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Research paper

Anticoccidial efficacy testing: *In vitro Eimeria tenella* assays as replacement for animal experiments

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ABSTRACT

Scope: Availability of an accurate *in vitro* assay is a crucial demand to determine sensitivity of *Eimeria* spp. field strains toward anticoccidials routinely. In this study we tested *in vitro* models of *Eimeria tenella* using various polyether ionophores (monensin, salinomycin, maduramicin, and lasalocid) and toltrazuril. Minimum inhibitory concentrations (MIC_{95}, $MIC_{50/95}$) for the tested anticoccidials were defined based on a susceptible reference (Houghton strain), Ref-1. *In vitro* sporozoite invasion inhibition assay (SIA) and reproduction inhibition assay (RIA) were applied on sensitive laboratory (Ref-1 and Ref-2) and field (FS-1, FS-2, and FS-3) strains to calculate percent of inhibition under exposure of these strains to the various anticoccidials ($\%I_{SIA}$ and $\%I_{RIA}$, respectively). The *in vitro* data were related to oocyst excretion, lesion scores, performance, and global resistance indices (GI) assessed in experimentally infected chickens.
Results: Polyether ionophores applied in the RIA were highly effective at MIC_{95} against Ref-1 and Ref-2 ($\%I_{RIA} \geq 95\%$). In contrast, all tested field strains displayed reduced to low efficacy ($\%I_{RIA} < 95\%$). $\%I_{RIA}$ values significantly correlated with oocyst excretion determined in the animal model ($p < 0.01$) for polyether ionophores. However, this relationship could not be demonstrated for toltrazuril due to unexpected lack of *in vitro* sensitivity in Ref-2 ($\%I_{RIA} = 56.1\%$). In infected chickens, toltrazuril was generally effective ($GI > 89\%$) against all strains used in this study. However, adjusted GI (GI_{adj}) for toltrazuril-treated groups exhibited differences between reference and field strains which might indicate varying sensitivity.
Conclusion: RIA is a suitable *in vitro* tool to detect sensitivity of *E. tenella* towards polyether ionophores, and may thus help to reduce, replace, or refine use of animal experimentation for *in vivo* sensitivity assays.

1. Introduction

Eimeria tenella (*E. tenella*) is an apicomplexan parasite and the causative agent of cecal coccidiosis in chickens. Cecal coccidiosis causes immense losses in poultry industry (McDougald and Fitz-Coy, 2013). Different strategies are used currently worldwide to control coccidiosis on poultry farms such as improvements in management and hygiene, using natural supposedly anticoccidial products or applying various types of anticoccidial vaccines (Quiroz-Castañeda and Dantán-González, 2015). However, preventive application of drugs in feed or drinking water remains the most widely distributed method to control coccidiosis worldwide (Chapman et al., 2010).

Anticoccidial agents are generally classified into either polyether ionophores or synthetic chemical anticoccidials (De Gussem, 2007). Modes of action and targeted developmental stages of *Eimeria* vary between different anticoccidials. Polyether ionophores arrest sporozoites by increasing intracellular Na^+ concentrations and activity of Na^+/K^+/ATPase (Wang et al., 2006). Besides, they affect merozoites by bursting the cell border, endoplasmic reticulum, and internal organelles (Mehlhorn et al., 1983). Toltrazuril, a triazine chemical member, acts upon all intracellular stages in either schizogony or gamogony cycles (Haberkorn and Stoltefuss, 1987).

Though different strategies are employed to reduce the emergence of anticoccidial resistance, resistant strains have been reported against almost all compounds introduced on the market (Stephan et al., 1997; Mattiello et al., 2000; Peek and Landman, 2003; Abbas et al., 2011) and resistance appears to be widespread.

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To select appropriate anticoccidial measures is a challenging demand for poultry farms in order to reduce losses caused by coccidia. Affordable and rapid assays to assess anticoccidial efficacy are essential to propose effective farm-specific treatment regimes. Several *in vitro* studies were conducted to compare anticoccidial sensitivities between known sensitive and field *E. tenella* strains applying different models. Counting intracellular sporozoites and schizonts after staining infected cell cultures and using phase-contrast microscopy has been utilized for this purpose (McDougald and Galloway, 1976; Smith et al., 1981; Zhu and McDougald, 1992). Jenkins et al. (2014) used quantitative PCR (qPCR) and semi-quantitative PCR to estimate intracellular sporozoite numbers after treatment with ionophores but without comparing their test directly to *in vivo* efficacy data. At present, *in vivo* anticoccidial sensitivity assays are considered the only reliable method for determination of sensitivity of *Eimeria* spp. (Dardi et al., 2013). However, these assays are time-consuming procedures and difficult from an ethical point of view since a large number of chickens needs to be experimentally infected and sacrificed in order to determine parasitological and pathological parameters (i.e. lesion score) (Chapman, 1998; Stephan et al., 1997). In the present study, we examined two quantitative *in vitro* models using qPCR to assess the efficacy of monensin, salinomycin, maduramicin, lasalocid, and toltrazuril against reference strains and field strains of *E. tenella*. The first is a sporozoite invasion inhibition assay (SIA), the second is a merozoite reproduction inhibition assay (RIA). Both models refer to previously described procedures representing general options to test for anticoccidial efficacy (Jenkins et al., 2014; Thabet et al., 2015). To assess their applicability for different anticoccidal compounds the assays were directly compared in the present study. Moreover, Both *in vitro* assays were compared to an *in vivo* sensitivity test with the same *E. tenella* strains, using the global resistance index (GI) as standard readout criterion (Stephan et al., 1997). *In vitro* and *in vivo* data were related to evaluate the suitability of the applied *in vitro* assays as a replacement for animal experiments.

2. Materials and methods

2.1. E. tenella strains

Two laboratory reference *E. tenella* strains (Ref-1: Houghton and Ref-2: Wisconsin (WIS)) with no history of exposure to anticoccidials and three *E. tenella* strains isolated from the field (FS-1, FS-2, and FS-3) were used in this study to compare their *in vitro* and *in vivo* sensitivity profiles. Sporulated oocysts of these strains were stored at +4 °C in 2% potassium dichromate solution at the Institute of Parasitology (Leipzig University, Germany), and maintained by subsequent passage every six months as described before (Shirley, 1977).

Excysted sporozoites were utilized to study *in vitro* sensitivity toward anticoccidials while sporulated oocysts were used in the *in vivo* study. Sporozoite excystation for *E. tenella* (reference and field strains) was conducted as described earlier (Raether et al., 1995). Oocysts were washed from potassium dichromate in phosphate-buffered saline (PBS, pH = 7.2), and surface sterilized using 12% sodium hypochlorite for 10 min. Oocysts were washed from sodium hypochlorite by diluting them 1:10 in PBS and centrifuged (1085 x g). Pellets were resuspended in PBS and the washing step was repeated three additional times. Mechanical breakage of oocyst walls was performed using 0.5 mm glass beads (BioSpec Products, Bartlesville, OK, USA). Then, enzymatic excystation of sporozoites was achieved 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. Sporozoites were purified by anion exchange chromatography (Schmatz et al., 1984). Sporozoites were centrifuged (2778 x g), washed three times with PBS (1.0 ml), and counted using a hemocytometer.

Each *E. tenella* strain was analysed by conventional PCR for purity using species-specific primer sets (Schnitzler et al., 1998) before each *in vitro* and *in vivo* experiment. Oocyst suspensions of all used strains had a sporulation rate more than 90%. Only sporulated oocysts were counted for preparation of infectious doses for *in vivo* sensitivity tests. All strains were stored for three to five months before use for animal experimentation.

2.2. Anticoccidials

Analytical grade reagents of the anticoccidials monensin sodium salt (Mon; 90–95% TLC), salinomycin monosodium hydrate (Sal; 93.8%), maduramicin ammonium (Mad; 97.9%), lasalocid A sodium salt solution (Las; 100 ng/μl), and toltrazuril (Tol; 99.7%) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Fresh stock solutions of Mon, Sal, Mad, and Tol (1000 μg/ml) were dissolved using dimethyl formamide (DMF) as a solvent and deionized water as diluent.

For *in vivo* anticoccidial sensitivity tests, feed premixes containing polyether ionophores at defined concentrations were prepared as follows: monensin sodium (Coxidin-200®, 110 ppm), salinomycin sodium (Sacox-120®, 60 ppm), maduramicin ammonium (Cygro-10G®, 5.5 ppm), and lasalocid sodium (Avatec-150G®, 100 ppm). Premixes were prepared at the Institute of Animal Nutrition, Nutrition Diseases & Dietetics (Leipzig University, Germany). Samples of feed were analysed (LUFA-Nord West, Hameln, Germany) for accurate anticoccidial concentrations. Toltrazuril (Baycox 2.5%, Bayer Animal Health, Germany) was added in drinking water on two consecutive days following the manufacturer's recommendations (0.28 ml in drinking water for the Tol group, equivalent to 7.0 mg, per kg body weight per day).

2.3. In vitro *anticoccidial sensitivity assays*

In vitro assays were performed as described by Thabet et al. (2015). Briefly, Madin-Darby bovine kidney (MDBK) cells (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were used as host cell culture for *E. tenella* sporozoite infections. 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 μg/ml; and amphotericin-B, 0.25 μg/ml; GE Healthcare, Germany). MDBK cells were inoculated with sporozoites and incubated with the various anticoccidials at different concentrations at 41 °C and 5% CO_2. Trypsin-versene (Lonza, Thermo Scientific, Germany) was added to detach adherent MDBK cells at the selected time point of incubation (see below).

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 nanodrop (Nanodrop 2000, Thermo Scientific, Germany) at 260 nm wavelength. DNA were diluted to a concentration of 20 ng/μl, and stored at −20 °C until use for qPCR.

2.3.1. Minimum inhibitory concentration ($MIC_{50/95}$ vs. MIC_{95}) determination

Strain Ref-1 (Houghton) served as reference to determine MIC of anticoccidials as previously described (Thabet et al., 2015). MDBK cells were inoculated with sporozoites of Ref-1 and extracellular (non-invasive) sporozoites were removed by washing cultures three times with PBS after 24 h p.i. Cell culture media were prepared