts collected from feces of animals immunized with LEEI exposed oocysts displayed similar $\%I_{RIA}$ values as the irradiated parental oocysts. This might indicate stable mutation leading to hereditary attenuation, which would be a relevant asset for development of a LEEI based vaccine. However, to confirm this assumption, repeated *in vivo* passages of progenies from LEEI attenuated oocysts and their evaluation are needed. Such data would require extensive animal experimentation

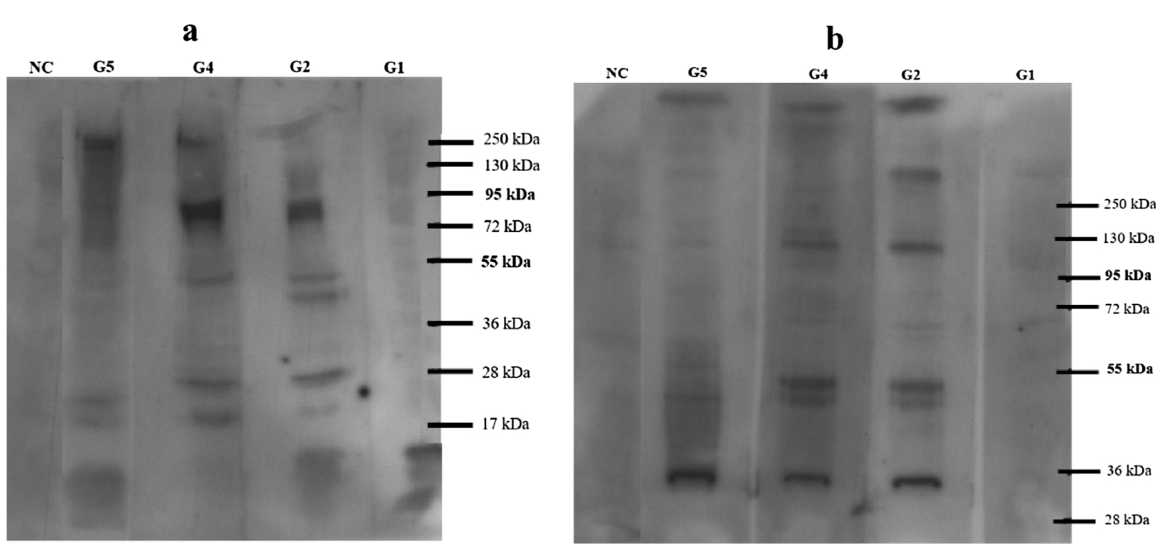

Fig. 7. Western blot probed with sera from vaccinated chicken collected at D21 (before challenge infection). Protein sizes in kDa are indicated on the right side. **8a**: Gradient gel (4–20%) was used to separate proteins of various sizes. Band sizes around 55 and 95 kDa (**Bold**) were of lower intensity in G5 sera compared with those in G2 and G4. **8b**: Using 10% linear gel similar band sizes were detected in G5 but not or to lesser extent in G2 and G4.

and are not available to date.

For the *in vivo* vaccination experiment Paracox®-8 was included as a reference product. Because *E. tenella* single species vaccines are not available and Paracox®-8 is well known to protect chicken against *E. tenella*, besides other *Eimeria* spp., we believe its use to benchmark LEEI attenuated oocysts for vaccination is reasonable and justified.

Immunisation with 0.5 kGy LEEI treated oocysts resulted in no clinical signs in chickens in contrast to oocysts exposed to a lower LEEI dose of 0.1 kGy (G3, G4). In the latter two groups blood stained litter was seen at D6 post-vaccination. Moreover, one chicken of G3 died due to cecal coccidiosis. Clinical cecal coccidiosis following infection by low dose (0.1 kGy) irradiated oocysts is probably attributable to the high infection dose of 1.0×10^4 oocysts/chicken that were apparently not sufficiently attenuated. The same number of high dose (0.5 kGy) irradiated oocysts did not cause any clinically obvious disease. Moreover, chickens vaccinated with 0.5 kGy irradiated oocysts excreted less oocysts seven days after application of the vaccination inoculum than did chicken of G3 and G4 (0.1 kGy) or those immunized with Paracox®-8. Performance parameters of chickens vaccinated with 0.5 kGy irradiated oocysts (G5) were not affected compared to the other vaccinated groups. In fact, weight gain was the highest and FCR the lowest in G5 during the first three weeks post-vaccination of all vaccinated groups. These results support our hypothesis that applying 0.5 kGy irradiated oocysts provide protection for chickens against *E. tenella* challenge not through causing subclinical infection but more as a vaccine.

Comparing pattern of oocyst excretion in samples collected directly from chickens or litter from G3 and G4 revealed variations despite using the same vaccination dose (0.1 kGy). Counting oocysts from freshly obtained feces reflects the intensity of infection at the time of sampling, while oocyst counting from litter smoothen the excretion pattern (Williams, 2002). Opg values of direct samples from G3 and G4 did not differ significantly after the challenge, *i.e.* the challenge did not seem to increase oocyst shedding. In other hand, the peak of oocyst excretion from litter samples in G3 was at D12, while G4 exhibited the first peak at D9 and a second peak at D20. Based on that, it seems that using LEEI at dose of 0.1 kGy on *E. tenella* oocysts did not reduce virulence of irradiated oocysts homogenously.

Chickens vaccinated with 0.5 kGy irradiated oocysts displayed lower serum antibody concentrations after vaccination than those vaccinated with 0.1 kGy exposed oocysts. This indicates lower infectivity and/or reproduction of 0.5 kGy irradiated parasites leading to less stimulation of the humoral reaction. Interestingly, there was no significant difference in serum *E. tenella* antibody concentration between G5 (0.5 kGy irradiated oocysts) and the Paracox®-8 group, while such difference was seen when the Paracox®-8 group was compared with G4 (0.1 kGy irradiated oocysts). Serum antibody values of G5 chicken (0.5 kGy irradiated oocysts) or Paracox®-8 vaccinated chicken with those of chicken primarily inoculated with virulent oocysts were clearly lower indicating variability of the immune reaction against attenuated and non-attenuated oocysts.

Western blot patterns suggest variable humoral response depending on the immunizing stimulus, which might contribute to the differing level of protection observed between groups. Interestingly, bands at ˜ 55 kDa and 95 kDa are of lower intensity or missing in Western blot lanes probed with pre-challenge sera of group G5 chicken (vaccinated with 0.5 kGy exposed oocysts) or G1 (Paracox®-8). Presence of these bands in G2 (virulent oocyts) and G4 (vaccinated with 0.1 kGy exposed oocysts) supported our assumption of suitability of 0.5 kGy LEEI for attenuation. Although the nature and function of the 55 kDa antigen in our study, that is obviously not expressed by parasites exposed to 0.5 kGy LEEI, are unknown, one may hypothesize that lack of this protein is related to attenuation. This assumption coincides with results of a previous study on the 56 kDa EtGAM56 antigen of *E. tenella* chicken passively immunized with antibody to EtGAM56 and showed distinctly (by 78%) reduced fecal oocyst excretion (Wiedmer et al., 2017). Since the proteins in these bands were not further characterized any assumption on their role in the immune response toward *E. tenella* would be hypothetical. Studying effect of LEEI on expression of microneme proteins might help to explain differences in Western blot patterns observed in the present study.

5. Conclusion

Exposure to LEEI attenuates *E. tenella* oocysts as demonstrated both *in vitro* and *in vivo*. Infection by oocysts exposed to 0.5 kGy reduced susceptibility of chicken to challenge without significantly impairing chicken productivity and health. It appears that LEEI induced attenuation is hereditary, however, this important aspect has to be further studied. A suitable *Eimeria* vaccine should contain multiple species to meet the requirements in the field, and therefore effects of LEEI on other *Eimeria* spp. should also be analyzed in future studies. Altogether, the present data open perspectives for a new technological approach for vaccine development against coccidia.

Authors' contribution

AT designed the study, wrote the manuscript, and performed the experiments. RS participated in *in vivo* studies. JF performed Western blot experiment. JF and JS participated in irradiating oocysts with LEEI. ML participated in designing *in vitro* experiment. BB participated in designing *in vivo* experiment. SU and AD critically revised the study design data interpretation and manuscript. All authors read and approved the final manuscript.

Ethical approval

The *in vivo* vaccination experiment was conducted in compliance with European and national legal requirements under registration and permission of the responsible authority (Landesdirektion Sachsen, Germany, file number TVV 53/16).

Conflict of interest

The authors declare that they have no competing interests.

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