



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.

Maria E. Nedişan and Adriana Györke contributed equally to this work.

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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)

Table 1 Experimental groups, *T. gondii* experimental infection, and applied treatments

Experimental group	Chickens (n)	<i>T. gondii</i> experimental infection			Treatments	
		Inoculum dose (n)	Route	<i>T. gondii</i> strain	Drug	Dose (in feed)
NC ^a	15	ND ^f	ND	ND	ND	-
WC ^b	15	12 cysts	Orally	Wild cat	ND	-
DC ^c	15	12 cysts	Orally	Domestic cat	ND	-
DC-D ^d	15	12 cysts	Orally	Domestic cat	Diclazuril	1.2 ppm
DC-A ^e	15	12 cysts	Orally	Domestic cat	<i>Artemisia annua</i> (artemisinin)	0.4% (68.24 ppm)

^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*

^f ND not done

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).

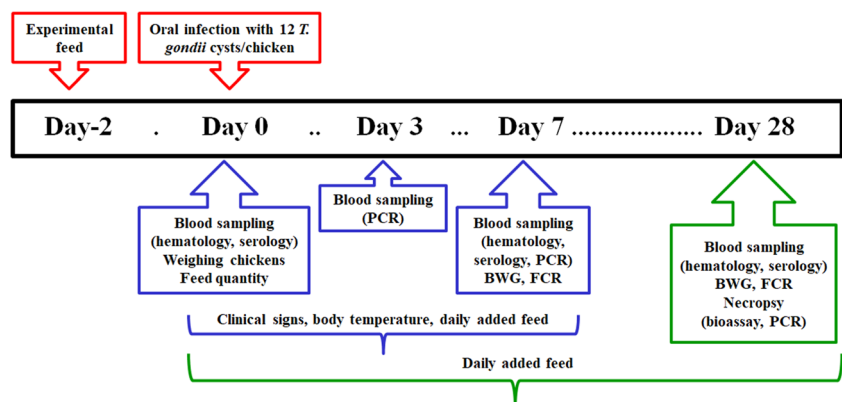
The wild cat strain was obtained from a road-killed animal. All heart was collected and artificial digestion performed (for details, refer to the “Mouse bioassay” section). Two mice were inoculated and euthanized by cervical dislocation at 4 weeks p.i. Mouse brains were examined by microscopy

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) (Nedişan 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,

Fig. 1 Study design. BWG, body weight gain; FCR, feed conversion ratio; PCR, polymerase chain reaction



the mice were euthanized. The brains were recovered and homogenized with a saline solution. A 20- μ L 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 (Clinacox® 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 μ L 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 μ L 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 (Schaes 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 μ L 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 digests 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 μ L 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 GTGCATCTGGATT-3') specific primers (Homan et al. 2000).

Amplification was performed in a final volume of 25 μ L consisting in 5 μ L PCR Master Mix (12.5 \times Green PCR Master Mix), 10 pmol of each primer (Tox4 and Tox5), 4 μ L of sample DNA, and 15 μ L 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 C1000™ 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 μ L) 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 post hoc tests and the Friedman test were 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 (Nedişan 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 the group infected with domestic cat strain (DC) (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

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 ^a	WC ^b	DC ^c	DC-D ^d	DC-A ^e
3 p.i. ^f	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)

^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*

^f p.i. post-infection

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 ($\chi_{(12)} = 3.45$, $P = 0.327$) 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-SAG2	BTUB	C22-8	C29-2	PK1	CS3	APICO	
Wild cat	II	II	III	-	-	III	-	-	II/III
Reference strain (TgCkGh1)	II	II	III	II	III	III	-	I	I/II/III

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			Heart			Breast muscle			Total		
	B ^a	D ^b	T ^c	B	D	T	B	D	T	B	D	T
NC ^d	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
DC ^e	2/5	3/5	3/5	2/5	1/5	2/5	2/5	2/5	3/5	4/5	4/5	5/5
WC ^f	2/5	2/5	4/5	1/5	2/5	2/5	0/5	1/5	1/5	3/5	4/5	5/5
DC-D ^g	3/5	0/5	3/5	0/5	4/5	4/5	0/5	2/5	2/5	3/5	5/5	5/5
DC-A ^h	1/5	1/5	2/5	1/5	0/5	1/5	0/5	0/5	0/5	2/5	1/5	2/5

PCR polymerase chain reaction

^a B bioassay

^b D tissue digest

^c T total (bioassay plus tissue digest)

^d NC negative control

^e WC group infected with *T. gondii* wild cat strain

^f DC group infected with *T. gondii* domestic cat strain

^g DC-D group infected with *T. gondii* DC strain and treated with diclazuril

^h DC-A group infected with *T. gondii* DC strain and treated with *A. annua*

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 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 (0.0)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)
WC ^b	3/7 (42.9)	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)

^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*

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).

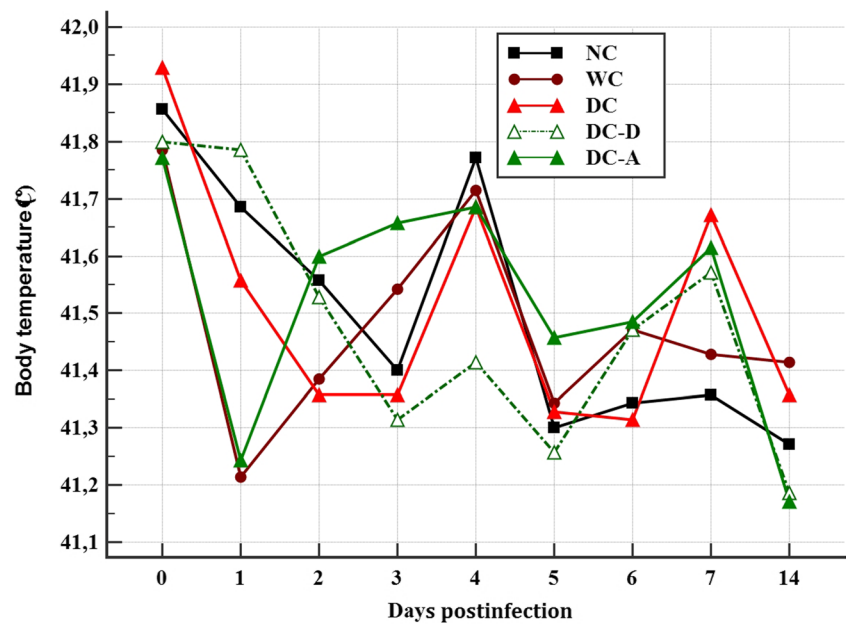
T. gondii DNA was detected overall in all infected and untreated chickens, irrespectively of the parasite strain. The rate of *T. gondii* DNA detection was comparable in bioassay (7/10) and tissue digests (8/10). Then, the *T. gondii* DNA was more frequently detected in the brain, than in the heart and 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 II/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

Fig. 2 Body temperature during the experiment



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 tovaquone 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

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The experiment was approved by the Animal Ethics Committee of UASVM CN (protocol no. 92/20.12.2017).

Consent to participate Not applicable.

Code availability Not applicable.

References

- Allen PC, Lydon J, Danforth H (1997) Effects of components of *Artemisia annua* on coccidia infections in chickens. *Poult Sci* 76(8):1156–1163. <https://doi.org/10.1093/ps/76.8.1156>
- Alves CF, Vitor RWA (2005) Efficacy of atovaquone and sulfadiazine in the treatment of mice infected with *Toxoplasma gondii* strains isolated in Brazil. *Parasite* 12(2):171–177. <https://doi.org/10.1051/parasite/2005122171>
- Bresciani KDS, Galvão ALB, de Vasconcellos AL, dos Santos TR, Kaneto CN, Viol MA, Gomes JF, Bilsland E (2016) Epidemiological aspects of feline toxoplasmosis. *Arch Vet Sci* 21(2):1–8. <https://doi.org/10.5380/avs.v21i2.39997>
- Breuer E, Efferth T (2014) Treatment of iron-loaded veterinary sarcoma by *Artemisia annua*. *Nat Prod Bioprospect* 4(2):113–118. <https://doi.org/10.1007/s13659-014-0013-7>
- Christensen K, Vizzier Thaxton Y, Thaxton JP, Scanes CG (2012) Changes in body temperature during growth and in response to fasting in growing modern meat type chickens. *Br Poult Sci* 53(4):531–537. <https://doi.org/10.1080/00071668.2012.715744>
- de Almeida GF, Horsted K, Thamsborg SM, Kyvsgaard NC, Ferreira JF, Hermansen JE (2012) Use of *Artemisia annua* as a natural coccidiostat in free-range broilers and its effects on infection dynamic and performance. *Vet. Parasitol* 186(3–4):178–187. <https://doi.org/10.1016/j.vetpar.2011.11.058>
- Desmonts GE, Remington JS (1980) Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. *J Clin Microbiol* 11(6):562–568
- Djurković-Djaković O, Nikolić A, Bobić B, Klun I, Aleksić A (2005) Stage conversion of *Toxoplasma gondii* RH parasites in mice by treatment with atovaquone and pyrrolidine dithiocarbamate. *Microbes Infect* 7(1):49–54. <https://doi.org/10.1016/j.micinf.2004.09.016>
- Drăgan L, Györke A, Ferreira JF, Pop IA, Dunca I, Drăgan M, Mircean V, Dan I, Cozma V (2014) Effects of *Artemisia annua* and *Foeniculum vulgare* on chickens highly infected with *Eimeria tenella* (phylum Apicomplexa). *Acta Vet Scand* 56:22. <https://doi.org/10.1186/1751-0147-56-22>
- Dubey JP (2010) *Toxoplasma gondii* infections in chickens (*Gallus domesticus*): prevalence, clinical disease, diagnosis and public health significance. *Zoonoses Public Health* 57(1):60–73. <https://doi.org/10.1111/j.1863-2378.2009.01274>
- Dubey JP, Frenkel JK (1973) Experimental toxoplasma infection in mice with strains producing oocysts. *J Parasitol* 59(3):505–512
- Dubey JP, Ruff MD, Camargo ME, Shen SK, Wilkins GL, Kwok OC, Thulliez P (1993) Serologic and parasitologic responses of domestic chickens after oral inoculation with *Toxoplasma gondii* oocysts. *Am J Vet Res* 54:1668–1672
- Dubey JP, Levy MZ, Sreekumar C, Kwok OCH, Shen SK, Dahl E, Thulliez P, Lehmann T (2004) Tissue distribution and molecular characterization of chicken isolates of *Toxoplasma gondii* from Peru. *J Parasitol* 90(5):1015–1018. <https://doi.org/10.1645/GE-329R>
- Dunay IR, Chan WC, Haynes RK, Sibley LD (2009) Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model. *Antimicrob Agents Chemother* 53(10):4450–4456. <https://doi.org/10.1128/aac.00502-09>
- Elmore SA, Jones JL, Conrad PA, Patton S, Lindsay DS, Dubey JP (2010) *Toxoplasma gondii*: epidemiology, feline clinical aspects, and prevention. *Trends Parasitol* 26(4):190–196. <https://doi.org/10.1016/j.pt.2010.01.009>
- FAO/WHO (2000) Food additives. Guidelines for the preparation of toxicological working papers for the Joint FAO/WHO Expert Committee on Food Additives
- Ferreira JFS, Gonzalez JM (2009) Analysis of underivatized artemisinin and related sesquiterpene lactones by high-performance liquid chromatography with ultraviolet detection. *Phytochem Anal* 56:91–97. <https://doi.org/10.1002/pca.1101>
- Geuthner AC, Koethe M, Ludewig M, Pott S, Schares G, Dausgchies A, Bangoura B (2014) Persistence of *Toxoplasma gondii* tissue stages in poultry over a conventional fattening cycle. *Parasitol* 141:1359–1364. <https://doi.org/10.1017/s003118201400078x>
- Geuthner AC, Koethe M, Ludewig M, Pott S, Schares G, Maksimov P, Dausgchies A, Bangoura B (2019) Development of an in vivo model for toxoplasma gondii infections in chickens and turkeys simulating natural routes of infection. *Vet Parasitol* 276:108956. <https://doi.org/10.1016/j.vetpar.2019.108956>
- Godoi FSLD, Nishi SM, Pena HFDJ, Gennari SM (2010) *Toxoplasma gondii*: diagnosis of experimental and natural infection in pigeons (*Columba livia*) by serological, biological and molecular techniques. *Rev Bras Parasitol Vet* 19(4):237–243. <https://doi.org/10.1590/S1984-29612010000400009>
- Györke A, Pop LM, Mircean M, Kalmár Z, Tăbăran AF, Paştiu AI, Dumitrache MO, Magdaş C, Balea A, Bărburaş D, Mircean V, Cozma V (2019) Metabolic and tissular effects of artemisinin supplemented diets in broiler chicken. *Pol J Vet Sci* 22(2):297–304. <https://doi.org/10.24425/pjvs.2019.129220>
- Hencken CP, Jones-Brando L, Bordón C, Stohler R, Mott BT, Yolken R, Woodard LE (2010) Thiazole, oxadiazole, and carboxamide derivatives of artemisinin are highly selective and potent inhibitors of

- Toxoplasma gondii*. J Med Chem 53(9):3594–3601. <https://doi.org/10.1021/jm901857d>
- Hiob L, Koethe M, Schares G, Goroll T, Dausgschies A, Bangoura B (2017) Experimental *Toxoplasma gondii* and *Eimeria tenella* co-infection in chickens. Parasitol Res 116(11):3189–3203. <https://doi.org/10.1007/s00436-017-5636-2>
- Homan WL, Vercammen M, De Braekeleer J, Verschueren H (2000) Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int J Parasitol 30(1):69–75. [https://doi.org/10.1016/S0020-7519\(99\)00170-8](https://doi.org/10.1016/S0020-7519(99)00170-8)
- Islamuddin M, Chouhan G, Farooque A, Dwarakanath BS, Sahal D, Afrin F (2015) Th1-biased immunomodulation and therapeutic potential of *Artemisia annua* in murine visceral leishmaniasis. PLoS Negl Trop Dis 9(1):3321. <https://doi.org/10.1371/journal.pntd.0003321>
- Khan A, Taylor S, Su C, Mackey AJ, Boyle J, Cole R, Glover D, Tang K, Paulsen IT, Berriman M, Boothroyd JC (2005) Composite genome map and recombination parameters derived from three archetypal lineages of *Toxoplasma gondii*. Nucleic Acids Res 33(9):2980–2992. <https://doi.org/10.1093/nar/gki604>
- Koethe M, Straubinger RK, Pott S, Bangoura B, Geuthner AC, Dausgschies A, Ludewig M (2015) Quantitative detection of *Toxoplasma gondii* in tissues of experimentally infected turkeys and in retail Turkey products by magnetic-capture PCR. Food Microbiol 52:11–17. <https://doi.org/10.1016/j.fm.2015.06.005>
- Landis JR, Koch GG (1977) The measurement of observer agreement for categorical data. Biometrics 33:159–174
- Lappin MR (2010) Update on the diagnosis and management of *Toxoplasma gondii* infection in cats. Top Companion Anim Med 25(3):36–41. <https://doi.org/10.1053/j.tcam.2010.07.002>
- Lindsay DS, Blagburn BL (1994) Activity of Diclazuril against *Toxoplasma gondii* in cultured cells and mice. Am J Vet Res 55(4):530–533
- Lindsay DS, Rippey NS, Blagburn BL (1995) Treatment of acute *Toxoplasma gondii* infections in mice with Diclazuril or a combination of Diclazuril and pyrimethamine. J Parasitol 81:315–318. <https://doi.org/10.2307/3283944>
- Malkwitz I, Berndt A, Dausgschies A, Bangoura B (2013) Long-term investigations on *Toxoplasma gondii*-infected primary chicken macrophages. Parasitol Res 112(9):3115–3122. <https://doi.org/10.1007/s00436-013-3486-0>
- Maxwell MH (1993) Avian blood leucocyte responses to stress. World Poultry Sci J 49:34–43. <https://doi.org/10.1079/WPS19930004>
- Mirzaalizadeh B, Sharif M, Daryani A, Ebrahimzadeh MA, Zargari M, Sarvi S, Mehrzadi S, Rahimi MT, Mirabediny Z, Golpour M, Montazeri M (2018) Effects of *Aloe vera* and *Eucalyptus* methanolic extracts on experimental toxoplasmosis in vitro and in vivo. Exp Parasitol 192:6–11. <https://doi.org/10.1016/j.exppara.2018.07.010>
- Montoya A, Miro G, Mateo M, Ramirez C, Fuentes I (2009) Detection of *Toxoplasma gondii* in cats by comparing bioassay in mice and polymerase chain reaction (PCR). Vet Parasitol 160:159–162. <https://doi.org/10.1016/j.vetpar.2008.10.029>
- Müller J, Balmer V, Winzer P, Rahman M, Manser V, Haynes RK, Hemphill A (2015) In vitro effects of new artemisinin derivatives in *Neospora caninum*-infected human fibroblasts. Int J Antimicrob Agents 46(1):88–93. <https://doi.org/10.1016/j.ijantimicag.2015.02.020>
- Nagamune K, Beatty WL, Sibley SD (2007) Artemisinin induces calcium-dependent protein secretion in the protozoan parasite *Toxoplasma gondii*. Eukaryot Cell 6(11):2147–2156. <https://doi.org/10.1128/ec.00262-07>
- Nedişan ME, Ursache AL, Dumitrache MO, Mircean V, Cozma-Petruş A, Györke A (2019) Intestinal toxoplasmosis in cats treated with Procox (case report). Sci Parasitol 20(1–2):19–24
- Ognean L, Cernea LC (2011) Practical applications in animal physiology. Ed. Academic Pres, Cluj-Napoca
- Ogolla KO, Gathumbi PK, Waruiru RM, Okumu PO, Chebet J, Kitala PM (2018) Efficacy of sulphachloropyrazine, amprolium hydrochloride, trimethoprim-sulphamethoxazole, and Diclazuril against experimental and natural rabbit coccidiosis. J Vet Med 5402469: 1–11. <https://doi.org/10.1155/2018/5402469>
- Opsteegh M, Schares G, Blaga R, van der Giessen J (2016) Experimental studies of *Toxoplasma gondii* in the main livestock species (GP/EFSA/BIOHAZ/2013/01) Final report. EFSA Support Public EN-995:161. <https://doi.org/10.2903/sp.efsa.2016.EN-995>
- Paştıu AI, Cozma-Petruş A, Mercier A, Balea A, Galal L, Mircean V, Pusta DL, Bogdan L, Györke A (2019) Prevalence and genetic characterization of *Toxoplasma gondii* in naturally infected backyard pigs intended for familial consumption in Romania. Parasit Vectors 12(1):586. <https://doi.org/10.1186/s13071-019-3842-8>
- Pop L, Györke A, Tăbăran AF, Dumitrache MO, Kalmár Z, Magdaş C, Mircean V, Zagon D, Balea A, Cozma V (2015) Effects of artemisinin in broiler chickens challenged with *Eimeria acervulina*, *E. maxima* and *E. tenella* in battery trials. Vet Parasitol 214(3–4): 264–271. <https://doi.org/10.1016/j.vetpar.2015.10.011>
- Quérel P, Pierre J, Hoang MD, Esnault E, Domenech J, Sibille P, Dimier-Poisson I (2013) Presence of dendritic cells in chicken spleen cell preparations and their functional interaction with the parasite *Toxoplasma gondii*. Vet Immunol Immunopathol 153:57–69. <https://doi.org/10.1016/j.vetimm.2013.02.007>
- Rodrigues FT, Moreira FA, Coutinho T, Dubey JP, Cardoso L, Lopes AP (2019) Antibodies to *Toxoplasma gondii* in slaughtered free-range and broiler chickens. Vet Parasitol 271:31–53. <https://doi.org/10.1016/j.vetpar.2019.06.007>
- Schares G, Bangoura B, Randau F, Goroll T, Ludewig M, Maksimov P, Opsteegh M (2017) High seroprevalence of *Toxoplasma gondii* and probability of detecting tissue cysts in backyard laying hens compared with hens from large free-range farms. Int J Parasitol 47(12): 765–777. <https://doi.org/10.1016/j.ijpara.2017.07.003>
- Schmidt S (2015) Top 5 leukogram patterns. Available at: <Available at: <http://www.cliniciansbrief.com> >
- Sergeant ESG (2018) Epitools epidemiological calculators. Ausvet. Available at: <http://epitools.ausvet.com.au>
- Su C, Zhang X, Dubey JP (2006) Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. Int J Parasitol 36(7):841–848. <https://doi.org/10.1016/j.ijpara.2006.03.003>
- Tajehmiri A, Issapour F, Moslem MN, Lakeh MT, Kolavani MH (2014) In vitro antimicrobial activity of *Artemisia annua* leaf extracts against pathogenic bacteria. Adv Stud Biol 6(3):93–97. <https://doi.org/10.12988/asb.2014.4525>
- Villena I, Durand B, Aubert D, Blaga R, Geers R, Thomas M, Perret C, Alliot A, Escotte-Binet S, Thébault A, Boireau P (2012) New strategy for the survey of *Toxoplasma gondii* in meat for human consumption. Vet Parasitol 183(3–4):203–208. <https://doi.org/10.1016/j.vetpar.2011.08.001>
- Watson PF, Petrie A (2010) Method agreement analysis: a review of correct methodology. Theriogenology 73:1167–1179. <https://doi.org/10.1016/j.theriogenology.2010.01.003>
- Yan C, Yue CL, Yuan ZG, Lin RQ, He Y, Yin CC, Xu MJ, Song HQ, Zhu XQ (2010) Molecular and serological diagnosis of *Toxoplasma gondii* infection in experimentally infected chickens. Vet Parasitol 173:179–183. <https://doi.org/10.1016/j.vetpar.2010.07.011>
- Zechner G, Bauer C, Jacobs J, Goossens L, Vertenten G, Taylor MA (2015) Efficacy of Diclazuril and toltrazuril in the prevention of coccidiosis in dairy calves under field conditions. Vet Rec 176: 126–126. <https://doi.org/10.1136/vr.102237>