Experimental infection with *Toxoplasma gondii* in broiler chickens (*Gallus domesticus*): seroconversion, tissue cyst distribution, and prophylaxis

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**Abstract**

*Toxoplasma gondii* is a widespread zoonotic protozoan that infects most species of mammals and birds, including poultry. This study aimed to investigate the course of *T. gondii* infection and the efficacy of diclazuril and *Artemisia annua* in preventing infection in experimentally infected chickens. Seventy-five 1-month-old chickens, female and male, were randomly divided into five groups (*n* = 15 each) as follows: (1) uninfected untreated (negative control, NC); (2) infected with *T. gondii* genotype II/III isolated from a wild cat (group WC); (3) infected with *T. gondii* genotype II isolated from a domestic cat (group DC); (4) infected with *T. gondii* domestic cat strain and treated with the anticoccidial diclazuril (group DC-D); and (5) infected with *T. gondii* domestic cat strain and treated with the medicinal plant *Artemisia annua* (group DC-A). Clinical signs, body temperature, mortality rate, weight gain, feed conversion ratio, hematological parameters, and the presence of *T. gondii*–specific IgY antibodies were recorded in all groups. Five chickens per group were euthanized 28 days post-infection (p.i.) and their brains, hearts, and breast muscle tested for *T. gondii* by mouse bioassay and polymerase chain reaction (PCR). No clinical signs related to the experimental infection were observed throughout the study period. *T. gondii*–specific antibodies were detected by day 28 p.i., but not in all infected chickens. Overall, *T. gondii* DNA was detected (bioassay or tissue digests) in all infected and untreated chickens (10/10), while viable parasite (bioassay) was isolated from 7 out of 10 chickens. The parasite was most frequently identified in the brain (7/10). There were no differences in the *T. gondii* strains regarding clinical infection and the rate of *T. gondii* detection in tissues. However, higher antibody titers were obtained in chickens infected with *T. gondii* WC strain (1:192) comparing with *T. gondii* DC strain (1:48). *A. annua* reduced replication of the parasite in 3 out of 5 chickens, while diclazuril did not. In conclusion, broiler chickens were resistant to clinical toxoplasmosis, irrespective of the strain (domestic or wild cat strain). The herb *A. annua* presented prophylactic efficacy by reduced parasite replication. However, further studies are required aiming at the efficacy of diclazuril and *A. annua* for the prevention of *T. gondii* infection in chickens using quantitative analysis methods.
Introduction

Toxoplasma gondii is a widespread zoonotic protozoan that infects mammals, including felids and humans, and birds. The definitive hosts, cats, and other felids are the only source of infective oocysts, while the intermediate hosts (mammals, birds, and humans) develop tissue cysts. The main sources of infection for humans are the consumption of raw or undercooked meat, as well as water and food (fruit, vegetables) contaminated with sporulated oocysts from the environment (Dubey 2010).

This apicomplexan parasite is an important pathogen with a varying prevalence in poultry, which depends on the raising system. A wide range of seroprevalence values have been reported, from 0% in broiler chickens (Rodrigues et al. 2019) to 100% in organic and backyard farm chickens (Dubey 2010). The backyard and free-range chickens represent a good indicator of soil contamination with T. gondii since they feed on the ground. Also, they play an important role in the epidemiology of T. gondii, as in the case of home slaughtering and improper offal disposal, they are the source of infection for cats (Dubey 2010).

Parasite isolation studies have shown that the prevalence of viable T. gondii in chickens from commercial indoor farms is low (Dubey 2010), but can be high in backyard chickens (Dubey et al. 2004; Schares et al. 2017). Therefore, consumption of undercooked infected chicken meat can be a potential source of infection with T. gondii for humans (Dubey 2010).

Despite numerous studies on T. gondii seroprevalence and parasite isolation in chickens, only a few clinical cases of chicken toxoplasmosis were reported worldwide (Dubey 2010). The strain of T. gondii has been shown to influence the course of infection in gallinaceous birds (Dubey et al. 1993; Koethe et al. 2015). Options for treatment of toxoplasmosis in chickens are limited. For small animals (i.e., cats), drugs such as clindamycin, azithromycin, and combinations of trimethoprim and a sulfonamide are used (Elmore et al. 2010; Lappin 2010; Bresciani et al. 2016). Diclazuril is a coccidiostat used for the prophylaxis of coccidiosis in broilers (Zechner et al. 2015; Ogolla et al. 2018). As well, experimental studies in mice have shown its efficacy against toxoplasmosis (Lindsay et al. 1995). Natural products from plant extracts have exhibited an anticoccidial activity in both in vitro and in vivo models (Mirzaalizadeh et al. 2018; de Almeida et al. 2012; Drăgan et al. 2014). Sweet wormwood plant (Artemisia annua) and its derivatives have shown efficacy in experimental models of toxoplasmosis in mice (Nagamune et al. 2007; Dunay et al. 2009; Hencken et al. 2010; Islamunddin et al. 2015; Müller et al. 2015).

The current study aimed to evaluate (i) the T. gondii clinical infection in chickens experimentally infected with a low dose of cysts of different strains (genotype II/III strain vs. genotype II strain), (ii) the rate of T. gondii infection in the brain, heart, and skeletal muscle, and (iii) the efficacy of diclazuril and A. annua for the prevention of T. gondii infection in chickens.

Materials and methods

Chickens

One-day-old chickens were purchased from S.C. AVIS S.A. Vadu Crișului and housed until 1 month old in batteries in dedicated facilities of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (UASVM CN). The chickens were fed with standard starter and growing feed supplemented with an anticoccidial drug (diclazuril). Diclazuril was withdrawn 2 days before the start of the experiment. Water and feed were provided ad libitum and the light was continuous. The experiment was approved by the Animal Ethics Committee of UASVM CN (protocol no. 92/20.12.2017).

Experimental design

Seventy-five 1-month-old chickens were randomly divided into five groups (n = 15 each) each with three replicates of five chickens per cage, as follows: (1) uninfected untreated (negative control, NC); (2) infected with T. gondii genotype II/III isolated from a wild cat (group WC); (3) infected with T. gondii genotype II isolated from a domestic cat (group DC); (4) infected with T. gondii domestic cat strain and treated with the anticoccidial diclazuril (group DC-D); and (5) infected with T. gondii domestic cat strain and treated with the medicinal plant Artemisia annua (group DC-A) (Table 1). The day of the infection was designated as day 0, when, according to the experimental group, twelve T. gondii tissue cysts per chicken were administered by oral gavage (Table 1). Diclazuril and A. annua were introduced into the diet 2 days before experimental infection (day −2) and continually administered until the end of the experiment (day 28 post-infection [p.i.]) (Fig. 1).

All chickens were monitored throughout the study period for any change in health status and mortality. Copro-parasitological examination by the flotation technique using a saturated salt solution (specific gravity 1.28) was performed on days 3 and 7 p.i. Body temperature (BT) was registered daily during the first-week p.i. Chickens were individually weighed at days 0, 7, and 28 p.i., to calculate the body weight gain (BWG). The quantity of feed given to the chickens was weighed daily per cage, and the feed conversion ratio (FCR)
was calculated as the ratio between the amount of feed consumed and the weight gain of the chickens (Pop et al., 2015).

Blood samples were collected from 7 chickens per group on days 0, 3, 7, and 28 p.i. The samples were analyzed for hematological parameters (days 0, 7, and 28) and direct (DNA) (days 3 and 7) and indirect (specific IgY antibodies) (days 0, 7, and 28) detection of *T. gondii*. Then, 5 chickens per group were euthanized on day 28 p.i. The brain, heart, and skeletal muscle were collected from each chicken and analyzed by mouse bioassay and PCR.

**T. gondii strains and experimental infection**

*T. gondii* strains isolated by mouse bioassay from a wild cat (*Felis silvestris*), and a domestic cat (*Felis catus*), were used for experimental infections (Table 1).

The wild cat strain was obtained from a road-killed animal. All heart was collected and artificial digestion performed (for details, refer to the “Mouse bioassay” section). Two mice were inoculated and euthanized by cervical dislocation at 4 weeks p.i. The brain, heart, and skeletal muscle were collected from each chicken and analyzed by mouse bioassay and PCR for the presence of *T. gondii* cysts and analyzed by PCR for confirmation. Then, the isolated strain was genotyped by the multiplex nested PCR-RFLP technique (Su et al. 2006; Khan et al. 2005), with the use of the following markers: GRA6, altSAG2, BTUB, APICO, C22, C29-2, PK1, and CS3. PCR products were digested with appropriate restriction enzymes for the different markers. The products obtained after digestion were visualized by electrophoresis using a 3% agarose gel.

The domestic cat was presented to our clinic with diarrhea and apathy. *T. gondii*-like oocysts were observed on parasitological examination of the feces, and *T. gondii* DNA was identified by PCR. The oocysts were sporulated in 2.5% potassium dichromate and then orally administered to four mice. The strain was previously genotyped by the PCR-RFLP technique and identified as type II (ToxoDB number 1) (Nedișan et al., 2019).

The *T. gondii* cysts used for experimental infection of chickens were obtained after the second passage in mice for each strain. Briefly, ten mice per strain were inoculated intraperitoneally (i.p.) with mouse brain cysts and after 1 month, for the presence of *T. gondii* cysts and analyzed by PCR for confirmation. Then, the isolated strain was genotyped by the multiplex nested PCR-RFLP technique (Su et al. 2006; Khan et al. 2005), with the use of the following markers: GRA6, altSAG2, BTUB, APICO, C22, C29-2, PK1, and CS3. PCR products were digested with appropriate restriction enzymes for the different markers. The products obtained after digestion were visualized by electrophoresis using a 3% agarose gel.

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the mice were euthanized. The brains were recovered and homogenized with a saline solution. A 20-μL drop of the homogenate was placed on a microscope slide and the cysts counted under a microscope (Djurković-Djaković et al. 2005). Finally, the number of *T. gondii* cysts in the suspension was adjusted to 12 cysts/0.5 mL (Table 1).

### Medication

Diclazuril (Clinacoxx® 0.5%, Huvepharma) and *A. annua* (leaf powder) were used for the prevention of *T. gondii* infection in chickens. Diclazuril was administered in the chicken diet to group DC-D at a concentration of 1.2 ppm (1.2 g/1000 kg feed) delivering 0.15 mg diclazuril/kg body weight (FAO/WHO, 2000).

*A. annua* plants were dried in the shade at ambient temperatures (20 °C). After drying, leaves were manually separated and ground to obtain *A. annua* leaf powder (Drăgan et al. 2014). The concentration of artemisinin (1.706% w/w) was evaluated by HPLC 3 months before the experiment (Ferreira and Gonzalez 2009). Broiler feed was prepared by adding 4 kg of *A. annua* leaf powder per 1000 kg of feed (0.4%) and fed daily to group DC-A. The amount of artemisinin in feed supplemented with 0.4% *A. annua* leaf powder was 68.24 ppm (Allen et al. 1997; Pop et al. 2015; Györke et al. 2019) delivering 8.53 mg artemisinin/kg body weight/day (FAO/WHO, 2000). The feed supplemented with diclazuril and *A. annua* was administered 2 days before the experimental infection (day −2) until the end of the experiment (day 28 p.i.).

### Hematological parameters

Red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit (PCV), and the total and differential white blood cell (WBC) counts were performed on days 0, 7, and 28 p.i. The concentration of Hb was determined by the spectrophotometer method (ELISA Bio-Rad 1100 microplate reader). After adding 4% ammonia solution to the blood sample, the absorbance was measured at 540 nm.

The PCV was established using the microhematocrit method. The capillary tubes were centrifuged at 12,000 rpm for 5 min. The values were determined with the aid of a microhematocrit reader.

The RBC and WBC counts were carried out manually by the hemocytometer method using the Natt-Herrick solution modified by the Prochaska (Ognean and Cernea 2011).

### Serology

Specific IgY antibodies against *T. gondii* were evaluated on days 0, 7, and 28 p.i. by MAT, TgSAG1-ELISA, and immunoblot using p30 antigen. Serum samples were sent to ANSES - Laboratoire de Santé Animale (Maisons-Alfort, France) for MAT and to Friedrich-Löffler-Institut (Germany) for TgSAG1-ELISA, and immunoblot. A positive sample in any of the used methods was considered positive in total serology.

**MAT**

*T. gondii*–specific IgY antibodies were detected by the modified agglutination test (MAT) (Desmonts and Remington 1980). Formalin-fixed whole RH tachyzoites were used as antigens. The antigen was provided by the National Reference Centre for Toxoplasmosis in Reims, France (Villena et al. 2012). The starting serum dilution was 1:6, and the sera were titrated to end-point in two-fold dilutions. All sera reactive at a serum dilution of 1:24 were considered positive (Dubey et al. 1993).

**TgSAG1-ELISA**

Chicken sera were also tested for antibodies against *T. gondii* tachyzoite surface antigen TgSAG1 as described by Schares et al. (2017). Affinity-purified TgSAG1 of *T. gondii* tachyzoites was diluted in bicarbonate buffer (0.1 M, pH 8.3) and used at a concentration of 30 ng/mL to sensitize ELISA plates. The plates were then washed with PBS supplemented with 0.05% (v/v) Tween® 20 (Serva, Heidelberg, Germany) (PBST). A blocking step with 1% casein in PBST (CasPBST; 30 min, 37 °C) followed. The plates were emptied and washed and 100 μL of serum, 1:200 diluted in CasPBST, was added for 30 min, 37 °C. After serum incubation and washing, a species-specific conjugate (goat anti-chicken IgY (H&L) peroxidase (POD), synonymous with anti-chicken IgY (H&L) POD, Rockland Immunochemicals, Dianova, Hamburg, Germany) was diluted 1:4000 in CasPBST. After washing with PBS-T (thrice) and distilled water (once), reactions were visualized using 1% tetra-methyl-benzidine (TMB) with 0.012% (v/v) H2O2 as the substrate. After 13–15 min at 37 °C, the reaction was stopped by the addition of 50 μL of 2 M H2SO4, and the O.D. in each well was read at 450 nm. ELISA index values (I) were calculated for each sample (S) based on the means of two O.D. values: IS = (O.D.S – O.D.NC)/(O.D.PC – O.D.NC). A cut-off optimized for maximum diagnostic specificity was applied (ELISA index 0.242) as previously described for the TgSAG1-ELISA (Schares et al. 2017).

**Immunoblot using p30 antigen**

The sera were analyzed according to the protocol described by Schares et al. (2017). Briefly, the p30 surface antigen was obtained from *T. gondii* RH strain cell culture–derived tachyzoites by affinity chromatography using the monoclonal antibodies. Purified p30 was incubated in non-reducing
sample buffer (2% [w/v] sodium dodecyl sulfate (SDS), 10% [v/v] glycerol, 62 mM Tris-HCl, pH 6.8) for 1 min (94 °C), separated in 12% (w/v) SDS polyacrylamide mini gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Germany). After the transfer, membranes were blocked using PBS-TG (PBS with 0.05% (v/v) Tween 20 (Sigma-Aldrich, Germany) and 2% (v/v) liquid fish gelatine (Serva, Germany)) and cut into 50 strips and examined. Chicken sera were diluted 1:100 in PBS-TG. Peroxidase-conjugated anti-chicken IgY (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used diluted 1:500 in PBS-TG. Positive and negative controls were used.

**Mouse bioassay**

Five of the fifteen chickens from each group were euthanized on day 28 p.i. Whole brain, whole heart, and half of the breast muscle were sampled separately from each chicken ($n = 75$). The tissue samples were bioassayed in 6–8-week-old female mice, CD1 line (one mouse/tissue; $n = 75$). Mice were housed and maintained following the regulations on the welfare and protection of laboratory animals, according to the national Romanian law 43/2014 and EU Directive 2010/63/EU. All mouse bioassays reported in this publication were approved by the Animal Ethics Committee of UASVM Cluj-Napoca (protocol no. 93/20.12.2017).

Each tissue sample was weighed, ground, and homogenized with digestion solution (0.25% trypsin from swine pancreas, Sigma-Aldrich code 93613; EDTA 0.025%; PBS) (50 mL for 20 g tissue). Tissue digestion was performed at 37 °C for 90 min. The digest was then centrifuged at 3000 rpm for 10 min. The supernatant was removed, and the sediment washed twice with PBS in repeated centrifugations. Finally, the supernatant was removed and the sediment (digest pellet) reconstituted in 2 mL of PBS, to which 200 μL of the antibiotic solution was added (penicillin-streptomycin, Sigma-Aldrich code P0781) (Montoya et al. 2009).

One mouse per tissue was i.p. inoculated with 1 mL tissue digest. Following inoculation, mice were housed in standard cages. Each cage was identified by the sample code and the date of inoculation. Inoculated mice were monitored for clinical signs. At 4 weeks p.i., mice were euthanized by cervical dislocation and brains harvested. Each mouse brain was checked by microscopy for the presence of *T. gondii* cysts (Paștiu et al. 2019) and analyzed by PCR (25 mg of the homogenized whole brain) for confirmation (Homan et al. 2000).

**PCR assays**

The buffy coat (WBCs) (days 3 and 7 p.i.), brain, heart, and breast muscle digestes from chickens (day 28 p.i.) and the brain homogenates from the mouse bioassays were analyzed by conventional PCR. WBCs were isolated by centrifugation at 1500–2000g for 10–15 min at room temperature. After centrifugation, the buffy coat containing the WBCs was collected with a plastic transfer pipet.

The DNA extraction was performed from 100 μL of the buffy coat, digest, and mouse brain homogenate using the commercial Isolate II Genomic DNA Kit (Bioline) according to the manufacturer’s instructions.

All DNA samples were analyzed with conventional PCR targeting the 529-bp DNA fragment of *T. gondii* using Tox4 (5′-CGCTGCAGGGAGGAAGACGAAAGTTG-3′) and Tox5 (5′-CGCTGCAGACACA GTGCATCTGATT-3′) specific primers (Homan et al. 2000).

Amplification was performed in a final volume of 25 μL consisting in 5 μL PCR Master Mix (12.5× Green PCR Master Mix), 10 pmol of each primer (Tox4 and Tox5), 4 μL of sample DNA, and 15 μL of ultrapure water. Positive (*T. gondii* domestic cat strain) and negative controls (ultrapure water) were used in each set of reactions. Amplification was performed with the C1000TM Thermal Cycler (Bio-Rad). The amplification program consisted of one initial cycle at 95 °C (5 min) and 37 cycles of denaturation at 95 °C (30 s) followed by hybridization (annealing) at 60 °C (30 s), extension at 72 °C (1 min), and a final extension cycle at 72 °C (5 min). In the end, PCR products (8 μL) were electrophoresed in 1.5% agarose gel in TAE buffer and stained with SYBR® Safe DNA gel stain (Invitrogen). Electrophoresis conditions were 100 V and 400 mA for 30 min. in TAE buffer. DNA fragments were visualized under UV light on an image analyzer (Bio-Rad BioDoc-It™ Imaging System) and compared to a 100-bp molecular weight marker (GeneRuler 100 bp DNA Ladder, Fermentas).

**Statistical analysis**

Arithmetic mean and standard error of the mean were calculated for numerical data. The data distribution was assessed by the Shapiro-Wilk test for normal distribution. If data followed a normal distribution, one-way analysis of variance with Tukey-Kramer as a post hoc test was used to examine differences within each experimental group at different time points, and repeated measures analysis of variance was used to compare experimental groups at specific time points (0, 7, and 28 days p.i.). In the case of the non-normal distribution of the data, the non-parametric Kruskal-Wallis with Conover as a post hoc test was used. The level of significance was set at 0.05. All statistical analyses were performed with the MedCalc Statistical Software version 19.0.7.

The degree of agreement between serological methods (MAT, TgsAg1-ELISA, immunoblot) was measured by Cohen’s kappa ($k$) statistic in EpiTools (Watson and Petrie 2010; Sergeant 2018). Also, the proportions of the positive
and negative agreements were calculated. The level of confidence was set at 0.95 and population status as unknown/mixed. The strength of agreement was defined based on the k value: < 0.00 poor agreement; 0.00–0.20 slight agreement; 0.21–0.40 fair agreement; 0.41–0.60 moderate agreement; 0.61–0.80 substantial agreement; and 0.81–1.00 almost perfect agreement (Landis and Koch 1977).

Results

T. gondii genotypes

The domestic cat strain was type II (ToxoDB number 1) and it was previously published (Nedișan et al. 2019). The wild cat strain, according to the RFLP analysis, was identified to belong to genotype II/III (Table 2).

T. gondii infection

T. gondii DNA was detected by PCR in the blood on day 3 p.i. in all experimentally infected groups, but at a different rate (Table 3). The group infected with wild cat strain (WC) presented a higher rate (71.4%; 5/7) of detection comparing with DC strain (28.5%; 2/7). The rate of T. gondii DNA detection decreased on day 7 p.i. in all groups (Table 3).

The results of T. gondii detection in chicken tissues (brain, heart, and breast muscle) on day 28 p.i. are presented in Table 4. T. gondii cysts were not identified by microscopic examination of the mouse brains.

Overall, T. gondii DNA was detected by PCR (in bioassay or in tissue digest) in all infected and untreated chickens regardless of the parasite strain. By bioassay, the rate of detection was of 80% (4/5) in chickens infected with DC strain and of 60% (3/5) in those infected with WC strain. Overall, the brain was the tissue of predilection for T. gondii in chickens 70% (7/10), followed by heart 40% (4/10) and breast 40% (4/10). However, in three chickens, T. gondii DNA was not detected in the brain, but in the heart or breast muscle.

None of the experimentally infected chickens had detectable T. gondii antibodies on day 7 p.i., and not all the chickens on day 28 p.i. regardless of the parasite strain (Table 5). The control group remained negative during the experiment. The total rate of seroconversion in infected groups was 57.2% and different accordingly to the serological test: 35.7% by MAT, 19.2% by TgSAG1-ELISA, and 42.3% by immunoblot. There was no statistically significant difference ($\chi^2 = 3.45, P = 0.327$) among T. gondii-infected groups. The highest serum titer was 1:48 for groups infected with T. gondii DC strain and 1:192 for group infected with T. gondii WC strain (Table 6).

Substantial agreement was observed between MAT and TgSAG1-ELISA, and moderate agreement for the other serological tests pairs (Table 7). Four out of 16 seropositive chickens have been detected positive in all three serological tests.

Clinical toxoplasmosis

No clinical signs related to the experimental infection were observed. None of the animals died, showed signs of apathy, or had increased body temperature (BT). At day 3 p.i., all groups, including animals from the NC, presented mild diarrhea and hematochezia. The copro-parasitological exam was negative. The BT was in the normal range (40.17–42.52) for the species in all experimental groups (Christensen et al. 2012) (Fig. 2).

Compared to control animals, all groups of infected animals irrespective of the strain or further treatment had both a lower weight gain and a higher FCR, although not statistically significant throughout the experiment (Supplementary file 1).

Table 2 Genotyping results of the T. gondii strain isolated from a wild cat from Romania

| Isolate                  | Genetic markers | Genotype |
|--------------------------|-----------------|----------|
|                          | GRA6 alt-       | BTUB     | C22-8 | C29-2 | PK1 | CS3 | APICO |
| Wild cat                 | II              | II       | III   | -     | -   | -   | -     |
| Reference strain (TgCkGh1) | II              | II       | III   | II    | III | III | -     |

Table 3 Detection of T. gondii DNA from white blood cells expressed as the number of positive chickens/total chickens and the percentage (%)

| Sampling day | Experimental group |
|--------------|---------------------|
|              | NC<sup>a</sup> WC<sup>b</sup> DC<sup>c</sup> DC-D<sup>d</sup> DC-A<sup>e</sup> |
| 3 p.i.<sup>f</sup> | 0/7 (0) 5/7 (71.4) 2/7 (28.5) 5/7 (71.4) 3/7 (42.8) |
| 7 p.i.       | 0/7 (0) 2/7 (28.5) 1/7 (14.2) 1/7 (14.2) 1/7 (14.2) |

<sup>a</sup>NC negative control  
<sup>b</sup>WC group infected with T. gondii wild cat strain  
<sup>c</sup>DC group infected with T. gondii domestic cat strain  
<sup>d</sup>DC-D group infected with T. gondii DC strain and treated with diclazuril  
<sup>e</sup>DC-A group infected with T. gondii DC strain and treated with A. annua  
<sup>f</sup>p.i. post-infection
The erythrocyte count (RBCs) and the concentration of Hb were in the normal range of the species during the entire experiment (Supplementary file 2). No significant differences were found among groups or days of sampling. Generally, WBC decreased significantly ($P < 0.001$) from day 0 to day 28 p.i. in all experimental groups. In contrast, the percentage of monocytes increased from day 0 to day 28 p.i. (Supplementary file 2) in WC ($F_{(2,17)} = 0.405$, $P = 0.673$), DC ($F_{(2,15)} = 8.625$, $P = 0.003$), and DC-A ($F_{(2,17)} = 2.637$, $P = 0.101$) groups and decreased in NC ($F_{(2,16)} = 0.790$, $P = 0.471$) and DC-D ($F_{(2,16)} = 2.597$, $P = 0.106$) groups, but no significant differences were noted among groups at any time point.

The efficacy of diclazuril and *Artemisia annua*

From the clinical and serological point of view, infected and treated chickens presented similar aspects with infected and untreated chickens (Supplementary files 1–2, Fig. 2, and Tables 5 and 6). Also, none of the mice presented clinical signs of toxoplasmosis and *T. gondii* cysts were not identified by microscopic examination of their brains.

Regarding the *T. gondii* DNA detection in tissues on day 28 p.i., the data varied according to the treatment (Table 4). Overall, the rate of *T. gondii* detection was lower in chickens treated with *A. annua* (40.0%; 2/5) comparing with that in untreated ones (5/5) or even with those treated with diclazuril (5/5).

Based on bioassay, the viable parasite was detected in 2 (from brain and heart) out of 5 chickens treated with *A. annua* and in 3 (from brain) out of 5 chickens treated with diclazuril, while in untreated ones in 4 (from the brain, heart, and breast muscle) out of 5 chickens (Table 4).

When bioassay was compared with PCR on tissue digests, in the *A. annua*–treated group, the detection rate was higher in bioassay. Contrary, in group treated with diclazuril, the detection rate was higher in tissue digests. In case of the untreated

### Table 4

Detection of *T. gondii* in chickens’ tissues by conventional PCR targeting the 529-bp DNA fragment of *T. gondii* (number of positive chickens/total chickens)

| Experimental group | Brain B | Heart D | Breast muscle T | Total  |
|--------------------|---------|---------|-----------------|--------|
| NC$^d$             | 0/5     | 0/5     | 0/5             | 0/5    |
| DC$^e$             | 2/5     | 3/5     | 3/5             | 5/5    |
| WC$^f$             | 2/5     | 2/5     | 4/5             | 5/5    |
| DC-D$^g$           | 3/5     | 0/5     | 3/5             | 5/5    |
| DC-A$^h$           | 1/5     | 1/5     | 2/5             | 2/5    |

*PCR* polymerase chain reaction

- **A**: bioassay
- **D**: tissue digest
- **T**: total (bioassay plus tissue digest)
- **NC**: negative control
- **WC**: group infected with *T. gondii* wild cat strain
- **DC**: group infected with *T. gondii* domestic cat strain
- **DC-D**: group infected with *T. gondii* DC strain and treated with diclazuril
- **DC-A**: group infected with *T. gondii* DC strain and treated with *A. annua*

### Table 5

*T. gondii*–specific IgY antibodies on day 28 post-infection by MAT, ELISA, and immunoblot expressed as the number of positive chickens/total chickens and the percentage (%)

| Experimental group | MAT  | TgSAG1-ELISA | Immunoblot | Total  |
|--------------------|------|--------------|------------|--------|
| NC$^a$             | 0/7  | 0/7          | 0/7        | 0/7    |
| WC$^b$             | 3/7  | 1/7 (14.3)   | 3/7 (42.9) | 5/7 (71.4) |
| DC$^c$             | 2/7 (28.6) | 1/7 (14.3) | 2/7 (28.6) | 3/7 (42.9) |
| DC-D$^d$           | 3/7 (42.9) | 1/6 (16.7)  | 2/6 (33.3) | 3/7 (42.9) |
| DC-A$^e$           | 2/7 (28.6) | 2/6 (33.3)  | 4/6 (66.7) | 5/7 (71.4) |

*NC*: negative control

- **WC**: group infected with *T. gondii* wild cat strain
- **DC**: group infected with *T. gondii* domestic cat strain
- **DC-D**: group infected with *T. gondii* DC strain and treated with diclazuril
- **DC-A**: group infected with *T. gondii* DC strain and treated with *A. annua*
Table 6  *T. gondii* IgY antibody titers obtained in MAT at 28 days post-infection in positive chickens expressed as the number of positive chickens/total tested chickens and percentage

| Group | Antibody titers | 1:24 | 1:48 | 1:96 | 1:192 |
|-------|----------------|------|------|------|------|
| NC*a | 0/7 (0%) | 0/7 (0%) | 0/7 (0%) | 0/7 (0%) |
| WC*b | 1/7 (14.3%) | 0/7 (0%) | 0/7 (0%) | 2/7 (28.6%) |
| DC*c | 1/7 (14.3%) | 1/7 (14.3%) | 0/7 (0%) | 0/7 (0%) |
| DC-D*d | 0/7 (0%) | 3/7 (42.8%) | 0/7 (0%) | 0/7 (0%) |
| DC-A*e | 1/7 (14.3%) | 1/7 (14.3%) | 0/7 (0%) | 0/7 (0%) |

*a*NC negative control  
*b*WC group infected with *T. gondii* wild cat strain  
*c*DC group infected with *T. gondii* domestic cat strain  
*d*DC-D group infected with *T. gondii* DC strain and treated with diclazuril  
*e*DC-A group infected with *T. gondii* DC strain and treated with *A. annua* group, the rate of detection was similar in bioassay and tissue digests (Table 4).

**Discussion**

The present study was designed to assess the clinical infection of experimental infection with a low dose of cysts of two different *T. gondii* strains in chickens, the rate of proliferation of *T. gondii* in tissues, and the efficacy of the prophylaxis with two different products: an anticoccidial drug, diclazuril, and a medicinal plant, *A. annua*. During the study period, no clinical signs attributable to *T. gondii* infection were observed. However, mild diarrhea was noticed on day 3 in all experimental groups that could be linked to a dysmicrobism of the intestinal flora caused by food change from starter to growing diet. Previously, other experimental infections showed that chickens are resistant to clinical toxoplasmosis and only a few clinical cases were reported (Dubey 2010). We noticed a slight increase in the WBC and monocytes in DC and WC groups at 7 days p.i. *T. gondii* has the capability to replicate in chicken macrophages, representing a reservoir for the parasite (Malkwitz et al. 2013; Quéré et al. 2013). The white blood cells followed a decreasing trend in all chickens, this leukogram pattern being consistent with a stress leukogram due to endogenous steroid release in stressful conditions (Maxwell 1993; Schmidt 2015).

Anti-*T. gondii* antibodies were detected on day 28 p.i. and not all experimentally infected chickens seroconvert (57.2%). We cannot exclude seroconversion before, as the serology was performed on days 7 and 28 p.i. Similar results were obtained by Godoi et al. (2010) with a seroconversion of 58.3% (by MAT, and IFAT) in experimentally infected pigeons with *T. gondii* oocysts. The rate of seroconversion in birds seems to vary with the infective dose and parasite strain (Geuthner et al. 2014; Geuthner et al. 2019).

*T. gondii* DNA was detected overall in all infected and untreated chickens, irrespectively of the parasite strain. The rate of *T. gondii* DNA detection was comparable in bioassay (7/10) and tissue digests (8/10). Then, the *T. gondii* DNA was more frequently detected in the brain, than in the heart and breast muscle. It is well known that a positive PCR in a tissue does not mean a viable parasite and that only the bioassay will prove that. Based on these results, uncooked meat from an infected chicken possesses a high risk for a consumer (Geuthner et al. 2019; Rodrigues et al. 2019).

The failure to detect *T. gondii* DNA in the same chicken in all examined tissues can be explained by the rare and inhomogeneous distribution of tissue cysts (Opsteegh et al. 2016). Also, the rate of infection depends on the inoculation dose, the route of infection, the parasitic stage, or the individual characteristics of the isolate. We used a low dose of cysts (12/chicken) that were orally inoculated, in order to reproduce a natural condition of infection. In a study performed by Dubey and Frenkel (1973), mice infected orally with 10 tissue cysts failed to establish infection, whereas higher doses (≥1000) resulted in successful infection. Then, for bioassay, we used the whole brain, whole heart, and half of the breast muscle, but only one mouse was bioassayed per tissue sample (in total three mice per chicken). If more than one mouse had been used for bioassay, the percentage of *T. gondii* isolation from experimentally infected chickens probably would have increased. In this study, there were no detected notable differences between the domestic and wild cat strains. This can be due to the fact that they were genotype II and genotype I/III.

Table 7  Agreement between different serological tests and total serology

|                  | MAT vs. TgSAG1-ELISA | MAT vs. immunoblot | TgSAG1-ELISA vs. immunoblot |
|------------------|----------------------|--------------------|-----------------------------|
| Kappa            | 0.67 (0.33–1.02)     | 0.46 (0.14–0.78)   | 0.53 (0.22–0.83)            |
| Proportion positive agreement | 0.73                  | 0.59                | 0.63                        |
| Proportion negative agreement | 0.95                  | 0.86                | 0.83                        |
| Overall proportion agreement | 0.91                  | 0.79                | 0.82                        |
respectively. Previous studies showed that the heart is the predilection tissue for *T. gondii* (Dubey 2010; Yan et al. 2010; Opsteegh et al. 2016), but we detected the parasite more frequently in the brain, and mainly in untreated chickens. In treated chickens, the rate of detection was the same in the brain and the heart. The concentration of *T. gondii* DNA in chicken tissues appears to be generally low. False-negative results in an unknown proportion of tissue samples cannot be excluded, even if extremely sensitive methods are applied (Hiob et al. 2017). However, combined results obtained in three different tissues by two different techniques suggest that the combination of the detection methods and on different tissues decreases the rate of false-negative results.

The administration of a drug in feed is a simple and effective way to prevent or treat diseases in large groups of animals, representing one of the two practical solutions in birds, along with the administration in water.

For centuries, *A. annua* has been a medicinal plant known to be an excellent antimalarial. Many other therapeutic attributes of this plant have been discovered, even coccidiostat, antimicrobial, or anticancer properties (Tajehmiri et al. 2014; Breuer and Efferth 2014).

Overall, *A. annua* seems to be a promising option in preventing *T. gondii* infection (2 chickens positive out of 5) when compared with untreated chickens (5/5), and even with those treated with diclazuril (5/5). It remains to be evaluated its efficacy in case of high-dose *T. gondii* infection. Also, the response to treatment can be strain dependent. A previous study showed that the susceptibility of *T. gondii* to atovaquone and to sulfadiazine was different according to the parasite strain (Alves and Vitor 2005).

When bioassay results are compared, diclazuril seems not to interfere with overall parasite dissemination, but rather with its viability (3 positive chickens out of 5). In vitro studies on cell cultures have shown that treatments with diclazuril resulted in >97% reduction in tachyzoite counts (Lindsay and Blagburn 1994). Also, in vivo studies revealed that diclazuril in doses between 0.5 and 10 mg/kg/day alone or in combination with pyrimethamine increases the survival rate of mice in acute toxoplasmosis, but did not prevent the formation of tissue cysts (Lindsay et al. 1995). Diclazuril was originally developed for prophylaxis of chicken coccidiosis in a dose of 1 ppm (0.125 mg/kg body weight). We used a dose of diclazuril of 0.15 mg/kg body weight and it was given 2 days before experimental infection. Also, the absorption of diclazuril from the gut is limited, 90% of the drug being excreted in the feces within 24 h.

Further studies are required aiming at the chicken’s response to *T. gondii* infection with different doses of cysts and with different strains. As regarding the efficacy of diclazuril and *A. annua* for prevention of *T. gondii* infection in chickens, further studies are needed using quantitative analysis of the parasite and different experimental models (dose of infection, strains, dose of the product).

In conclusion, broiler chickens were resistant to clinical toxoplasmosis, irrespective of the strain (domestic or wild cat strain). There were no differences in the rate of *T. gondii* detection in chickens depending on the parasite strain. Moreover, both strains replicated in all infected, but were detected at a higher rate in the brain. Not all infected chickens did seroconvert on day 28 p.i. regardless of the strain and serological test. The herb *A. annua* and the coccidiostat diclazuril revealed a prophylactic efficacy; however, further studies are required aiming at the efficacy of diclazuril and *A. annua* for the prevention of *T. gondii* infection in chickens using quantitative analysis methods.
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Compliance with ethical standards
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Ethics approval The experiment was approved by the Animal Ethics Committee of UASVM CN (protocol no. 92/20.12.2017).

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