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Toxoplasma gondii infections in chickens – performance of various antibody detection techniques in serum and meat juice relative to bioassay and DNA detection methods

G. Schares [a,*], M. Koethe [b], B. Bangoura [c], A.-C. Geuthner [c], F. Randau [c], M. Ludewig [b], P. Maksimov [a], M. Sens [a], A. Bärwald [a], F.J. Conraths [a], I. Villena [d,e], D. Aubert [d,e], M. Opsteegh [f], J. Van der Giessen [f]

[a] *Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, National Reference Centre for Toxoplasmosis, Greifswald-Insel Riems, Germany*
[b] *Institute of Food Hygiene, Faculty of Veterinary Medicine, University Leipzig, Germany*
[c] *Institute of Parasitology, Faculty of Veterinary Medicine, University Leipzig, Germany*
[d] *EA 3800, UFR Medecine, University of Reims Champagne Ardenne, Reims, France*
[e] *Laboratory of Parasitology, National Reference Centre on Toxoplasmosis, Hospital Maison Blanche, Centre Hospitalier Universitaire de Reims, Reims, France*
[f] *National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands*

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ABSTRACT

Chickens, especially if free-range, are frequently exposed to *Toxoplasma gondii*, and may represent an important reservoir for *T. gondii*. Poultry products may pose a risk to humans, when consumed undercooked. In addition, chickens are regarded as sensitive indicators for environmental contamination with *T. gondii* oocysts and have been used as sentinels. The aim of the present study was to determine the suitability of commonly used antibody detection methods, i.e. the modified agglutination test (MAT), IFAT and ELISA to detect *T. gondii*-infected chickens. Samples of experimentally and naturally infected chickens were used. The infection state of all chickens was determined by Magnetic-Capture (MC-) real-time PCR (RT PCR). Naturally exposed chickens were additionally examined by mouse bioassay and conventional RT PCR on acidic pepsin digests (PD-RT PCR). Blood serum and meat juice of various sources were tested for antibodies to *T. gondii*. In naturally infected chickens, there was substantial agreement between the mouse bioassay and MC-RT PCR or the mouse bioassay and conventional PD-RT PCR. PD-RT PCR was slightly more sensitive than MC-RT PCR, as all (26/26) bioassay-positive chickens also tested positive in at least one of the tissues tested (heart, drumstick). By MC-RT PCR, 92.3% (24/26) of the naturally infected bioassay-positive chickens were positive. The diagnostic sensitivity of MC-RT PCR was clearly related to the organ examined. Based on a quantitative assessment of the MC-RT PCR results in experimentally infected chickens, brain and heart tissues harbored an at least 100 times higher parasite concentration than breast, thigh or drumstick musculature. In naturally infected chickens, only three out of 24 birds, which were MC-RT PCR-positive in heart samples, also tested positive in drumstick musculature. Under experimental conditions, the agreement between MC-RT PCR and the serological techniques revealed 100% diagnostic sensitivity and specificity. Under field conditions, examinations of sera by ELISA, IFAT and MAT showed good performance in identifying chickens that were positive in either a mouse bioassay, MC-RT PCR, or PD-RT PCR as illustrated by diagnostic sensitivities of 87.5%, 87.5% and 65.2%, respectively, and diagnostic specificities of 86.2%, 82.8% and 100%, respectively. The examination of meat juice samples from breast, drumstick or heart musculature revealed similar or even better results in the ELISA. The results in the MAT with meat juice from breast musculature were less consistent than those of ELISA and IFAT because a number of negative chickens tested false-positive in the MAT. The MAT performed similar to ELISA and IFAT when applied to test meat juice samples collected from heart, thigh or drumstick musculature.

1. Introduction

Toxoplasma gondii is a protozoan parasite which ranks among the most important foodborne pathogens worldwide (Havelaar

* Corresponding author at: Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, Südufer 10, 17493 Greifswald – Insel Riems, Germany. Fax: +49 38351 7 1526.
E-mail address: gereon.schares@fli.de (G. Schares).

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et al., 2010; Scallan et al., 2011, 2015; Torgerson and Mastroiacovo, 2013). Human toxoplasmosis includes congenitally and postnatally acquired toxoplasmosis (Schluter et al., 2014). Congenital toxoplasmosis can be transmitted from a recently infected mother to the fetus and may cause abortion or the birth of severely affected children (e.g. hydrocephalus, seizures, mental or growth retardation). Congenitally infected persons that are asymptomatic at birth can also develop symptoms of toxoplasmosis later in life (e.g. ocular toxoplasmosis). In most cases, post-natally acquired *T. gondii* infections – either acquired through undercooked infected meat or by oral uptake of oocysts shed by felids – have no severe consequences (Robert-Gangneux and Darde, 2012). However, there is increasing evidence indicating that a large number of ocular uveitis cases in humans are caused by postnatal *T. gondii* infection (Maenz et al., 2014). Moreover, persistent or recently acquired *T. gondii* infections in immunocompromised patients (e.g. transplant patients) may cause life-threatening disease (Robert-Gangneux and Darde, 2012).

Chickens, especially if free-range, are frequently exposed to infection, although reports on toxoplasmosis in chickens are rare (reviewed by Dubey (2010)). As chickens are ground-feeding and susceptible hosts for *T. gondii*, they have been used as sentinels to monitor the potential contamination of farms with this parasite (More et al., 2012; Dubey et al., 2015). Chicken meat is usually heated long enough to ensure the inactivation of *T. gondii*. However, there are specific dishes (e.g. chicken carpaccio, chicken sashimi, barbecued chicken) or products (sausages), for which the tissue may be either not, or not sufficiently, treated to inactivate the parasite. In addition, because tasting meat during cooking (Kapperud et al., 1996; Cook et al., 2000) or poor kitchen hygiene (Kapperud et al., 1996) have been reported as risk factors for human infection, handling chicken meat during slaughter and in the kitchen may represent a possible route of infection for humans. From an epidemiological point of view, the improper handling of slaughter remnants, especially on backyard farms, may favor the transmission from infected chickens to domestic cats and thus further propagation of the infection to intermediate hosts including humans through oocysts shed by cats. A recent study on risk factors for *T. gondii* infections in chickens kept outdoors confirmed the importance of cats in the transmission of *T. gondii* to poultry (Schares et al., 2017a).

In many epidemiological studies, serum or plasma was used to determine specific antibodies against *T. gondii*. The results have been used to estimate the burden of infections in this animal species or on farms (reviewed by Dubey (2010)) to assess the potential risk for consumers (Dubey et al., 2005), to identify chickens with viable *T. gondii* infections (Dubey et al., 2002, 2016; Lehmann et al., 2006; Shwab et al., 2014) or to assess risk factors for infection in this livestock species (Zhu et al., 2008; Millar et al., 2012; Magalhaes et al., 2016; Salant et al., 2016; Schares et al., 2017a).

It is often advantageous to detect specific antibodies not only in blood serum or plasma, but also in meat juice. When testing meat juice, the antibody response can be directly linked to a meat sample and it is not necessary to take blood from the birds prior to or during slaughter. Blood sampling is more time-consuming and laborious, and sometimes requires the support or consent of the owner of the slaughtered animal. Since meat juice sampling is easier and because more animals can usually be sampled, this analyte is better suited for large-scale epidemiological studies and may help to increase statistical power.

Analysing meat juice samples is common practice in the pig industry, for example in the Danish or the German salmonella monitoring programs. A number of studies have shown that porcine meat juice can also be used to detect *T. gondii*-specific antibodies (Wingstrand et al., 1997; Lunden et al., 2002; Dubey et al., 2005; Berger-Schoch et al., 2011; Meemken and Blaha, 2011; Forbes et al., 2012; Meemken et al., 2014; Bacci et al., 2015; Slany et al., 2016; Felin et al., 2017). Moreover, a commercial ELISA has been validated for meat juice to determine specific antibodies to *T. gondii* in sheep (Glor et al., 2013). Total lysate antigen or other crude antigen preparations of *T. gondii* are often employed for antibody detection (Wingstrand et al., 1997; Lunden et al., 2002; Dubey et al., 2005; Glor et al., 2013), but some studies used purified or single antigens such as the major *T. gondii* tachyzoite Surface Antigen 1 (TgSAG1, P30, SRS29B) to avoid cross-reactions with pathogens related to *T. gondii* (Berger-Schoch et al., 2011; Meemken and Blaha, 2011; Meemken et al., 2014; Slany et al., 2016).

In the present study, we aimed to validate commonly used methods for the detection of serum antibodies to *T. gondii*, i.e. the modified agglutination test (MAT), IFAT and an ELISA based on TgSAG1 to detect *T. gondii* infection in chickens. In addition, the suitability of meat juice from different tissues to detect *T. gondii* infections was investigated.

In contrast to other studies, we used materials from experimentally as well as naturally infected chickens. To establish experimental infections, we used both oocysts and tissue cysts as they represent the relevant stages in natural infections of chickens. For oocysts infections, we selected *T. gondii* strains which are common in Europe, i.e. type II and type III strains (Howe and Sibley, 1995; Schares et al., 2008), but we also included oocysts and tissue cysts of a recently isolated *T. gondii* type II strain (CZ-Tiger; Jurankova et al., 2013), which might show different infection characteristics compared with a strain such as ME49, which has been passaged already for a long time.

To determine the true infection status in chickens, we applied Magnetic-Capture (MC-) real-time PCR (RT PCR) in experimentally infected chickens and a combination of a mouse-bioassay, MC-RT PCR and conventional RT PCR on acidic pepsin muscle digests (PD-RT PCR) in naturally infected chickens. Our results confirmed the suitability of meat juices for the detection of specific antibodies to *T. gondii* in chickens but also showed limitations when particular serological tests or meat juices were applied. Moreover, results from mouse bioassays and RT PCRs provided further insights into the predilection sites of *T. gondii* in chickens, which only partially corroborate previous findings of others.

2. Materials and methods

2.1. Parasite strains and experimental infection of chickens

Chickens (breed ISA JA 757) were experimentally infected by oocysts, tissue cysts or tachyzoites. All experiments in chickens had been approved by the responsible authority (Landesdirektion Leipzig, Germany, trial no. TVV 29/10). Care and maintenance of animals were in accordance with governmental and institutional guidelines.

2.1.1. Oocysts

Oocysts of three different *T. gondii* strains were used in the study: the type II *T. gondii* strain CZ-Tiger (Jurankova et al., 2013) was kindly provided as oocysts by Walter Basso, Institute of Parasitology, University of Zurich, Switzerland. Further strains, initially available as tachyzoites, namely type II *T. gondii* ME49 (Lunde and Jacobs, 1983) and type III *T. gondii* NED (Howe and Sibley, 1995), were maintained as tachyzoites in VERO cell cultures in Iscove's Modified Dulbecco's Medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphotericin B at 37 °C and 5% CO_2 as previously described (Geuthner et al., 2014). To harvest tachyzoites, cell culture supernatants were centrifuged at 2000*g* for 5 min, the resulting pellet was resolved in PBS and the

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tachyzoite concentration was determined by using a Neubauer chamber. To generate tissue cysts for oral cat infection, CD1 mice were inoculated i.p. with either 2000 ME49 or 1500 NED strain tachyzoites per mouse. In case of the CZ-Tiger strain, CD1 mice were orally inoculated with 100 oocysts. Cats were fed with infected mouse brains (i.e. one brain per cat) to passage the parasites and yield sufficient numbers of oocysts. In detail, two cats each were fed with brain of a mouse which had been infected with the CZ-Tiger strain 4 weeks earlier, another two cats were fed with the brains of mice which had been infected with the NED strain 4 weeks earlier and two other cats each received two ME49 strain-infected mouse brains (mice infected approximately 7 months earlier). Subsequently, oocysts were harvested from cat faeces by sodium chloride flotation. Briefly, faecal samples were screened daily by flotation using saturated sodium chloride solution, starting on day 3 p.i. Samples positive for *T. gondii* oocysts were subjected to oocyst purification. Oocysts were purified as follows: the faecal sample was dissolved in tap water, centrifuged at 1100*g* (10 min), the supernatant discarded, the sediment resuspended in 50 ml of saturated sodium chloride solution (specific density 1.2 g/ml), and the supernatant containing oocysts collected. The supernatant was diluted 1:10 with tap water and washed three times to obtain a concentrated oocyst solution. After sporulation at room temperature, oocysts were stored in 2% potassium dichromate solution until used. Three different doses were used for infecting birds, i.e. 1×10^3 (CZ-Tiger, ME49, NED), 1×10^5 (CZ-Tiger, ME49), 1×10^6 (CZ-Tiger, ME49) (Table 1). NED strain oocysts were not available in sufficient numbers to apply them in doses higher than 1×10^3. This was also the reason why we included an additional group inoculated with NED 1×10^6 tachyzoites as described in Section 2.1.3. (Table 1). All experiments in cat and mice had been approved by the responsible authority (Landesdirektion Leipzig, Germany, trial no. TVV 29/10). Care and maintenance of animals were in accordance with governmental and institutional guidelines.

2.1.2. Tissue cysts

To obtain brain tissue cysts for chicken infections, CD1 mice were infected with 10–100 oocysts orally or 100–2000 tachyzoites i.p. each of the CZ-Tiger, ME49 or NED strains. Mouse infections were verified by light microscopical examination of squashes of a small aliquot of brain (approximately 20 mg of cerebrum, cerebellum and brain stem). For infection, birds each received the remainder of one microscopically positive mouse brain orally (Table 1).

2.1.3. Tachyzoites

The *T. gondii* type III strain NED (Howe and Sibley, 1995) was used to inoculate poultry i.v. into the wing vein (in 0.1 ml of sterile isotonic saline solution (B. Braun Melsungen AG, Melsungen, Germany) per bird). Tachyzoites for infection of the animals were grown in VERO cells as described in Section 2.1.1.

The observation period usually lasted 5 weeks in all infected groups. Additionally, in the case of tachyzoite infection, six inoculated and six non-inoculated birds were observed for a total of 10 weeks (Table 1). After the observation period, blood was collected for serological analysis and animals were euthanised and their tissues (brain, heart, breast, thigh and drumstick musculature) were stored frozen at −20 °C until used.

2.2. MC-RT PCR

MC-RT PCR was essentially performed as published (Opsteegh et al., 2010) with some slight modifications.

2.2.1. Preparation of crude DNA extract

Up to 100 g of breast (28.8–100 g), thigh (18.4–100 g), or drumstick muscle (7.2–100 g), free of fat and connective tissue, were cut into pieces of approximately 1 cm^3. New, sterile, single-use scalpels were used for every sample to prevent cross-contamination. Hearts (0.9–12.4 g) were prepared in total by either using a laboratory grinder or sterile single-use scalpels to cut the tissue into very small pieces. Brains (0.2–1.3 g) were also prepared in total and cut by scalpels analogously. The cut muscle tissue was transferred into a stomacher bag with filter (BagPagePlus, 400 ml, Interscience, France) while hearts and brains were placed into sterile 15 ml polypropylene tubes. Each sample was homogenised in 2.5 volumes of lysis buffer, consisting of 100 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0, 200 mM NaCl, 0.2% SDS, and 1.2 U/ml of proteinase K using a stomacher bag (for muscle tissue; 2 min, high speed) or rigorous manual shaking (for hearts and brains). Samples were digested overnight in a rocking water bath (85 rpm) at 55 °C. After digestion, lysates were homogenised again for 1 min. Then, up to 50 ml of homogenate (for breast and thigh always 50 ml, for drumstick 25.2–50 ml, for heart 3.2–43.4 ml and for brain 0.7–4.6 ml) were transferred in a 50 ml sterile polypropylene tube and centrifuged for 45 min at 3500*g*.

2.2.2. Removal of free biotin

Up to 12.0 ml of supernatant (for breast, thigh and drumstick always 12 ml, for heart 3.0–12.0 ml and for brain 0.5–4.4 ml) were

Table 1
Number of chickens used in experimental *Toxoplasma gondii* infections.

Group	Infection stage, dose	Strain	Number of animals with an observation period of		Total
			10 weeks p.i.	5 weeks p.i.	
Controls			6	18	24
Inf-1A	Oocysts, 1×10^3	CZ-Tiger	–	6	6
		ME49	–	6	6
		NED	–	5	5
Inf-1B	Oocysts, 1×10^5	CZ-Tiger	–	6	6
		ME49	–	6	6
Inf-1C	Oocysts, 1×10^6	CZ-Tiger	–	6	6
		ME49	–	4	4
Inf-2	Tissue cysts, 1 mouse brain per bird	CZ-Tiger	–	6	6
		ME49	–	5	5
		NED	–	5	5
Inf-3	Tachyzoites, 1×10^6	NED	6	6	12
Total	–	–	12	79	91

Inf, infection.

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transferred to a clean 15 ml polypropylene tube and incubated in a water bath at 100 °C for 10 min to inactivate proteinase K. Streptavidin sepharose (binding capacity 300 nmol/ml; GE Healthcare, VWR, Germany) was washed three times in PBS, pH 7.2. After cooling the crude extract samples in a cold water bath to temperatures below 40 °C, 50 µl of washed streptavidin sepharose were added per sample. The samples were incubated for 45 min at room temperature while rotating at 10 rpm to allow for streptavidin–biotin binding. After biotin precipitation, the tubes were centrifuged for 15 min at 3500*g*, and up to 10 ml of biotin-free supernatant (for breast, thigh and drumstick always 10 ml, for heart 2.0–9.0 ml and for brain 0.2–2.7 ml) were transferred to a clean 15 ml polypropylene tube. For each individual sample the volume of biotin-free supernatant was recorded and used to calculate the estimated number of parasites per 100 g of tissue, relative to a standard curve for a *T. gondii* tachyzoite dilution series in 100 g of muscle tissue.

2.2.3. Sequence-specific magnetic capture

Ten picomoles of biotin-labelled capture oligonucleotides Tox-CapF and Tox-CapR (Opsteegh et al., 2010) were added to each biotin-free supernatant. The supernatants were heated to 95 °C for 15 min to denature DNA. The tubes were then incubated at 55 °C for 45 min in a shaking water bath (hybridisation of the capture oligonucleotides with *T. gondii* DNA). The tubes were then allowed to cool down to room temperature while rotating at 10 rpm for 15 min. An aliquot of hyBeads Streptavidin (Hyglos, Bernried, Germany) was washed in 1 ml of Binding & Washing (B&W) buffer (5 mM Tris HCl pH 7.5, 0.5 mM EDTA pH 8.0, 1 M NaCl) and subsequently resuspended in 1 vol of B&W buffer. Per sample, 80 µl of washed beads and 2 ml of 5 M NaCl were added, and the samples incubated by rotating (10 rpm) at room temperature for 60 min. The complexes of streptavidin beads and biotin-labelled capture oligonucleotide with hybridised *T. gondii* DNA were isolated using the DynaMag-15 magnet (Invitrogen). The tube was placed into the magnetic field for 10 min horisontal shaking at 90 min^{-1} and the supernatant removed by decanting it. The beads were resuspended in 500 µl of B&W buffer, transferred to a clean 1.5 ml tube, then washed in 100 µl of B&W buffer using the DynaMag-2 magnet (Invitrogen), and finally resuspended in 50 µl of distilled water in a 1.5-ml tube. The bead suspension was heated to 100 °C for 10 min to release *T. gondii* DNA. The tube was finally placed into the magnetic field of the DynaMag-2 magnet (Invitrogen) and the supernatant immediately transferred to a clean 1.5 ml tube. The beads remaining in the tube were discarded.

A positive (10^3 tachyzoites in 100 g of breast muscle) and a negative (100 g of breast muscle without tachyzoites) extraction control were included every time the procedure was performed.

2.2.4. RT PCR on 529-bp repeat element

PCR amplification was performed in 96-wells plates using the StepOnePlus thermal cycler instrument (Life Technologies, Darmstadt, Germany). The 27.5 µl reaction mixtures consisted of 1× concentrated reaction buffer, 5.5 mM MgCl2, dNTP-Mix (0.2 mM dATP, dCTP, dGTP, 0.4 mM dUTP), 0.01 U/µl uracil-N-glycosylase, 0.025 U/µl TrueStart Hot Start DNA polymerase (Fermentas/Thermo Scientific, St. Leon-Rot, Germany), 0.9 µM of each primer (Tox-SC forward: 5′-GAGGGGGTGGCGTGGTT-3′ and Tox-SC reverse: 5′-GCGTCGTCTCGTCTRGAT-3′), 0.2 µM of Tox-TP1 (5′-6-FAM-CCGGCTTGGCTGCTTTTCCT-BHQ1-3′) and 2.5 µl of template DNA. The reaction mixture was initially incubated at 50 °C for 10 min to allow uracil-N-glycosylase (UNG) to destroy any remaining uracil-containing DNA and 10 min initial denaturation at 95 °C to inactivate UNG and to activate TrueStart DNA polymerase. This was followed by 45 amplification cycles that consisted of a denaturation step at 95 °C for 15 s, an annealing step at 50 °C for 30 s, and an extension step at 72 °C for 15 s. After completion of all cycles, the samples were cooled to 15 °C. The temperature ramp rate was set to 100%. Fluorescence was measured during each annealing step. A standard series of DNA from serially diluted tachyzoites (10^6 to 10^2 per 100 g of meat) was included in each run for the calculation of a standard curve and for assessing PCR efficiency. For each sample, the quantity of *T. gondii* genome equivalents was calculated by comparing Cycle of transition values (Ct values) of samples with the standards (StepOnePlus software, LifeTechnologies). In general, RT PCR results were expressed as Ct values. Results with Ct values <35 were regarded as positive. Results with Ct values >40 were regarded as negative. If the Ct value ranged between 35 and 40, the respective amplification curves were visually inspected. If they diverged strongly from those of the positive controls, the samples were regarded as negative.

2.3. RT PCR on acid pepsin digests (PD-RT PCR)

The RT PCR was performed as described (Legnani et al., 2016; Schares et al., 2017b). In brief, for DNA extraction 200 µl of pepsin-digested tissue (heart, drumstick; described in Section 2.5) were treated with proteinase K by scaling up the volumes used for the initial digestion (1440 µl of T1 lysis buffer buffer, 200 µl of Proteinase K; Macherey-Nagel, Germany). After digestion (56 °C, 3 h), 230 µl of the suspension were taken and DNA extracted using the Nucleospin Tissue kit as recommended by the supplier (Macherey-Nagel). *Toxoplasma gondii*-specific DNA was amplified in a TaqMan RT PCR (Legnani et al., 2016; Schares et al., 2017b) using primers and a probe targeting the 529 bp repeat of *T. gondii* (Talabani et al., 2009).

2.4. Serological tests

2.4.1. Collection of sera and meat juice

When the chickens were slaughtered, blood was collected and allowed to clot. The samples were then centrifuged, sera collected and stored frozen at −20 °C until use.

If experimentally infected animals were sampled, meat juice was collected after thawing the sampled musculature, i.e. prior to processing for MC-RT PCR. Meat juice from naturally infected chickens was usually collected from fresh muscle tissues by using the fluid that had remained in the sample bags after the tissue had been removed from the bags for further processing. If no fluid was available in the bags, breast tissue samples were frozen and the fluid collected after thawing. To avoid the inactivation of the parasite in drumstick or heart muscle tissue (i.e., in samples that had to be analysed in the bioassay) meat juice recovery after freezing was not performed.

2.4.2. MAT

The MAT for the detection of *T. gondii*-specific IgG antibodies was performed as previously described using antigen produced by the Laboratory of Parasitology, Centre Hospitalier Universitaire de Reims, Reims, France (Dubey and Desmonts, 1987). Each serum or fluid sample was two-fold serially diluted. A titre of 1:6 (serum) or 1:1 (meat juice) was applied as the positive cut-off.

2.4.3. IFAT

The IFAT was done as recently described (Schares et al., 2017a). Briefly, 10 µl of a suspension of cell culture-derived *T. gondii* RH strain tachyzoites (5×10^6 ml^{-1}) in PBS were used to sensitise IFAT slide wells. Slides were air-dried, stored frozen at −20 °C until used, fixed with ice-cold acetone for 10 min before use and incubated in PBS for 10 min. Chicken sera were titrated in PBS in two-fold steps, starting at a serum dilution of 1:50 or at a body

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fluid dilution of 1:5. Anti-chicken IgG (directed against the heavy and light chain (H&L)), synonymous to anti-chicken IgY (H&L) produced in goat and coupled to FITC (Rockland Immunochemicals, Limerick, PA, USA) diluted 1:50 in PBS, 0.2% Evans Blue, was used to detect primary antibodies. The slides were examined using an Olympus IX50 microscope (Olympus, Hamburg, Germany). Only complete peripheral fluorescence of the tachyzoite was considered specific. A titre of 1:50 (serum) or 1:5 (meat juice) was used as the positive cut-off.

2.4.4. TgSAG1-ELISA$_{SH}$

Chicken sera were tested for antibodies against the native *T. gondii* tachyzoite surface antigen TgSAG1 as described (Schares et al., 2017a). In brief, affinity purified TgSAG1 of *T. gondii* tachyzoites (Hosseininejad et al., 2009; Maksimov et al., 2011) 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 100 µl of serum, 1:200 diluted in CasPBST, or meat juice, 1:20 diluted in CasPBST, were added for 30 min, 37 °C. The plates were then washed with PBST. A species-specific conjugate (goat anti-chicken IgG (H&L) peroxidase (POD), synonymous to 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 visualised using 1% tetra-methyl-benzidine (TMB) with 0.012% (v/v) H_2O_2 as the substrate. After 15 min at 37 °C, the reaction was stopped by addition of 50 µl of 2 M H_2SO_4 and the O.D. in each well was read at 450 nm. Each sample was tested in duplicate. Positive (PC) and negative control (NC) sera (Schares et al., 2017a) were tested in quadruplicate on each plate. To normalise the results, ELISA index values (I) were calculated for each sample (S) based the means of two O.D. values: IS = (O.D.S − O.D.NC)/(O.D.PC − O.D.NC). A cut-off optimised for maximum diagnostic specificity was applied (ELISA index 0.242) as previously described for the TgSAG1-ELISA$_{SH}$ (Schares et al., 2017a).

2.5. Mouse bioassay

The mouse bioassay was conducted as described (Schares et al., 2017a). Briefly, IFNɣ-knockout mice (GKO, IFNɣ -/-, C.129S7(B6)-Ifngtm1Ts/J) or IFNɣ-receptor-knockout mice (GRKO, IFNɣreceptor -/-; B6.129 Sv/Ev-IfngrtmAgt) were used. Initially it was planned to use only GKO mice. Due to temporal problems in breeding these mice, some experiments were performed with GRKO. Evaluation of data produced in experiments conducted in parallel with both mouse strains revealed no statistically significant differences in the susceptibility of GKO and GRKO mice for *T. gondii* (Schares et al., 2017a). The mice were inoculated with pepsin-digested heart and drumstick musculature (two mice for each kind of tissue). Pepsin digestion was performed as described (Dubey, 1998; More et al., 2012). Mice were monitored for 42 days. If a mouse developed signs of toxoplasmosis (ruffled hair, apathy), it was euthanised according to the Federation for Laboratory Animal Science Associations (FELASA, Germany) regulations and necropsied. Brain tissue was examined by conventional RT PCR (see Section 2.3) to detect *T. gondii* DNA. Peritoneal fluid (10 µl) and a lung homogenate (10 µl) prepared by homogenising half of the lung in 0.5 ml of DMEM by mortar and pestle were analyzed by light microscopy for the presence of tachyzoites. All mouse experiments (bioassays) reported in this publication were approved by the "Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei" of the German Federal State of Mecklenburg-Vorpommern. Care and maintenance of animals were in accordance with governmental and institutional guidelines.

2.6. Statistical analysis

Differences in the serological responses of experimentally infected chickens were analysed using the Kruskal–Wallis test for multiple comparisons, two-tailed (Statistica 13 Academic, StatSoft (Europe) GmbH, Hamburg, Germany). To analyse the differences in MC-RT PCR results between different tissues in experimentally infected chickens, a pairwise multiple comparison procedure (Dunn's Method, (Dunn, 1961)) was used as implemented in SigmaPlot for Windows Version 11.0 (Systat Software, Inc., San Jose, CA, USA). Generally, $P < 0.05$ was regarded as statistically significant.

To assess agreement, Kappa values (i.e. Cohen's Kappa values, (Cohen, 1960)) were determined using an online tool (http://vassarstats.net/kappa.html). Diagnostic sensitivity and diagnostic specificity including confidence intervals (95% CI) were determined using tools that were available online (http://vassarstats.net/clin1.html). To assess the overall diagnostic performance of a test, Youden's index was calculated by the following formula using EXCEL spreadsheet functions: Sensitivity + Specificity − 1 (Youden, 1950).

3. Results

3.1. Experimental T. gondii infection in chickens

Sera and meat juice samples collected from muscle tissues of experimentally infected chickens were examined by antibody detection techniques. In addition, tissues (brain, heart, breast, thigh and drumstick muscles) were examined by MC-RT PCR and the results compared with those of the serological techniques.

3.1.1. Serological finding in TgSAG1-ELISA, IFAT and MAT

The success of infection after experimental inoculation was confirmed by serology using the TgSAG1-ELISA$_{SH}$, IFAT or MAT. All control animals remained negative in the serological tests.

Table 2
Proportions of serologically positive animals in tests to detect antibodies against *Toxoplasma gondii* in experimentally inoculated chickens.

Serological test	Non-inoculated controls (positives/total examined)	Inoculated animals (positives/total examined), inoculation with[a]			
		Oocysts	Tissue cysts	Tachyzoites	Total
TgSAG1-ELISA$_{SH}$	0% (0/24)	97.4% (38/39)	87.5% (14/16)	75.0% (9/12)	91.0% (61/67)
IFAT	0% (0/24)	100.0% (39/39)	93.8% (15/16)	91.7% (11/12)	97.0% (65/67)
MAT	0% 0/24)	97.4% (38/39)	87.5% (14/16)	50.0% (6/12)	86.6% (58/67)

MAT, Modified Agglutination Test.
[a] For details on the infection dose and the strains, please refer to Table 1.

When chickens were inoculated with oocysts, 38 (TgSAG1-ELISA$_{SH}$ and MAT) or 39 (IFAT) of the 39 birds seroconverted (Table 2). After infection with tissue cysts, 14 or 15 of 16 birds tested serologically positive in the TgSAG1-ELISA$_{SH}$, MAT or IFAT (Table 2). After tachyzoite infection, from nine to 11 of 12 animals (5 or 10 weeks p.i.) tested serologically positive (Table 2). Overall, the highest number of experimentally inoculated chickens (97.0%, 65/67) tested positive by IFAT, followed by TgSAG1-ELISA$_{SH}$ (91.0%, 61/67) while the lowest number of inoculated chickens were detected as seropositive by MAT (86.6%, 58/67) (Table 2).

3.1.2. Differences in serological findings related to experimental infection stage, dose and strain

The serological response of chickens was different between inoculated groups (Fig. 1).

3.1.2.1. Inoculations with oocysts. Chickens inoculated with oocysts of the CZ-Tiger strain showed a dose-dependent response. Chickens inoculated with the lowest dose (10^3 oocysts) showed, by ELISA and MAT, antibody levels that did not significantly differ statistically from those observed in the non-inoculated chicken group (Kruskal-Wallis test for multiple comparisons) (Fig. 1A and C). In the IFAT (Fig. 1B), all chickens inoculated with oocysts of the CZ-Tiger strain reacted positive and the results were statistically significantly different from those of the non-inoculated controls. Chickens inoculated with oocysts of the ME49 strain were serologically positive in all serological tests, independent of the inoculation dose (Fig. 1A–C). In contrast, all chickens inoculated with 10^3 oocysts of the NED strain yielded serological results that were not statistically significantly different from those observed for non-inoculated control chickens.

3.1.2.2. Inoculations with tissue cysts. Among the chickens inoculated with tissue cysts (one mouse brain per animal), only those inoculated with tissue cysts of the CZ-Tiger strain, but not those inoculated with ME49 or NED strain tissue cysts, developed antibody responses. The results in the CZ-Tiger strain-infected group were statistically significantly different from those obtained with sera of non-inoculated control chickens (Fig. 1A–C).

3.1.2.3. Inoculations with tachyzoites. Weak responses were observed with sera from all animals inoculated with tachyzoites of the NED strain. The results were not statistically significantly different from those of non-inoculated control chickens (Fig. 1A–C).

3.1.3. Differences in parasitic loads between tissues in experimentally infected chickens as determined by MC-RT PCR

None of the non-inoculated control animals tested positive in the MC-RT PCR (0/24) (Table 3). None of the tachyzoite-inoculated chickens (0/12) was positive in the MC-RT PCR, also, while 89.5% (35/39) of those inoculated with oocysts and 68.8% (11/16) of those inoculated with tissue cysts tested positive in at least one of the tissues sampled (Table 3). Brain and heart of chickens represented tissues that most often tested MC-RT PCR positive compared with thigh, breast and drumstick musculature (Table 3).

Relative to standard concentrations of tachyzoites, the Ct values were used to estimate the number of parasites per 100 g of tissue (Fig. 2). The highest loads of *T. gondii* genome equivalents normalised to 100 g of tissue in proteinase K solubilised MC-RT PCR-positive chicken organs were observed in brain (median 3.01×10^6), followed by heart (median 9.91×10^5), thigh (median 5.46×10^3), drumstick (median 4.86×10^3) and breast musculature (median 3.53×10^3). Differences in estimated parasite genome loads were statistically significant between heart or brain and all remaining tissues (Dunn's method, $P < 0.05$).

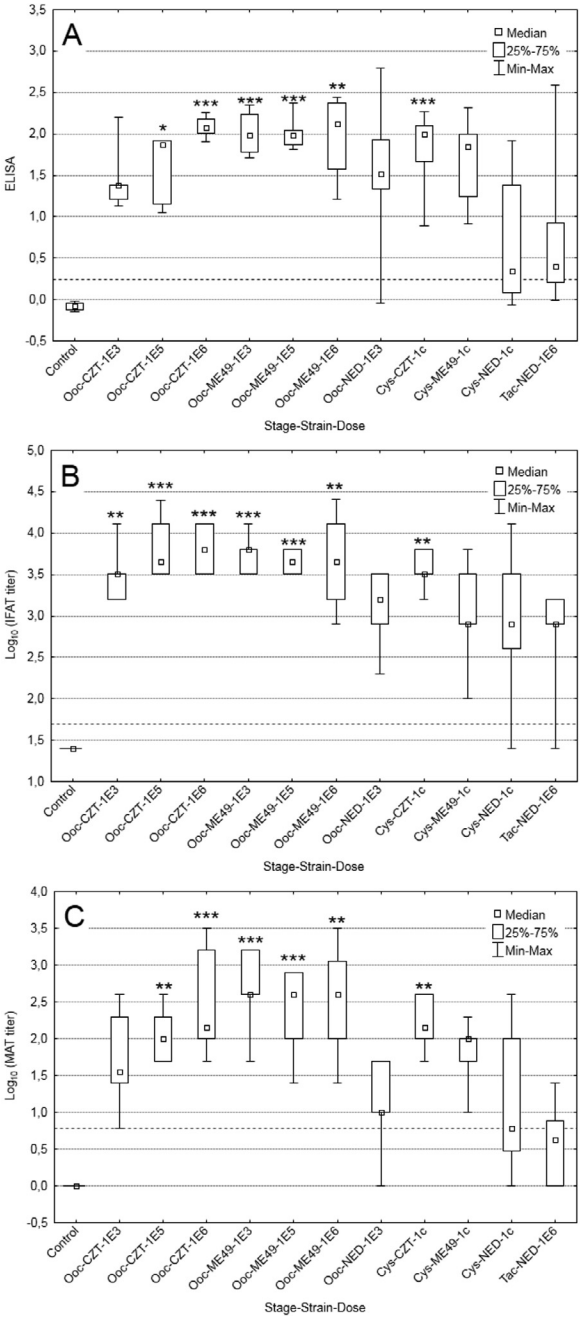

Fig. 1. Serological responses of chickens (median, 25–75% percentiles, minimum and maximum values) inoculated with *Toxoplasma gondii* oocysts of the CZ-Tiger strain 5 weeks after inoculation (Ooc-CZT-1E3, Ooc-CZT-1E5, Ooc-CZT-1E6: inoculation with 10^3, 10^5 or 10^6 oocysts), oocysts of the ME49 strain (Ooc-ME49-1E3, Ooc-ME49-1E5, Ooc-ME49-1E6: inoculation with 10^3, 10^5 or 10^6 oocysts), oocysts of the NED strain (Ooc-NED-1E3: inoculation with 10^3 oocysts), tissue cysts from one mouse brain infected with CZ-Tiger strain (Cys-CZT-1c), tissue cysts from one mouse brain infected with ME49 strain (Cys-ME49-1c) or 10^6 tachyzoites of NED strain (Tac-NED-1E6, 5 or 10 weeks p.i.); (A) ELISA-Index in the in an ELISA using the *T. gondii* tachyzoite Surface Antigen 1 as an antigen (TgSAG1-ELISA), (B) IFAT titer (log10), (C) Modified Agglutination Test (MAT) titer (log10). For each group of animals, the median, 25% and 75% percentiles, the minimum and maximum values are displayed. Statistically significant differences relative to negative controls are indicated (Kruskal-Wallis test for multiple comparisons, two-tailed: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$). Dotted lines represent the positive cut-offs applied in the respective tests.

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Table 3
Proportions of Magnetic-Capture real-time PCR *Toxoplasma gondii* DNA-positive findings in tissues of experimentally inoculated chickens.

Tissue	Experimental infection				Control
	Oocysts	Tissue cysts	Tachyzoite	Total – experimental infection	Not experimentally infected
Brain	79.5% (31/39)	56.3% (9/16)	0.0% (0/12)	59.7% (40/67)	0% (0/24)
Heart	87.2% (34/39)	56.3% (9/16)	0.0% (0/12)	64.2% (43/67)	0% (0/24)
Thigh	61.5% (24/39)	25.0% (4/16)	0.0% (0/12)	41.8% (28/67)	0% (0/24)
Breast	53.8% (21/39)	25.0% (4/16)	0.0% (0/12)	37.3% (25/67)	0% (0/24)
Drumstick	52.6% (20/38)	25.0% (4/16)	0.0% (0/12)	36.4% (24/66)	0% (0/24)
All tissues	89.7% (35/39)	68.8% (11/16)	0.0% (0/12)	68.7% (46/67)	0% (0/24)

3.1.4. Experimental T. gondii infection in chickens – detection of specific antibodies in various meat juices relative to findings in MC-RT PCR

The results of the serological tests for various analytes (sera, meat juice) were compared with those of the MC-RT PCR (i.e. MC-RT PCR positivity in one of the tested tissues). Because the reference status of inoculated chickens that had tested negative in MC-RT PCR was uncertain, these chickens were excluded from analyses. Overall, the ELISA represented the most sensitive test for the detection of MC-RT PCR-positive experimentally infected chickens (100% diagnostic sensitivity, regardless of the analyte), while MAT showed a lower diagnostic sensitivity (91.3–97.8%) for all muscle tissue fluids. In addition, MAT had also a low diagnostic specificity when meat juice samples were examined, especially in juice from breast muscle, as 25% (6/24) of non-inoculated chickens reacted positive with this analyte. Overall, the IFAT showed the highest Youdens's indices (i.e. taking into account both sensitivity and specificity) followed by those observed in ELISA and MAT (Table 4).

3.2. Natural T. gondii infection in chickens

To confirm the findings obtained with experimentally infected chickens, blood serum and meat juice samples were collected from muscle tissues (heart, breast, drumstick) of naturally infected chickens (details on the selection of chickens have been reported recently (Schares et al., 2017a)). The collected analytes were examined by antibody detection techniques (ELISA, IFAT, MAT) and the results compared with those of direct parasite detection.

3.2.1. Comparison of direct parasite detection methods in naturally infected chickens

Relative to the mouse bioassay, the results of MC-RT PCR or PD-RT PCR (i.e. a RT PCR on acidic pepsin tissue digests) showed a high level of agreement characterised by estimated Kappa values of 0.769 (95% CI: 0.609–0.929) or 0.869 (95% CI: 0.744–0.993), respectively. The PD-RT PCR was slightly more sensitive than the MC-RT PCR, because all (100%, 26/26) bioassay-positive chickens also tested positive in at least one of the examined tissues (heart, drumstick). In MC-RT PCR, only 92.3% (24/26) of the bioassay-positive chickens were positive (Table 5). However, overall more tissues tested positive in the MC-RT PCR ($n = 34$, i.e. 28 heart positives and six drumstick positives, Table 5) than in the PD-RT PCR ($n = 31$, i.e. 30 heart positives and one drumstick positive, Table 5). Three of the five chickens negative in the bioassay but positive in MC-RT PCR had also tested positive in the PD-RT PCR. Of the remaining two MC-RT PCR positive but bioassay negative animals, one tested positive in drumstick only and one in heart only. The remaining PD-RT PCR positive animal tested positive in heart tissue only.

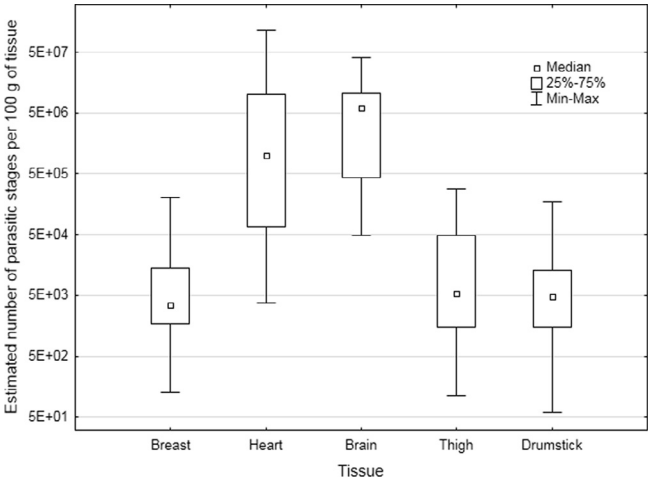

Fig. 2. Magnetic-Capture real-time PCR (MC-RT PCR) results for samples of chickens experimentally infected with *Toxoplasma gondii* oocysts or tissue cysts, 5 weeks after inoculation (median, 25–75% percentiles, minimum and maximum values). The parasite genome equivalents in MC-RT PCR-positive samples homogenised by treatment with Proteinase K were estimated using a standard curve for a *T. gondii* tachyzoite dilution series in 100 g of muscle tissue. The differences in parasite genome equivalents in solubilised heart or brain tissues and all remaining tissues digests were statistically significant (Dunn's method; $P < 0.05$).

3.2.2. Diagnostic performance of TgSAG1-ELISA$_{SH}$, IFAT and MAT relative to direct detection when sera are used as an analyte

Relative to a reference standard on direct detection of *T. gondii* (i.e. chickens with heart or drumstick tissues positive either in a mouse bioassay, MC-RT PCR or PD-RT PCR), the serological analysis by TgSAG1-ELISA$_{SH}$ showed the highest Youden's index, which was reflected by a high diagnostic sensitivity (87.5%, 28/32) and a high diagnostic specificity (86.2%, 25/29) (Table 6). Only the MAT was superior in terms of diagnostic specificity (100%, 29/29), but the analysis by MAT revealed a low diagnostic sensitivity (65.6%, 21/32) at the same time (Table 6).

3.2.3. Suitability of various meat juices to replace serum in antibody assays

The suitability of an analysis of various body fluids for specific antibodies against *T. gondii* was compared relative to the reference standard on direct detection of *T. gondii* (i.e. chickens with heart or drumstick tissues positive either in a mouse bioassay, MC-RT PCR

Table 4
Summary of the characteristics of serological tests relative to Magnetic-Capture real-time PCR in experimentally infected chickens, stratified for analytes. For the analysis, all experimentally inoculated chickens were excluded for which infection had not been confirmed by Magnetic-Capture real-time PCR.

Serological test	Analyte	% diagnostic sensitivity, 95% confidence interval (positive/reference positive[a])	% diagnostic specificity, 95% confidence interval (negative/reference negative[b])	Youden's index
TgSAG1-ELISA$_{SH}$	Serum	100, 90.4–100 (46/46)	100, 82.8–100 (24/24)	1.00
	Heart meat juice	100, 90.4–100 (46/46)	91.7, 71.5–98.5 (22/24)	0.92
	Breast meat juice	100, 90.4–100 (46/46)	100, 82.8–100 (24/24)	1.00
	Thigh meat juice	100, 90.4–100 (46/46)	95.8, 76.9–99.8 (23/24)	0.96
	Drumstick meat juice	100, 90.4–100 (46/46)	100, 82.8–100 (24/24)	1.00
IFAT	Serum	100, 90.4–100 (46/46)	100, 82.8–100 (24/24)	1.00
	Heart meat juice	95.7, 84.0–99.2 (44/46)	95.8, 76.9–99.8 (23/24)	0.92
	Breast meat juice	100, 90.4–100 (46/46)	100, 82.8–100 (24/24)	1.00
	Thigh meat juice	100, 90.4–100 (46/46)	100, 82.8–100 (24/24)	1.00
	Drumstick meat juice	100, 90.4–100 (46/46)	100, 82.8–100 (24/24)	1.00
MAT	Serum	100, 90.4–100 (46/46)	100, 82.8–100 (24/24)	1.00
	Heart meat juice	95.7, 84.0–99.2 (44/46)	87.5, 66.5–96.7 (21/24)	0.83
	Breast meat juice	91.3, 78.3–97.2 (42/46)	25.0, 10.6–47.1 (6/24)	0.16
	Thigh meat juice	95.7, 84.0–99.2 (44/46)	79.2, 57.3–92.1 (19/24)	0.75
	Drumstick meat juice	91.3, 78.3–97.2 (42/46)	87.5, 66.5–96.7 (21/24)	0.79

MAT, Modified Agglutination Test.
[a] A chicken is regarded as reference positive, if at least one of the tissues tested positive by Magnetic-Capture real-time PCR.
[b] All non-inoculated control chickens were regarded as reference negative.

Table 5
Comparison of *Toxoplasma gondii* mouse bioassay and Magnetic-Capture (MC-) real-time PCR (RT PCR) or RT PCR on on acidic pepsin digested chicken tissue (PD-RT PCR).

Test	PCR result		# bioassay-positive chickens			# bioassay-negative chickens
			Total positive	Heart positive	Drumstick positive[a]	
MC-RT PCR	# positive chickens	Total	24	24	3	5[b]
		Heart and drumstick	5	5	1	0
		Heart only	19	19	2	4
		Drumstick only	0	0	0	1
	#negative chickens		2	2	0	30
PD-RT PCR	# positive chickens		26	26	0	4[b]
		Heart and drumstick	1	1	0	0
		Heart only	25	25	0	4
		Drumstick only	0	0	0	0
	# negative chickens		0	0	0	31

[a] All chickens that tested bioassay-positive in drumstick tissue were also positive in heart tissue by bioassay.
[b] Three of the five chickens negative in bioassay but positive in MC-RT PCR had also tested positive in the RT PCR on acidic pepsin digest (PD-RT PCR).

or PD-RT PCR). The sensitivity of the TgSAG1-ELISA$_{SH}$ was similar for serum and meat juice samples, while the diagnostic specificity was even higher in meat juice preparations and ranged between 96.6% (28/29) and 100% (29/29) when fluids from heart, drumstick or breast muscle were examined (Table 6). In meat juices the IFAT showed higher diagnostic specificity values than with serum; diagnostic sensitivity values ranged between 56.3% (18/32) and 77.4% (24/31) when IFAT was applied to meat juices (Table 6). In the MAT the use of meat juices collected from heart and drumstick muscle improved the diagnostic performance, mainly due to a higher diagnostic sensitivity (ranging from 83.9% (26/31) to 90.3 (28/31)). However, the analysis of meat-juice collected from breast musculature caused an especially large number of false positive reactions, resulting in a low diagnostic specificity of 37.0% (10/27) (Table 6). Overall, the TgSAG1-ELISA$_{SH}$ performed optimally with meat juices as this test always revealed Youden's indices higher than 0.8 independent of the source of meat juice (Table 6). When applied to meat juices the IFAT showed lower diagnostic sensitivities compared with its application to serum, resulting in Youden's indices between 0.56 and 0.67 (Table 6). Diagnostic sensitivity in the MAT applied to meat juice was generally better than the MAT performed with serum. However, as mentioned above, in meat juice from breast muscle a low diagnostic specificity of 37.0% was observed, while the analysis of diagnostic specificities in meat juice from heart and drumstick revealed high levels of 100% or 92.9%, respectively (Table 6).

4. Discussion

Serological methods seem to be valuable tools to predict viable infection with *T. gondii* in chickens as shown by several investigators (Casartelli-Alves et al., 2014; Dubey et al., 2015; 2016). Relative to the mouse bioassay, a newly established ELISA, i.e. the TgSAG1-ELISA$_{SH}$, provided sensitive and specific information on the presence of a viable *T. gondii* infection in chickens reared outdoors in backyard or large organic farms (Schares et al., 2017a).

Here, we aimed to confirm the suitability of blood serum and meat juice samples of different origins to detect *T. gondii* infections in chickens by ELISA (TgSAG1-ELISA$_{SH}$), IFAT or MAT. In contrast to other studies, we were able to use serum, meat juice and tissues of experimentally as well as naturally infected chickens. Moreover, we determined the putative 'true' infection status by MC-RT PCR in experimentally infected chickens, and by a combination of a mouse bioassay, MC-RT PCR and PD-RT PCR (i.e. a conventional RT PCR on acidic pepsin digests of muscle samples) in naturally infected chickens.

In all serological tests applied to sera from experimentally oocyst-inoculated chickens, seroconversion was observed if doses $\geq 10^4$ oocysts had been used for inoculation. Previous studies in pigs also revealed dose-dependent effects in the serological detectability of oocyst-induced infection (Forbes et al., 2012). With respect to inoculations with tissue cysts, only tissue cysts of a type II *T. gondii* strain recently isolated from the Czech Republic

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Table 6
Performance of *Toxoplasma gondii* tests used to examine chicken sera and meat juices relative to direct positive detection by mouse bioassay, Magnetic-Capture real-time PCR or real-time PCR on acidic pepsin digested chicken tissues.

Test	Analyte	% diagnostic sensitivity, 95% confidence interval (positive/reference positive)	% diagnostic specificity, 95% confidence interval (negative/reference negative)	Youden's index
TgSAG1-ELISA$_{SH}$	Serum	87.5, 70.1–95.9 (28/32)	86.2, 67.4–95.5 (25/29)	0.74
	Heart meat juice	87.1, 69.2–95.8 (27/31)	96.6, 80.4–99.8 (28/29)	0.84
	Drumstick meat juice	87.5, 70.1–95.9 (28/32)	100, 85.4–100 (29/29)	0.88
	Breast meat juice	87.1, 69.2–95.8 (27/31)	100, 85.4–100 (29/29)	0.87
IFAT	Serum	87.5, 70.1–95.9 (28/32)	82.8, 63.5–93.5 (24/29)	0.70
	Heart meat juice	77.4, 58.5–89.7 (24/31)	89.7, 71.5–97.3 (26/29)	0.67
	Drumstick meat juice	56.3, 37.9–73.2 (18/32)	100, 85.4–100 (29/29)	0.56
	Breast meat juice	62.5, 43.8–78.3 (20/32)	100, 85.4–100 (29/29)	0.63
MAT	Serum	65.6, 46.8–80.8 (21/32)	100, 85.4–100 (29/29)	0.66
	Heart meat juice	83.9, 65.5–93.9 (26/31)	100, 83.4–100 (25/25)	0.84
	Drumstick meat juice	84.4, 66.5–94.1 (27/32)	92.9, 75.0–98.8 (26/28)	0.77
	Breast meat juice	90.3, 73.1–97.5 (28/31)	37.0, 20.1–57.5 (10/27)	0.27

MAT, Modified Agglutination Test.

(Jurankova et al., 2013) induced a statistically significant seroconversion. Inoculation with tissue cysts of other strains (ME49, NED) or tachyzoites (NED) caused only slightly elevated antibody levels, which did not significantly differ statistically from those in control animals, although all sera tested positive in the respective tests except a single serum sample collected from a bird inoculated with NED oocysts that tested negative in the MAT. It has to be noted here that due to multiple comparisons, a highly stringent statistical test had been applied, i.e. Kruskal-Wallis test for multiple comparisons, two-tailed. In addition, it is impossible to compare strains based on tissue cyst inoculation because per bird one mouse brain was used and parasitic cysts in the individual brains had not been enumerated prior to inoculation. The weak serological response in chickens inoculated by tachyzoites (in a dose of 10^6) was not due to a low viability of tachyzoites as their viability had been confirmed by a cell culture test (data not shown). However, the weak antibody response is in accord with our observation, that no infection could be detected in tachyzoite-inoculated chickens by MC-RT PCR. This finding corroborates results that demonstrated a low detectability of parasitic DNA in various organs of chickens and turkeys after tachyzoite inoculation (Geuthner et al., 2014). Altogether, these findings suggest that the route of infection (e.g. oral or parenteral), the parasitic stage (e.g. oocysts, tissue cysts or tachyzoites), or the individual characteristics of the isolate (e.g. genotype or prolonged cell cultivation) play an important role, in addition to the inoculation dose, in inducing a viable infection and a clear antibody response in chickens. Future experiments should include natural routes of infection, i.e. oral routes by inoculating with oocysts and tissue cysts, and by using *T. gondii* isolates that have not been passaged in the laboratory for a long time.

Mouse or cat bioassays are regarded as "gold standards" to detect viable *T. gondii* infections (Dubey et al., 2016). Since the opportunities to perform these bioassays are limited, a further objective of the present study was to determine the value of various PCR methods (i.e. a MC-RT PCR and PD-RT PCR) that can be used as an alternative for the detection of viable infections in chickens. In addition, a combination of bioassay and PCR methods may have enabled us to better define the true infection status of individual chickens and thus to validate antibody detection methods.

In chickens experimentally infected with oocysts or tissue cysts, the MC-RT PCR revealed a low (25.0%) to high (87.2%) proportion of positive findings in inoculated animals. The diagnostic sensitivity of the MC-RT PCR was clearly related to the examined organ. Based on a quantitative assessment of the MC-RT PCR results, brain and heart tissues harboured an at least 100 times higher parasite concentration than breast, thigh or drumstick musculature. This confirms previous studies that identified brain and heart of turkeys as predilection organs (Koethe et al., 2015). However, our finding of brain and heart as predilection organs in chickens fits only partially with recent findings in chickens used as sentinels for detection of oocyst contaminations on pig farms (Dubey et al., 2015). *Toxoplasma gondii* in seroconverting sentinel chickens could be isolated from heart tissues of 26 chickens with a MAT titre of $\geq$1:100, but only from three brains of the same chickens. This may suggest that chicken brain is not a predilection site for *T. gondii*. However, previous studies (summarised in Dubey (2010)) reported higher proportions of *T. gondii* isolation from chicken brain (49.2%, 67/136), but also in these experiments heart turned out to be the most important predilection site with 89.5% (129/144) of positive findings (Dubey, 2010). Some differences between our and previous studies regarding the importance of brain as a predilection organ may be due to experimental conditions (e.g. the particular traits of the infecting parasites or the duration of infection but also prolonged transportation times affecting the viability of parasites in particular types of tissues).

To compare the diagnostic characteristics of antibody detection methods in experimentally inoculated chickens, we used the MC-RT PCR result on five different tissue samples of these birds as a reference. We do not know if MC-RT PCR negative experimentally inoculated chickens were truly non-infected or if the low proportion of positive findings – especially due to no positive findings in birds inoculated with tachyzoites – reflects technical limitations of MC-RT PCR in detecting low levels of infection. Due to the uncertainty on the infection state of MC-RT PCR negative inoculated chickens we had to exclude the data of these birds when we

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compared the suitability of antibody detection methods to identify experimentally infected chickens. Consequently, it is possible that we overestimated diagnostic sensitivity of the antibody detection methods. Nevertheless, the comparative evaluation of antibody detection techniques is valid because for these comparisons only birds were used for which the infection state was definitively known.

In field chickens, there was an excellent agreement between the PCR results and the mouse bioassay. An excellent agreement between MC-RT PCR and bioassay was not unexpected because in both bioassay and MC-RT PCR large volumes of tissues are digested and analysed which increases the chance to detect infection. In the case of PD-RT PCR, the excellent agreement between the bioassay and this PCR was not expected per se. However, in contrast to usual protocols (usually only small aliquots of tissues are subjected to DNA extraction) we used DNA extracted from an aliquot of the acidic pepsin digest for analysis. This treatment obviously had ensured good homogenisation and most likely also a breakdown of tissue cysts which allowed us to also find *T. gondii* DNA in a small aliquot of the sample. Only in drumstick tissues, i.e. tissues with low parasite loads, was MC-RT PCR superior to the conventional RT-PCR on acidic pepsin digests.

Both the PCR and bioassay results were used to define a reference standard to validate various antibody detection methods (TgSAG1-ELISA$_{SH}$, IFAT, MAT) in samples collected from experimentally and naturally infected chickens. Since serum is not always available for epidemiological studies, it was one of the aims of this study to test also the applicability of fluids collected from the musculature (meat juice) of the birds under examination. The combination of several direct detection techniques to define a reference standard most likely decreased the likelihood that false negative samples had entered the reference panel in the present study. Due to the excellent agreement between the direct detection techniques applied here, the number of false positive samples in the panel was also low, because almost all reference positive chickens (with the exception of two) had been identified as positive by more than one technique.

Overall, the ELISA performed optimally with Youden's indices reflecting both excellent diagnostic sensitivity and specificity relative to direct detection, when samples from experimentally or naturally infected chickens were used. Validation of the IFAT relative to direct detection also revealed optimal diagnostic characteristics when applied to various analytes. However, in chickens from the field, meat juice examinations by IFAT revealed limitations regarding diagnostic sensitivity which resulted in lower Youden's indices. In the MAT, most comparisons revealed high levels of diagnostic sensitivities and specificities. However, results obtained with breast meat juice from experimentally or naturally infected chickens revealed only low levels of diagnostic specificity (i.e. false-positive MAT reactions). The observation of false-positive MAT reactions in meat juice collected from this type of tissue is surprising. There may be a general propensity of meat juice to cause false-positive reactions in MAT, because the analysis of blood sera from negative control animals or positive reference standards always revealed an excellent specificity and no false-positive samples. Overall, the use of meat juice seems to be suitable to replace serum as an analyte at least for IFAT and ELISA.

Our titrations to find suitable dilutions for IFAT and ELISA (data not shown) revealed that meat juice had to be used at 10 times greater concentration than serum, which corroborates the results of others who examined blood serum and meat juice of pigs (Wingstrand et al., 1997; Meemken and Blaha, 2011) or sheep (Glor et al., 2013). However, in the MAT, the 10-fold reduced concentration of antibodies in meat juice may have been the reason for reduced sensitivity. Even the positive cut-off of 1:1 (i.e. a positive reaction in undiluted meat juice) could not completely compensate for this difference in antibody concentrations between meat juice and serum. In addition, the use of undiluted meat juice may cause specificity problems. Therefore, based on our findings, the use of a MAT for the examination of meat juices cannot be recommended.

Although the MAT has shown excellent performance in experimentally infected pigs (Forbes et al., 2012; Hill et al., 2006), viable *T. gondii* infections in naturally infected pigs were not always correlated with MAT titres $\geq$1:10 (Hill et al., 2006). This highlights the importance of validating antibody detection tests, not only using samples from experimentally infected animals, but also with material collected from naturally infected animals. The latter probably provide a more realistic panel of materials, better reflecting field situations including varying doses, variations in time between infection and sampling, and the possibility of co-infections, potentially causing false-positive reactions. One of the additional parameters not sufficiently addressed by experimental infection is trickle infections (i.e. repeated exposures to low doses), which could cause viable infections but may induce only low levels of antibodies, so that particularly highly sensitive (but also specific) antibody detection assays are needed for a correct diagnosis of these infections.

An ELISA or IFAT may not always be superior to a MAT, although this was the outcome of our study under the particular circumstances surrounding it. Other researchers, who investigated the antibody response to *T. gondii* in pigs, found that the diagnostic performance of a MAT was superior to that of an ELISA (Dubey et al., 1995). However, in contrast to MAT, the ELISA and partially the IFAT, offer more and better opportunities for optimisation, for example by testing different antigen compositions, improving antigen purification, selecting optimal secondary antibodies and antigen, antibody and analyte concentrations by checkerboard titration.

In conclusion, the ELISA, IFAT and MAT performed well in identifying mouse bioassay, MC-RT PCR or PD-RT PCR positive chickens, regardless of whether blood serum or meat juice from different sources of muscle tissue were applied. The MAT showed a relatively low specificity when used with meat juice samples collected from breast musculature, but performed well with fluids from heart, thigh or drumstick muscles, although the overall results were less consistent than those obtained by ELISA. There was substantial agreement between the mouse bioassay, MC-RT PCR or RT PCR on acidic tissue digests (PD-RT PCR) in the detection of viable *T. gondii* infections in chickens. Heart was confirmed as a predilection site for *T. gondii* in experimentally and naturally infected chickens.

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