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SHORT COMMUNICATION

Cross-reactivity of anti-*Eimeria tenella* antibody fragments on merozoites and sporozoites of different chicken *Eimeria* species

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Abstract *Eimeria tenella*-specific antibodies were examined for the cross-reactivity on the sporozoites and merozoites of *E. tenella*, *Eimeria maxima*, *Eimeria acervulina* and *Eimeria brunetti* in an indirect fluorescence antibody test. Two of nine antibodies showed cross-reactivity with sporozoites of *E. maxima*, *E. acervulina* and *E. brunetti*; however, the localization of specific fluorescence differed between species. No antibody binding was observed on merozoites. The suitability of these antibodies to alter the infectivity of *Eimeria* sporozoites and/or merozoites must be verified in cell culture models and in vivo experimental infections.

Introduction

Avian coccidiosis is caused by intestinal protozoa belonging to several species of the genus *Eimeria*. Coccidiosis results in more or less extensive destruction of the avian intestinal epithelium and causes reduced feed efficiency, depression in body weight gain and increased animal loss, thus significantly altering animal health and economic profit for the poultry industry.

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The development of alternative methods to control coccidiosis, e.g. food modulation and recombinant antibodies, remains an important task in the search for strategies that may be suited to replace the conventional methods using drugs.

Although coccidiosis is regarded as one of the most important infections in chicken, the incidence of clinical disease is generally low in intensive poultry farms despite the crowding of animals. This is due mainly to the proper application of prophylactic anti-coccidial drugs (Shirley and Bedrnik 1997); however, with the increased prevalence of drug resistant parasites, there is increasing interest in vaccines and recombinant antibodies (Shirley et al. 2005). The aim of the present study is to evaluate the anticoccidial effect of some recombinant antibody fragments against *Eimeria tenella* which have been expressed in transgenic pea.

Materials and methods

Eimeria species sporozoites

Sporulated oocysts of *E. tenella* (strain LE-01 Eten-05/1), *Eimeria brunetti* (strain LE-04 Ebru-06/1), *Eimeria acervulina* (strain LE-02 Eacer-05/1) and *Eimeria maxima* (strain LE-03 Emax-05/1) were maintained at the Institute of Parasitology, University of Leipzig, Germany, by using single oocyst infection and subsequent passage every 6 months as described before (Khalafalla and Daugschies 2010; Shirley 1977, 1995).

Sporozoites of *Eimeria* species were excysted as described by Tomley (1997). Briefly, after surface sterilization with bleach, the oocyst walls were broken using 0.5 mm glass beads (BioSpec Products, Bartlesville, OK, USA). The sporozoites were recovered from sporocysts by

enzymatic excystation [0.25% trypsin (*w*/*v*) (Carl Roth, Karlsruhe, Germany], 10 mM $MgCl_2$ (*w*/*v*) and 1% sodium taurocholic acid (*w*/*v*) (Sigma, Taufkirchen, Germany) at 41°C for 60–90 min. The excysted sporozoites were purified by centrifugation in 60% (*v*/*v*) isotonic Percoll™ solution (density, 1.129 g/ml; Pharmacia Fine Chemicals, Uppsala, Sweden) by a 1-min spin at 10,000×g. The pelleted sporozoites were collected carefully from the bottom and washed three times with phosphate-buffered saline (PBS) (pH 7.6; Na_2HPO_4 13.48 g/L; NaH_2PO_4, 0.78 g/L; NaCl 4.25 g/L) as described previously (Dulski 1990; Tomley 1997).

Eimeria species merozoites

Two-week-old chickens were infected per os with 2×10^5 sporulated oocysts of each species. The infected birds were killed humanely by anaesthetic euthanization after the prepatent period (*E. acervulina* 80 h, *E. tenella* 112 h, *E. maxima* 110–112 h and *E. brunetti* 110–112 h) to collect the merozoites (Shirley 1995). The upper part of the small intestine from the gizzard to the yolk sac diverticulum (*E. acervulina*), ceca (*E. tenella*), the middle part of small intestine (*E. maxima*) or the lower part of small intestine, rectum and ceca *(E. brunetti*) were prepared separately.

The intestinal portions were cut into small pieces and placed separately in the incubation medium for 30 min at 41°C on a heated plate with stirring at 250 rpm. The incubation medium consisted of 1× Hank's balanced salt solution (HBSS; Biochrom, Germany) with 10 mM $MgCl_2$ (Roth, Karlsruhe, Germany), 0.25% Trypsin (Roth, Karlsruhe, Germany) and 1% Na taurocholate (T-4009, Sigma, Taufkirchen, Germany). This medium was sterilized by filtration. The merozoite suspension was filtered through gauze to remove large particles. Then the filtrate was centrifuged at 700×g for 5 min and resuspended in 1× PBS. The merozoite pellet was passed through an equal volume of DE-52 (diethylaminoethyl cellulose, preswollen microgranular anion exchanger; Whatman International Ltd., Maidstone, England) and purified as described (Hollingdale and Kilejian 1979; Schmatz et al. 1984; Ono et al. 1991).

The following primary antibodies were used:

- Using the phage display antibody library, Novoplant GmbH generated nine anti-*Eimeria* scFv antibody fragments which were fully described by Zimmermann et al. (2009). These antibodies were expressed either transiently in agrobacteria-infiltrated tobacco leaves or stably in seeds of transgenic pea plant. The antibodies were kindly supplied by the company Novoplant (Novoplant GmbH, Am Schwabeplan 1b, 06466 Gatersleben, Germany) and were denominated Ab1, Ab2, Ab3, Ab4, Ab5, Ab6, Ab7, Ab8 and Ab9.
- As control, a rabbit anti-*Eimeria bovis* calmodulin domain protein kinase antiserum was kindly provided by Dr. V. Dyachenko (Institute of Parasitology, University of Leipzig, Germany).

The following secondary antibodies were applied:

- FITC-conjugated goat anti-rabbit IgG (H+L) (code 111-0950003, Dianova GmbH, Jackson Immunoresearch Laboratories, Hamburg, Germany).
- Unconjugated monoclonal mouse anti-polyhistidine IgG clone His-1 (Sigma Aldrich, Saint Louis, MO, USA)
- FITC-conjugated goat anti-mouse IgG (Fcg-fragment specific, 115-095-008, Dianova GmbH, Jackson Immunoresearch Laboratories, Hamburg, Germany)
- Polyclonal rabbit anti-EbCDPK (*E. bovis* calcium dependent protein kinase) IgG

Freshly excysted sporozoites and merozoites of *Eimeria* spp. were purified as described above and fixed on 12-well slides (epoxy-coated, 5 mm diameter, X1XER302W#MNZ, Diagnostika, Menzel Glaeser, Braunschweig, Germany). The slides were fixed with 4% buffered formaldehyde [paraformaldehyde (*w*/*v*) in PBS] for 10 min at room temperature. A second group of slides was fixed with 100% methanol for 10 min at −20°C. All slides were washed three times in PBS and left to dry. The formaldehyde-fixed slides were blocked with PBS containing 100 mM (*w*/*v*) glycin for 10 min in a humid chamber (petri dishes with wetted tissue). After three washes in PBS, both formaldehyde and methanol fixed slides were blocked with PBS containing 2% (*w*/*v*) bovine serum albumin (BSA) (Roth, Karlsruhe, Germany).

After three washes in PBS, 20 µl of each diluted primary antibody [1:10, 1:50, 1:100, 1:500 in 1×PBS containing 1% BSA (*w*/*v*)] were dropped onto the antigen spots. The slides were incubated for 1 h at room temperature in a humid chamber. The slides were washed three times in PBS and left to dry. All tested antigen spots were additionally incubated with 1:100 diluted monoclonal anti-polyhistidine antibody and subsequently incubated for 60 min at room temperature in a humid chamber. The slides were washed in 1×PBS three times and left to dry.

Conjugated antibody (FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG) was added at dilution of 1:100 in incubation buffer containing 0.02% Evan's blue as a counter stain. The slides were incubated in a humid chamber for 60 min at room temperature and again washed three times in PBS and left to dry.

All spots were covered with 20 µl of DAPI (DAPI nucleic acid stain, Molecular Probes, Invitrogen, Karlsruhe, Germany) solution (300 nM) for 3 min in the dark at room temperature. After rinsing with PBS, the slides were mounted in 90% glycerol (*v*/*v*), 10% PBS (*v*/*v*) and 1 mg/ml p-

phenylenediamine. The slides were examined under an inverted microscope (Leica DM IRB, Wetzlar GmbH, Bensheim, Germany). The control reactions were made as described above using the following antibodies:

First control: monoclonal anti-polyhistidine antibody plus FITC-conjugated goat anti-mouse IgG (without application of antibody fragments)
Second control: only FITC-conjugated goat anti-mouse IgG
Third control: rabbit anti-EbCDPK IgG plus FITC-conjugated goat anti-rabbit IgG

Imaging and picture processing

For examination and digital image capture, an inverted microscope (Leica DM IRB, Bensheim, Germany) equipped with a cooled camera head (Nikon DS-5Mc) and LCD (Nikon DS-L1, Nikon, Japan) was used. Fluorescent as well as differential interference contrast microscopy pictures were taken under identical adjustments in respect of magnification power. These pictures were further processed and overlaid using the computer software package CorelDRAW® Graphics Suite 12.

Results and discussion

Some antibodies with inhibitory effect on various *Eimeria* stages have already been identified (Danforth et al. 1985; Lillehoj and Choi 1998; Labbe et al. 2005). Such antibodies produced in plants used for animal feeding could offer a simple and inexpensive biopharmaceutical means for coccidiosis control. Plant-based antibodies (plantibodies) are supposed as alternative options to control several animal diseases, for example foot and mouth disease (Santos et al. 2005), rinderpest (Khandelwal et al. 2003a, b), rotavirus infection (Saldana et al. 2006) and infectious bronchitis virus (Zhou et al. 2003). Plant-based recombinant chicken sIgA was expressed in tobacco leaves and induced immune protection against coccidiosis (Wieland et al. 2006).

There are several potential advantages of using plant technology for the production of vaccines; most notably, the overall costs can be greatly reduced compared with competing systems. This is mainly due to the relatively low cost afforded by oral delivery of antigen in large amounts. Despite the obvious advantages of plant-based vaccines, there are still severe biosafety limitations as well as concerns in the public on genetically modified plants.

Potential biosafety risks are transgene spread in the environment, recombinant protein accumulation in the ecosystem, contamination of food and feed chains with transgenes and their products. Also, aspects of product quality and safety are discussed in this respect (Commandeur et al. 2003); however, these potential risks can be controlled by various physical and biological means (Commandeur et al. 2003).

The current study was conducted to evaluate the inhibitory effect of nine recombinant antibody fragments against *E. tenella* which have been expressed in transgenic pea. If efficacious, these antibody fragments might be applied later as a food constituent to protect chicken from infection with coccidia. Indirect fluorescence antibody test was performed to evaluate the binding reaction of these antibody fragments with *E. tenella*.

Binding with sporozoites of *E. tenella* was recorded for seven of nine antibody fragments (Ab1, Ab4, Ab5, Ab6, Ab7, Ab8 and Ab9) (Table 1). Of the seven Ab fragments that bound to sporozoites of *E. tenella*, only two (Ab4 and Ab5) displayed cross-reactivity with the sporozoites of *E. brunetti*, *E. maxima*, and *E. acervulina*. In contrast, no specific antibody binding reaction could be observed on merozoites of all examined *Eimeria* spp. (Table 1).

Failure of immunofluorescent staining of merozoites is suggesting that the target antigens used for the preparation of these antibody fragments are not expressed on merozoites reflecting the different antigen patterns of merozoites and sporozoites of *Eimeria* spp. (Reduker and Speer 1986; Speer et al. 1989; Tabares et al. 2004). Merozoites and sporozoites of *Eimeria* spp. are different in their ultrastructures as the refractile bodies are not found in the first generation merozoites and are replaced by several smaller

Table 1 Biniding of anti-*Eimeria tenella* antibody fragments to sporozoites and merozoites of *Eimeria* species

Antibody fragments		Ab1	Ab2	Ab3	Ab4	Ab5	Ab6	Ab7	Ab8	Ab9
E. tenella	Sporozoites	+	−	−	+	+	+	+	+	+
	Merozoites	−	−	−	−	−	−	−	−	−
E. brunetti	Sporozoites	−	−	−	+	+	−	−	−	−
	Merozoites	−	−	−	−	−	−	−	−	−
E. acervulina	Sporozoites	−	−	−	+	+	−	−	−	−
	Merozoites	−	−	−	−	−	−	−	−	−
E. maxima	Sporozoites	−	−	−	+	+	−	−	−	−
	Merozoites	−	−	−	−	−	−	−	−	−

spherical bodies (Hammond et al. 1970; Danforth and Augustine 1989). Antigens unique to sporozoites or merozoites may thus represent stage-specific differentiation, as has been reported in *Toxoplasma gondii* (Kasper et al. 1984; Kasper and Ware 1985) and *Plasmodium* spp. (Toure-Balde et al. 2009).

Fluorescence was observed on the outer surface of the sporozoites of *E. tenella* for Ab1, Ab6, Ab7, Ab8 and Ab9, and thus these antibody fragments are obviously reacting with surface antigen. Cross-reactivity of Ab4 and Ab5 was observed with sporozoites of *E. acervulina*, *E. brunetti* and *E. maxima*. This indicates the presence of common antigens (Danforth and Augustine 1983). It is well-known that immunity to *E. tenella* does not confer resistance to *E. maxima* or other species (McDougald 1998), and thus it appears improbable that certain antibody fragments even if they bind across the species barrier, will have general protective effects against coccidia.

None of the tested antibody fragments showed positive binding reaction with merozoites. This property will be a disadvantage because sporozoites invading host cells in spite of the presence of antibody fragments will induce rapid multiplication of the parasite and eventually oocyst excretion. A single oocyst, single sporocyst or single sporozoite is able to give rise to a clonal population of the respective *Eimeria* species (Shirley and Harvey 1996). For production of effective recombinant antibodies against multistage protozoan parasites such as *Eimeria* spp., it would be advantageous if they would target antigens of different stages. This would include sporozoite antigen to protect against the first invasive stages and merozoite antigen to prevent asexual multiplication which might appear if sporozoites escape the first line of defence.

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