Aus dem Institut für Parasitologie
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In-vivo-Modelle der *Toxoplasma gondii*-Infektion von Huhn und Pute

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Meinen Eltern, meiner Schwester, meiner Omi

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Abkürzungsverzeichnis

%	Prozent
>	größer als, mehr als
Abb.	Abbildung
Anon.	anonym
AVEC	Verband der Geflügelverarbeiter und des Geflügelhandels in den EU-Ländern, engl. Association of Poultry Processors and Poultry Trade in the EU countries
CFT	Komplement-Fixationstest
DNA	Desoxyribonukleinsäure, engl. deoxyribonucleic acid
d. h.	das heißt
DT	Sabin-Feldman-Dye-Test
ELISA	Enzyme-linked Immunosorbent Assay
engl.	englisch
griech.	griechisch
i.c.	intracerebral
IFAT	Indirekter Fluoreszenz-Antikörpertest
IgG	Immunglobulin G
IgY	Immunglobulin Y
IHAT	Indirekter Hämagglutinationstest
i.m.	intramuskulär
i.p.	intraperitoneal
i.v.	intravenös
KELA	kinetischer ELISA
LAT	Latex-Agglutinationstest
MAT	modifizierter Agglutinationstest
MC-qPCR	magnetic-capture real-time PCR
n. u.	nicht untersucht
OECD	Organisation für wirtschaftliche Zusammenarbeit und Entwicklung, engl. Organisation for Economic Co-operation and Development
PCR	Polymerase-Kettenreaktion, engl. polymerase chain reaction
p.i.	post infectionem
s.c.	subcutan
T. gondii	*Toxoplasma gondii*
z. B.	zum Beispiel

1. Einleitung

Geflügel wird als einer der bedeutendsten Zwischenwirte im Lebenszyklus von *Toxoplasma* (*T.*) *gondii* angesehen, zum einen aufgrund der erheblich höheren Lebenserwartung von Geflügel im Vergleich zu Nagetieren, zum anderen durch die Resistenz gegenüber klinischer Toxoplasmose (DUBEY 2010a). Der Konsum von rohem oder nicht durchgegartem Fleisch mit *T. gondii* ist der größte Risikofaktor einer Infektion des Menschen (COOK et al. 2000; WILKING et al. 2016), insbesondere der Verzehr von nicht ausreichend erhitzten Geflügelfleisch darf nicht unterschätzt werden (ALVARADO-ESQUIVEL et al. 2006, 2008; SAID et al. 2017). Im Rahmen einer sich ändernden Lebensweise wird Geflügelfleisch vermehrt roh in Form von Fleischerzeugnissen wie kurzgereifte Rohwürste oder Rohschinken konsumiert, wobei Gewebezysten in Abhängigkeit von der Pökeldauer und Salzkonzentration infektiös bleiben (DUBEY 1997a; POTT et al. 2012, 2013).

Insbesondere in der Freiland- und ökologischen Haltung infizieren sich Nutztiere häufiger mit *T. gondii* als in Innenhaltungen (KIJLSTRA et al. 2004; ZHU et al. 2008; YAN et al. 2009; MAKSIMOV et al. 2011; ALVARADO-ESQUIVEL et al. 2012; MILLAR et al. 2012; XU et al. 2012; GUO et al. 2015a; OPSTEEGH et al. 2016; SCHARES et al. 2017a; RODRIGUES et al. 2019). Ob Gewebezysten nach einer Infektion über die gesamte Mastperiode bei Auslauf- und ökologischer Haltung von bis zu mindestens 14 bzw. 20 Wochen bei Puten und 11 Wochen bei Hühnern (ANON. 2008, 2018) bis zur Schlachtung persistieren und somit ein Risiko einer Infektion des Menschen bergen, oder die Gewebezysten eliminiert werden, wie bei anderen Zwischenwirten wie beispielsweise Rindern beschrieben (BEVERLEY et al. 1977), ist bislang nicht bekannt. Zur Risikoabschätzung einer Übertragung von *T. gondii* auf den Menschen beim Verzehr von tierischen Erzeugnissen ist darüber hinaus das Wissen über die Gewebeverteilung unerlässlich. Bei Säugetieren sind die Hauptprädilektionsstellen umfangreich untersucht. Unterschiede in der Verteilung der Gewebezysten variieren stamm- und dosisabhängig (DUBEY 1988; ESTEBAN-REDONDO et al. 1999; JUNGERSEN et al. 1999; OPSTEEGH et al. 2010; DUBEY et al. 2016). Für Geflügel existieren in diesem Zusammenhang durch den Einsatz unterschiedlicher Stämme derzeit nur inkonsistente Daten (DUBEY et al. 1993a; KANETO et al. 1997; SEDLÁK et al. 2000; BANGOURA et al. 2013; ZÖLLER et al. 2013). Des Weiteren lag der Fokus in zahlreichen Feldstudien auf den für den humanen Verzehr kaum relevanten Organen Herz und Gehirn (DUBEY 2010a; SCHARES et al. 2017a).

Ziel dieser Dissertation war es daher, im ersten Teil die Persistenz von *T. gondii* in verschiedenen Geweben von Huhn und Pute unter Berücksichtigung der üblichen Mastperioden zu ermitteln. Im zweiten Teil der Arbeit wurde unter Simulation der natürlich auftretenden Infektionswege ein Infektionsmodell bei Huhn und Pute unter Nutzung drei verschiedener *T. gondii*-Stämme - als Vertreter der in Europa überwiegend vorkommenden klonalen Linien II und III - entwickelt. Zudem wurde der Einfluss von Infektionsdosen und Infektionsstadien auf die Verteilung von *T. gondii* im

Gewebe von Huhn und Pute evaluiert. Die Untersuchung der Gewebeverteilung von *T. gondii* erfolgte in allen Teilen dieser Arbeit mittels verschiedener PCR-Methoden. Zusätzlich wurde der Einfluss der Infektionsparameter wie Dauer der Infektion, Infektionsstamm, -dosis und -stadium auf die Serokonversion untersucht.

2. Literatur

2.1. *Toxoplasma gondii*

2.1.1. Erreger

Toxoplasma (*T.*) *gondii* ist ein weltweitverbreitetes Protozoon aus dem Stamm der Apicomplexa und der Familie der *Sarcocystidea*. Erstmalig wurde *T. gondii* 1908 von Nicolle und Manceaux in Nordafrika aus dem afrikanischen Nagetier *Ctenodactylus gundi* isoliert und als *Leishmania* beschrieben (NICOLLE und MANCEAUX 1908). Im gleichen Zeitraum isolierte Splendore *T. gondii* aus einem Kaninchen in Brasilien (SPLENDORE 1908). Der Name *Toxoplasma* beruht auf der halbmondartigen Form des Parasiten (griech. *toxon:* Bogen; *plasma:* Gebilde) und wurde 1909 von Nicolle und Manceaux vorgeschlagen (NICOLLE und MANCEAUX 1909).

2.1.2. Entwicklungszyklus

Der fakultativ heteroxene Entwicklungszyklus von *T. gondii* ist in Abbildung 1 dargestellt. Als Endwirt von *T. gondii* sind Katzen (FRENKEL et al. 1970) und andere Feliden (JEWELL et al. 1972; MARCHIONDO et al. 1976; MILLER et al. 1972; AKUZAWA et al. 1987; DUBEY et al. 1988; DORNY und FRANSEN 1989; OCHOLI et al. 1989; ARAMINI et al. 1998; LUKEŠOVÁ und LITERÁK 1998; JURÁNKOVÁ et al. 2013; SMITH und KOK 2006) beschrieben. Im Endwirt kommt es nach Aufnahme von Gewebezysten im Magen und Dünndarm des Endwirtes zur Freisetzung von Bradyzoiten und nachfolgender ungeschlechtlicher Vermehrung in den Dünndarmepithelzellen, genauer einer Endodyogenie mit sich direkt anschließender Endopolygenie (DUBEY und FRENKEL 1972). In der darauffolgenden Gamogonie erfolgt die Bildung der Mikro- und Makrogameten (DUBEY und FRENKEL 1972). Die Befruchtung der Makrogameten durch die beweglichen Mikrogameten führt zur Bildung von Zygoten (DUBEY 2010b) und Entwicklung der 10 bis 12 µm großen unsporulierten Oozysten (DUBEY et al. 1970). Diese werden durch Ruptur der Dünndarmepithelzellen in den Darminhalt freigesetzt und mit dem Kot in die Umwelt ausgeschieden. Sowohl SHEFFIELD und MELTON (1969) als auch FRENKEL et al. (1969) stellten fest, dass Oozysten unmittelbar nach der Ausscheidung mit dem Kot in die Umwelt noch nicht infektiös sind. In Abhängigkeit von Umgebungstemperatur und Sauerstoffverfügbarkeit sporulieren Oozysten innerhalb von ein bis fünf Tagen und erhalten mit der Sporulation ihre Infektiosität (DUBEY et al. 1970; DUBEY 1986). Nach abgeschlossener Sporulation enthalten die 11 bis 13 µm großen infektiösen Oozysten zwei Sporozysten mit jeweils vier Sporozoiten (DUBEY et al. 1970). Aufgrund des strukturellen Aufbaus der Oozysten von *T. gondii* werden sie dem *Isospora*-Typ zugeordnet (FRENKEL et al. 1970; SHEFFIELD und MELTON 1970).

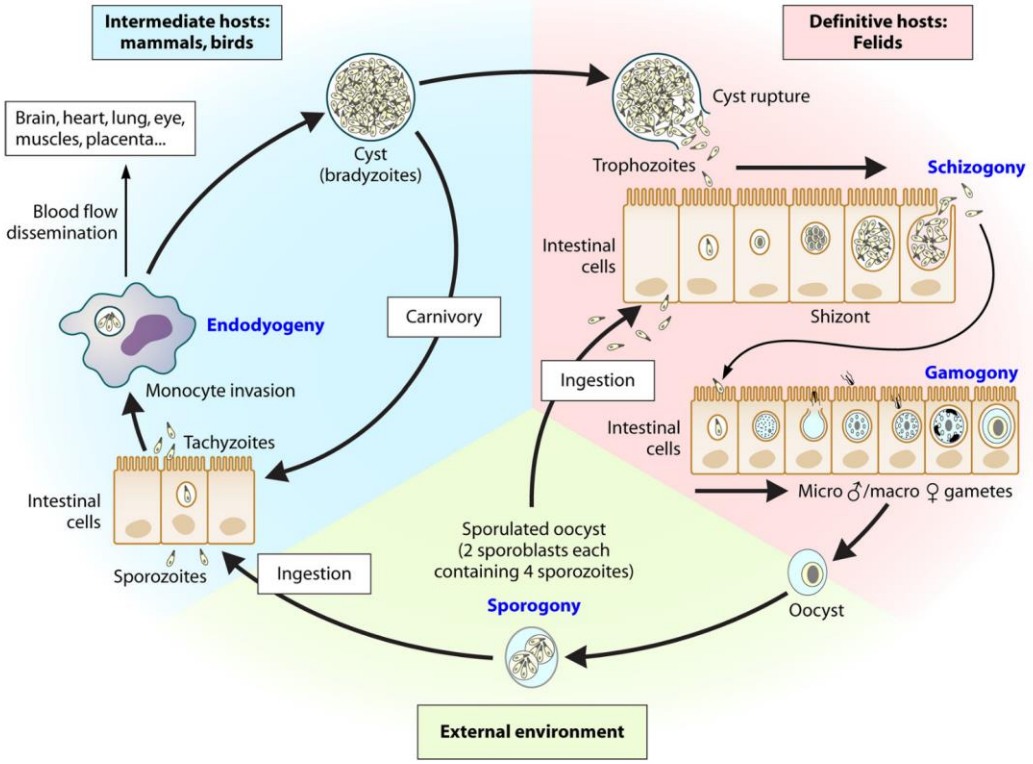

Abbildung 1: Lebenszyklus von *T. gondii* (ROBERT-GANGNEUX und DARDE 2012)

Die Infektion des Endwirts kann auch über die perorale Aufnahme von sporulierten Oozysten sowie in seltenen Fällen auch durch Aufnahme von Tachyzoiten bei Verzehr akut infizierter Zwischenwirte erfolgen (DUBEY et al. 1970; FREYRE et al. 1989; DUBEY 1998, 2002, 2005; POWELL et al. 2001). Nach Aufnahme von Oozysten dringen die Sporozoiten in Zellen der Dünndarmepithelzellen ein und wandeln sich in Tachyzoiten um, welche im Wirt disseminieren. Es findet eine sich wiederholende asexuelle Vermehrung der Tachyzoiten (griech. *tachy*: schnell) in Form einer Endodyogenie in Zellen verschiedenster Gewebe des Endwirtes statt, woran sich die Bildung von Bradyzoiten in Gewebezysten anschließt (FREYRE et al. 1989). Unter bestimmten Umständen, z.B. wenn Zysten aufgrund einer Immunsuppression reaktiviert werden, können Bradyzoiten erneut zu Tachyzoiten konvertieren, welche sich vermehren und eine akute Parasitämie ohne erneute Infektion verursachen können, sowie Dünndarmepithelzellen befallen. Dadurch kann es zu einem sogenannten „*Reshedding*“, also einer erneuten, verzögerten geschlechtlichen Vermehrung mit Oozystenausscheidung kommen.

Die Präpatenz variiert in Abhängigkeit vom aufgenommenen infektiösen Stadium zwischen drei bis acht Tagen nach Infektion mit Bradyzoiten (DUBEY und FRENKEL 1972; WALLACE 1973; DUBEY 2001; BÁRTOVÁ et al. 2003), 26 bis 40 Tage nach Infektion mit Tachyzoiten (DUBEY und FRENKEL 1976;

DUBEY 2002) und 21 bis 49 Tage nach Oozysteninfektion (DUBEY et al. 1970; WALLACE 1973; DUBEY 1996; FREYRE et al. 1989). Die Patenz variiert bei Infektionen mit Gewebezysten von vier bis 20 Tage (DUBEY und FRENKEL 1972; WALLACE 1973; DUBEY 2001; BÁRTOVÁ et al. 2003), fünf bis zehn Tage bei Oozysteninfektionen (WALLACE 1973; DUBEY 1996) und von zwei bis zwölf Tagen bei Infektionen mit Tachyzoiten (DUBEY et al. 1970; DUBEY 1998, 2002).

Als Zwischenwirte für *T. gondii* sind warmblütige Tiere einschließlich des Menschen beschrieben (MILLER et al. 1972; DUBEY 2010b), aber auch Feliden dienen als Zwischenwirt (DUBEY 1997b; DUBEY et al. 2010, 2013a, 2013b; YANG et al. 2017, 2019; DONG et al. 2019). Der Zwischenwirt kann sich durch die orale Aufnahme von infektiösen Oozysten oder durch Verzehr von Zysten-haltigem Gewebe anderer Zwischenwirte infizieren (MILLER et al. 1972). Die Vermehrung im Zwischenwirt erfolgt ausschließlich über Sporo- und Bradyzoiten. Andererseits sind, wenn auch selten, Infektionen von Zwischenwirten mit Tachyzoiten möglich, wie zum Beispiel durch die Aufnahme von Milch infizierter Säugetiere, wie Schafe, Ziegen und Ratten (RIEMANN et al. 1975; TENTER 2009; COSTA und LANGONI, 2010; DUBEY et al. 2014; SAAD et al. 2018).

Nach oraler Aufnahme von infektiösen Oozysten, Bradyzoiten in Gewebezysten oder Tachyzoiten durch Zwischenwirte dringen die entsprechenden Infektionsstadien in die Dünndarmmukosa ein. Im Falle von Oozysten und Gewebezysten handelt es sich um die Sporozoiten bzw. Bradyzoiten, welche sich im Dünndarmepithel zu Tachyzoiten differenzieren (DUBEY et al. 1997). Wie oben für den Endwirt beschrieben, vermehren sich die Tachyzoiten durch Endodyogenie in den verschiedensten Gewebetypen, woran sich die Bildung von Bradyzoiten und damit Gewebezysten anschließt (TENTER et al. 2000). Letzteres wird als Evasion der zunehmenden Immunreaktion des Wirtes interpretiert. Die Zystenbildung beginnt bereits drei bis sieben Tage nach Infektion (DUBEY und FRENKEL 1976; RANI et al. 2019). Innerhalb der Gewebezysten findet eine langsame Endodyogenie der Bradyzoiten (griech. *brady*: langsam) statt. *T. gondii* hat in dieser Phase eine hohe Affinität zu Nerven- und Muskelgewebe (DUBEY et al. 1998; TENTER et al. 2000; DUBEY 2010b). Folglich werden überwiegend diese Gewebetypen als Prädilektionsstellen für die Bildung von Gewebezysten erachtet, welche im Zwischenwirt persistieren. Es ist von einer lebenslangen Persistenz der Gewebezysten in einigen Zwischenwirt-Spezies auszugehen (TENTER et al. 2000). Andere Zwischenwirte eliminieren gebildete Gewebezysten über einen langen Zeitraum. So isolierten DUBEY et al. (1984) und DUBEY (1988) Zysten aus Geweben von Schweinen 171 bis mindestens 875 Tage nach oraler Oozysteninfektion. Eine ähnliche Persistenzdauer von Gewebezysten zeigte DUBEY (1985) in essbaren Geweben von Equiden. In Gewebe von Rindern persistierten Gewebezysten mindestens 1191 Tage nach Oozysteninfektion (DUBEY und THULLIEZ 1993).

Infektionsmaterial für experimentelle Studien lässt sich unter Beachtung des Entwicklungszyklus von *T. gondii* auf verschiedenen Wegen gewinnen. Die Fähigkeit zur Vermehrung von Tachyzoiten durch

Endodyogenie in Zellen verschiedenster Gewebe lässt sich zur kontinuierlichen In-vitro-Generierung von Tachyzoiten in Zellkultur nutzen (GUIMARÃES und MEYER, 1942; HUGHES et al. 1986; EVANS et al. 1999; SUBAUSTE 2012). Auch die Vermehrung und intraperitoneale Gewinnung von Tachyzoiten aus akut infizierten Mäusen ist möglich (DUBEY und FRENKEL 1972; DEMPSTER 1984). Für die Gewinnung von Oozysten ist die Passage in naiven Feliden, in der Regel Hauskatzen, notwendig. Dafür wird zumeist Gehirngewebe von chronisch infizierten Mäusen an Katzen verabreicht, da für diesen Infektionsweg die höchste Empfänglichkeit des Endwirtes und damit verbundene Oozystenausscheidung beschrieben ist (WALLACE 1973; DUBEY und FRENKEL 1976; DUBEY 2001, 2006).

WALLACE (1973) und DUBEY und FRENKEL (1976) konnten bei einer Infektion von Katzen mit Oozysten eine Oozystenausscheidung bei nur 16 % bzw. 21 % der infizierten Katzen zeigen. Folglich sind Infektionen von Feliden mit sporulierten Oozysten möglich, wenn auch im Vergleich zu Infektionen mit Gewebezysten weniger erfolgversprechend. Ein weiterer Nachteil ist die sehr lange Präpatenz nach Oozystenverabreichung von mehr als 19 Tagen (DUBEY et al. 1970; WALLACE 1973; FREYRE et al. 1989; DUBEY 1996) und die damit verbundenen, langen Passagezeiträume.

Im Prinzip kann jede Warmblutspezies zur Gewinnung von Gewebezysten von *T. gondii* genutzt werden. Die Generierung von Gewebezysten erfolgt jedoch in der Regel in der Maus, da Labornager als In-vivo-Modell seit Jahrzehnten erfolgreich etabliert sind. Mäuse und andere Labornager können zur Produktion von Gewebezysten mit Tachyzoiten, Sporozoiten (sporulierten Oozysten) oder Bradyzoiten entweder oral oder auch intraperitoneal infiziert werden (DUBEY 2010b).

2.1.3. Genetik und Virulenz

Historisch erfolgte die Einteilung der Stämme nach der Virulenz in der Maus (DUBEY 2010b). Stämme vom Typ I sind als hochvirulent in der Maus beschrieben, wobei sie keine Gewebezysten in der Maus induzieren und bereits in geringen Infektionsdosen von weniger als 10 Tachyzoiten letal wirken (DUBEY 1980; DARDÉ et al. 1992). Stämme vom Typ II und Typ III sind als avirulent im Mausmodell bei letalen $Dosen_{50\%}$ von 10^2 bis zu 10^5 Tachyzoiten charakterisiert, wobei Typ III-Stämme auch Wochen nach Infektion zum Tod führen können (DARDÉ et al. 1988, 2014).

HOWE und SIBLEY (1995) charakterisierten *T. gondii* erstmalig molekularbiologisch und gruppierten *T. gondii* in drei klonale Linien anhand des mittels PCR-RFLP ermittelten Genotyps, welcher im Zusammenhang zur Virulenz in Menschen und Tieren steht. Isolate, deren Genotyp in den sechs untersuchten Markern keiner der drei klonalen Linien zugeordnet werden kann, werden als atypisch bezeichnet (DARDÉ et al. 2014). Diese atypischen Stämme werden überwiegend in Isolaten aus Südamerika nachgewiesen (AJZENBERG et al. 2004; FERREIRA et al. 2006; KHAN et al. 2006; DUBEY et

al. 2007a; PENA et al. 2008; GUO et al. 2015b; VIEIRA et al. 2018). KHAN et al. (2011) wiesen eine vierte klonale Linie von *T. gondii* in Wildtierisolaten aus Nordamerika mittels Sequenzierung nach.

In Europa kommen vornehmlich Stämme vom Typ II sowohl im Menschen als auch im Tier vor. Humane Toxoplasmose wird hauptsächlich durch Typ II-Stämme verursacht (AJZENBERG et al. 2002; NOWAKOWSKA et al. 2006; PEYRON et al. 2006; FEKKAR et al. 2011; MAKSIMOV et al. 2012; HERRMANN et al. 2014; PAGLIUCA et al. 2017; JOKELAINEN et al. 2018). Aus Proben in Europa wurden in einem breiten Spektrum an Wirtsspezies ebenfalls überwiegend Typ II-Stämme isoliert (DUBEY et al. 2005b, 2006c; DE SOUSA et al. 2006; DUMÈTRE et al. 2006; SCHARES et al. 2008, 2017a; RICHOMME et al. 2009; AUBERT et al. 2010; HALOS et al. 2010; DE CRAEYE et al. 2011; JOKELAINEN et al. 2011; FREY et al. 2012; HERRMANN et al. 2013).

Aber auch Typ III-Stämme sind in Europa beim Menschen (AJZENBERG et al. 2002; MESSARITAKIS et al. 2008; HERRMANN et al. 2014; JOKELAINEN et al. 2018) und Tier (DE SOUSA et al. 2006; DUBEY et al. 2006c; HALOS et al. 2010; HERRMANN et al. 2010, 2012; DE CRAEYE et al. 2011; CALERO-BERNAL et al. 2015) beschrieben.

Dagegen werden Stämme vom Typ I in Europa nur selten (AJZENBERG, 2010; FEKKAR et al. 2011; HERRMANN et al. 2014) isoliert, wie der Nachweis von 5,9 % Typ I-Stämme in Wildtieren aus Spanien durch CALERO-BERNAL et al. (2015) zeigt.

Atypische *T. gondii*-Stämme werden in Europa deutlich seltener als in Südamerika und als Typ II- und Typ III-Stämme beschrieben, kommen jedoch vor. HERRMANN et al. (2010, 2012) wiesen sowohl in Füchsen aus Sachsen-Anhalt und Brandenburg als auch in Katzen aus Deutschland atypische *T. gondii*-Stämme nach. In drei schweren Fällen kongenitaler Toxoplasmose konnten AJZENBERG et al. (2002) atypische *T. gondii*-Stämme isolieren.

2.1.4. Nachweismöglichkeiten

2.1.4.1. Bioassay

Der Goldstandard zum Nachweis von infektiösen *T. gondii*-Stadien ist bis zum jetzigen Zeitpunkt der Bioassay (DUBEY 2010b; OPSTEEGH et al. 2010; BIER et al. 2019), wobei Katzen oder Mäusen das zu testende Material, Gewebe oder Kot, verabreicht wird.
Bei der Prüfung auf infektiöse Gewebezysten erfolgt, ab dem dritten bis zum 21. Tag nach oraler Verabreichung an *T. gondii*-naive Katzen, die Untersuchung des Kots auf eine Oozystenausscheidung (DUBEY 1995). Im Falle einer Oozystenausscheidung ist der Beweis erbracht, dass infektiöse Gewebezysten im untersuchten Gewebe enthalten waren (DUBEY et al. 1970; FRENKEL et al. 1970). Auch die Untersuchung von Katzenkot auf sporulierte und damit infektiöse Oozysten ist auf diesem

Weg möglich, jedoch ist die deutlich verlängerte Präpatenz von mindestens 19 Tagen (DUBEY et al. 1970; WALLACE 1973; FRENKEL et al. 1976) und eine verminderte Empfänglichkeit gegenüber Oozysteninfektionen beim Endwirt (WALLACE 1973; DUBEY und FRENKEL 1976; DUBEY 2006) ein Nachteil gegenüber der Infektion mit zystenhaltigen Geweben. Katzen werden daher in der Regel für Bioassays von Gewebe genutzt (DUBEY 1985, 1988; DUBEY und THULLIEZ 1993; RANI et al. 2019). Dies hat auch den Vorteil, dass an Katzen deutlich größere Mengen an Gewebe verfüttert werden können als an Mäuse (DUBEY 2010b). Als nachteilig sind jedoch die vergleichsweisen hohen Kosten beim Einsatz von Katzen und der Tierschutzaspekt zu erwähnen.

Mäusen können die verschiedenen Arten des zu testenden Probenmaterials auf unterschiedlichen Wegen appliziert werden. Die orale Verabreichung hat sich für die Untersuchung von Kot auf Oozysten bewährt (ARAMINI et al. 1998; DUBEY 2005, 2010b). Gewebe zur Prüfung auf *T. gondii*-Zysten kann sowohl oral als auch subkutan oder intraperitoneal verabreicht werden (DUBEY 2010b). Um größere Gewebemengen in Mäusen zu untersuchen, wird das Gewebe üblicherweise mit Trypsin oder Pepsin verdaut und dann die Zysten angereichert (DUBEY et al. 1984, 2015; DUBEY 1985, 1988, 2010b; RANI et al. 2019).

Unabhängig von der Verabreichungsform erfolgt nach einer Inkubationszeit von sechs bis acht Wochen die Tötung der Maus, eine serologische Untersuchung auf *T. gondii*-Antikörper und eine mikroskopische und gegebenenfalls molekularbiologische Untersuchung des Gehirns auf Gewebezysten (Dubey 2010b).

2.1.4.2. Polymerase-Kettenreaktion

Die Polymerase-Kettenreaktion (engl. *polymerase chain reaction*, Abk. PCR) ist ein häufig genutztes Verfahren zum Nachweis von *T. gondii*-DNA. Zahlreiche Zielsequenzen im *T. gondii*-Genom sind für den Nachweis beschrieben und sind in Tabelle 1 zusammengefasst.

Tabelle 1: Häufig genutzte Zielgene zum Nachweis von *T. gondii* mittels PCR

Zielgen	Erstbeschreibung des Zielgens zum Nachweis von *T. gondii*
B1-Gen	BURG et al. (1989)
529 bp repeat element	HOMAN et al. (2000)
Internal transcribed spacer (ITS1)	HURTADO et al. (2001), JAUREGUI et al. (2001)
18S rRNA-Gen	CAZENAVE et al. (1991)
TGR1E-Sequenz	CRISTINA et al. (1992)
P30-Gen	SAVVA et al. (1990)

In der PCR-Diagnostik von *T. gondii* haben sich Zielsequenzen basierend auf „*multi-copy*"-Genen wie das von BURG et al. (1989) beschriebene B1-Gen und das von HOMAN et al. (2000) beschriebene „529 bp *repeat element*" etabliert (WYROSDICK und SCHAEFER 2015).
BURG et al. (1989) postulierte, dass aufgrund des 35-fachen Vorliegens des B1-Gens, dieses eine geeignete Zielsequenz zur Detektion von *T. gondii* darstellt. Zur Verbesserung der Sensitivität der PCR erweiterten zahlreiche Autoren bestehende B1-Gen-Nachweise um eine nested PCR (HO-YEN et al. 1992; JOSS et al. 1993; WASTLING et al. 1993; ZÖLLER et al. 2013; SARKARI et al. 2014). Aufgrund des 200 bis 300-fachen Vorkommens des „529 bp *repeat element*" im Genom, weisen darauf basierende PCR-Methoden dennoch eine besonders hohe Sensitivität im Vergleich zu B1-Gen-basierten Assays auf (HOMAN et al. 2000; EDVINSSON et al. 2006; STERKERS et al. 2010; BELAZ et al. 2015).
COSTA et al. (2000) und REISCHL et al. (2003) entwickelten auf Grundlage des B1-Gens und des „529 bp *repeat element*" real-time PCRs zum Nachweis von *T. gondii*. Neben Bestimmung der Parasitenlast durch die Möglichkeit der Quantifizierung, hat die real-time PCR den Vorteil der Vermeidung von falsch-positiven Reaktionen durch Kontaminationen mit amplifizierter DNA, da die Reaktionsgefäße für die Detektion der DNA nicht geöffnet werden müssen (COSTA et al. 2000; REISCHL et al. 2003).

Großen Einfluss auf die Sensitivität des Nachweises von *T. gondii* mittels PCR, insbesondere von Gewebezysten, hat nicht nur das eingesetzte PCR-System, sondern auch die zur DNA-Extraktion eingesetzte Probenmenge, aufgrund der inhomogenen Verteilung der Zysten im Gewebe (ESTEBAN-REDONDO et al. 1999; JURÁNKOVÁ et al. 2014a; RANI et al. 2019).
Bei der Anwendung von kommerziell erhältlichen Kits zur DNA-Extraktion ist die Probenmenge in der Regel auf wenige Milligramm an Untersuchungsmaterial begrenzt. Im Bioassay können 500 g und mehr Material an eine Katze verfüttert oder bis zu 100 g verdautes Gewebe pro Maus verabreicht werden (DUBEY 2010b). Mit steigender Probenmenge erhöht sich die Nachweisrate von *T. gondii* aus Gewebe (RANI et al. 2019), folglich weisen Bioassays verglichen mit PCR-Methoden eine höhere Sensitivität auf (DA SILVA und LANGONI 2001; GARCIA et al. 2006; HILL et al. 2006; TSUTSUI et al. 2007; MONTOYA et al. 2009; BEZERRA et al. 2012).

Eine sequenzspezifische DNA-Extraktion durch „*magnetic-capture*" von *T. gondii*-spezifischen DNA-Sequenzen ermöglicht den Einsatz größerer Probenmengen auch für PCR-Untersuchungen (OPSTEEGH et al. 2010). Hierbei wird aus bis zu 100 g Probenmaterial die DNA sequenzspezifisch mittels magnetischer Partikel extrahiert und anschließend durch real-time PCR des „529 bp *repeat element*" analysiert (*magnetic-capture* real-time PCR, Abk. MC-qPCR) (OPSTEEGH et al. 2010; ALGABA et al. 2017). Kleine Mengen Gewebeproben als limitierender Faktor für einen sensitiven PCR-Nachweis werden somit umgangen, wodurch vergleichbare oder bessere Ergebnisse gegenüber dem Bioassay generiert werden (OPSTEEGH et al. 2010, 2016; ALGABA et al. 2017).

Trotz einer vergleichbaren Leistungsfähigkeit von Bioassay und MC-qPCR hinsichtlich ihrer Sensitivität (ALGABA et al. 2017), ist eine generelle Einschränkung der PCR eine fehlende Beurteilung der Infektiosität nachgewiesener Stadien im Gegensatz zum Bioassay (DUBEY 2010b).

2.1.4.3. Serologie

Serologische Testverfahren für das Geflügel detektieren Immunglobulin Y (IgY). In der Literatur wird synonym fälschlicherweise häufig noch der Begriff Immunglobulin G (IgG) verwendet, obwohl Geflügel keine dem Säuger-IgG entsprechenden Antikörper bildet (SCHADE et al. 2005). Allgemein ist IgY erstmals vier Tage nach einer Infektion im Serum von Hühnern nachweisbar (SCHADE et al. 2005). Nach Infektionen mit *T. gondii* treten spezifische Antikörper und somit eine Serokonversion nach ein bis zwei Wochen bei Huhn und Pute auf (DUBEY et al. 1993a, 1993b; SEDLÁK et al. 2000; MAKSIMOV et al. 2018).

Zahlreiche serologische Verfahren zum Nachweis von *T. gondii*-Antikörpern sind etabliert (Tab. 2). Jedoch eignet sich nur ein Teil davon für die Anwendung beim Geflügel. Vielfach genutzte und geeignete Verfahren bei Huhn und Pute sind der modifizierte Agglutinationstest (MAT), *Enzyme-linked Immunosorbent Assay* (ELISA) und der indirekte Fluoreszenz-Antikörpertest (IFAT) (CASARTELLI-ALVES et al. 2014; DUBEY et al. 2016; OPSTEEGH et al. 2016; MAKSIMOV et al. 2018).

Der MAT (FULTON und TURK 1959; DESMONTS und REMINGTON 1980; DUBEY und DESMONT 1987) ist eines der am häufigsten bei Huhn und Pute angewandten Testverfahren zur serologischen Diagnostik von *T. gondii*-Infektionen. In experimentellen *Toxoplasma*-Infektionen von Hühnern und Puten zeigt der MAT mit einer Sensitivität von 92,5 % bis 100 % sowie einer Spezifität von 100 % eine gute Eignung zum Nachweis von *Toxoplasma*-Antikörpern (DUBEY et al. 1993a, 1993b, 2016; OPSTEEGH et al. 2016).
Der ELISA, als weiteres bei Huhn und Pute angewandtes serologisches Testverfahren, detektiert *T. gondii*-Antikörper auf der Basis von Tachyzoiten-Antigenen oder rekombinanten Oberflächen- bzw. Sekretionsantigenen. Die verfügbaren ELISAs zeigen in experimentell infizierten Hühnern und Puten Sensitivitäten von 81 % bis 100 % (BIANCIFIORI et al. 1986; DUBEY et al. 1993a, 1993b; KOETHE et al. 2011; OPSTEEGH et al. 2016; SCHARES et al. 2017a; MAKSIMOV et al. 2018), folglich kann auf eine Eignung zum Nachweis von *T. gondii*-Antikörpern bei Huhn und Pute geschlossen werden.

Bei Huhn und Pute wird auch der IFAT basierend auf Tachyzoiten-Oberflächenantigenen eingesetzt. Sowohl MAKSIMOV et al. (2018) als auch SEDLÁK et al. (2000) zeigten die Anwendbarkeit des IFAT zum Nachweis von *T. gondii*-Antikörpern mit Sensitivitäten von 100 % bei Huhn und Pute nach Infektionen mit Tachyzoiten oder Oozysten.

Tabelle 2: Serologische Nachweisfahren für *T. gondii*-Antikörper und deren Anwendung bei Huhn und Pute

Nachweisverfahren	**Studien, in denen das genannte Testverfahren bei Huhn und/oder Pute angewendet wurde**
Sabin-Feldman-Dye-Test (DT)	DROBECK et al. (1953); ERICHSEN und HARBOE (1953); HARBOE und REENAAS (1957); EYLES et al. (1959); ZÀSTĚRA et al. (1965); GRZYWIŃSKI (1967); MILLER et al. (1972); RUIZ und FRENKEL (1980); DUBEY et al. (1993a, 1993b); LITERÁK und HEJLÍČEK (1993)
Komplement-Fixationstest (CFT)	ERICHSEN und HARBOE (1953); HARBOE und REENAAS (1957) UMIŃSKI et al. (1961); ROBERTSON et al. (1963); BEAUREGARD et al. (1965); GRZYWIŃSKI (1967); FRENKEL (1981)
Indirekter-Hämagglutination-Test (IHAT)	FRENKEL (1981); AGANGA und BELINO (1984); GHORBANI et al. (1990); DUBEY et al. (1993a, 1993b); HARFOUSH und TAHOON (2010); YAN et al. (2010); BELTRAME et al. (2012); CASARTELLI-ALVES et al. (2014)
Latex-Agglutinationtest (LAT)	DUBEY et al. (1993a, 1993b); ZIA-ALI et al. (2007); BUTTY (2009); MATSUO et al. (2014)
Indirekter Fluoreszenz-Antikörpertest (IFAT)	SEDLÁK et al. (2000); SREEKUMAR et al. (2001); ABRAHAMS-SANDÍ und VARGAS-BRENES (2005); BRANDÃO et al. (2006); BÁRTOVÁ et al. (2009); CHUMPOLBANCHORN et al. (2009); MILLAR et al. (2012); MORÉ et al. (2012); CASARTELLI-ALVES et al. (2014); AYINMODE und JONES-AKINBOBOLA (2015); MAKSIMOV et al. (2018)
Enzyme-linked Immunoabsorbent Assay (ELISA)	BIANCIFIORI et al. (1986); DUBEY et al. (1993a, 1993b); ZHU et al. (2008); MILLAR et al. (2012); CASARTELLI-ALVES et al. (2014); HAMIDINEJAT et al. (2014); OPSTEEGH et al. (2016); LIU et al. (2017); SCHARES et al. (2017a, 2017b); MAKSIMOV et al. (2018)
Modifizierter Agglutinationstest (MAT)	DUBEY et al. (1993a, 1993b, 2016); YAN et al. (2010); DUBEY (2010a); CASARTELLI-ALVES et al. (2014); HAMIDINEJAT et al. (2014); OPSTEEGH et al. (2016)
Kinetischer ELISA (KELA)	KOETHE et al. (2011)

Falsch-positive Ergebnisse der serologischen Testverfahren beim Geflügel sind aufgrund von Kreuzreaktionen mit Antikörpern gegen eng verwandte Kokzidien-Spezies möglich. Jedoch haben nur wenige Autoren die angewandten Nachweisverfahren auf Spezifität für *T. gondii*-Antikörper des Geflügels im Vergleich zu z.B. Anti-*Eimeria*-Antikörper geprüft (DUBEY et al. 1993a, 1993b; KOETHE et al. 2011; MAKSIMOV et al. 2011). Dabei variierte der Anteil falsch-positiver Nachweise für *T. gondii* zwischen den verschiedenen Testverfahren. So wiesen KOETHE et al. (2011) im kinetischen ELISA (KELA) in 10 % der Seren von mit *Hammondia hammondi* oder *Eimeria* spp. infizierten Puten Kreuzreaktionen nach, jedoch nicht bei Infektionen mit *Neospora caninum*. Im Gegensatz dazu beobachtete MAKSIMOV et al. (2011) im TgSAG1-ELISA keine falsch-positiven Reaktionen von *Hammondia hammondi*-Antikörpern bei Enten und Gänsen, sehr wohl jedoch im IFAT. DUBEY et al. (1993a, 1993b) detektierte im MAT und ELISA keine falsch-positiven Reaktionen mit Seren von mit *Eimeria spp.* infizierten Hühnern und Puten.

Der Latex-Agglutinationstest (LAT) erweist sich aufgrund unzureichender Sensitivität als nicht anwendbar bei Huhn und Pute (DUBEY et al. 1993a, 1993b). Ebenfalls ungeeignet für die Diagnostik von *Toxoplasma*-Infektionen bei Huhn und Pute sind sowohl der in der Humandiagnostik häufig angewandte Sabin-Feldman-Dye-Test (DT), der Komplement-Fixationstest (CFT) als auch der Indirekte-Hämagglutinationstest (IHAT) (ERICHSEN und HARBOE, 1953; ROBERTSON et al. 1963; MILLER et al. 1972; FRENKEL 1981; DUBEY et al. 1993a, 1993b; YAN et al. 2010).

2.2. Bedeutung für den Menschen

2.2.1. Humane Toxoplasmose

Der Mensch dient *T. gondii* ausschließlich als Fehl-Zwischenwirt. Infektionen sind weltweit verbreitet mit Seroprävalenzen von 4 % bis zu 92 % in Abhängigkeit der untersuchten Bevölkerungsgruppen und Herkunftsländer (PAPPAS et al. 2009; DUBEY 2010b).
Akute Infektionen verlaufen bei immunkompetenten Personen in der Regel symptomlos und selbstlimitierend. Bei ungefähr 10 % der akut infizierten immunkompetenten Personen treten unspezifische erkältungsähnliche Symptome wie Lymphadenopathie mit Fieber, Erschöpfung und Halsschmerzen auf (MONTOYA und LIESENFELD 2004; HALONEN und WEISS 2013). Okuläre Toxoplasmose in Form einer Chorioretinitis wird als Folge einer akuten oder kongenitalen Infektion des Menschen beobachtet (MONTOYA und REMINGTON 1996; HOLLAND 1999). Die chronische Form der Toxoplasmose verläuft beim immunkompetenten Menschen überwiegend symptomlos. Jedoch kann die Reaktivierung einer chronischen Infektion im immunsupprimierten Patienten zu lebensbedrohlichen Symptomen wie Enzephalitis, Pneumonie bis hin zum septischen Schock führen (MONTOYA und LIESENFELD 2004; DUBEY 2010b; HALONEN und WEISS 2013; ABBASI FARD et al. 2020). Selten erkranken auch immunkompetente Patienten schwer an akuter Toxoplasmose (DEMAR et al. 2012; SOBANSKI et al. 2013).

T. gondii-Infektionen in der Schwangerschaft mit diaplazentarer Infektion des Fetus treten zum einen bei einer akuten Infektion serologisch negativer, immunkompetenter Frauen auf. Gleichermaßen ist eine Reaktivierung einer chronischen Toxoplasmose durch Immunsuppression in der Schwangerschaft oder durch Reinfektion mit einem virulenteren *T. gondii*-Stamm bekannt (DUBEY 2010b; HALONEN und WEISS 2013; MALDONADO und READ 2017; KARANOVIC et al. 2019). Während die immunkompetente Schwangere zumeist keine Symptome ausprägt (MONTOYA und LIESENFELD 2004), sind die Symptome einer kongenital erworbenen Toxoplasmose beim Fetus vielfältig in Abhängigkeit vom Infektionszeitpunkt während Schwangerschaft (MONTOYA und LIESENFELD 2004; DUBEY 2010b; HALONEN und WEISS 2013). Beschrieben sind schwerste Missbildungen wie Hydrocephalus, intracraniale Kalzifizierungen, Blindheit und auch Chorioretinitis bis hin zu Aborten (MALDONADO und READ 2017).

2.2.2. Risikofaktoren

Eine Infektion des Menschen mit *T. gondii* kann auf vielfältigem Wege durch die Aufnahme von Gewebezysten oder Oozysten erfolgen.
Die Infektion mit Oozysten ist durch den Verzehr von kontaminierten Lebensmitteln, insbesondere Obst und Gemüse, möglich (LASS et al. 2012; MARCHIORO et al. 2016; CARADONNA et al. 2017). Epidemiologische Studien von KAPPERUD et al. (1996), BARIL et al. (1999) und TEWELDEMEDHIN et al. (2019) bestätigen Zusammenhänge zwischen dem Verzehr von ungewaschenem Obst und Gemüse und einer *T. gondii*-Infektion in der Schwangerschaft. COOK et al. (2000) sieht den Kontakt mit Oozysten-kontaminiertem Erdboden bei der Gartenarbeit als einen nicht unerheblichen Risikofaktor für die Infektion mit *T. gondii* in der Schwangerschaft. Ein weiterer, nicht zu unterschätzender Infektionsweg ist die Aufnahme von Oozysten-kontaminiertem Trinkwasser. Dies zeigt das Auftreten von Ausbrüchen akuter humaner Toxoplasmose, verursacht durch kontaminiertes Trinkwasser mit insgesamt 536 erkrankten Personen (BOWIE et al. 1997; DE MOURA et al. 2006).

Diskrepanzen fallen in der Literatur bezogen auf das Risiko, welches von der Haltung beziehungsweise dem Kontakt zu Katzen ausgeht, auf. KAPPERUD et al. (1996), BARIL et al. (1999), JONES et al. (2009), WILKING et al. (2016) und auch TEWELDEMEDHIN et al. (2019) zeigten für Katzenkontakt beziehungsweise Katzenhaltung ein hohes Risiko für eine Infektion mit *T. gondii*. Dahingegen konnten COOK et al. (2000), ALVARADO-ESQUIVEL et al. (2006) und SAID et al. (2017) keine signifikanten Zusammenhänge zwischen einer *T. gondii*-Infektion und Katzenkontakt nachweisen.

Als eines der größten Risiken wird jedoch die Aufnahme von nicht durchgegartem Fleisch mit infektiösen Gewebezysten (KAPPERUD et al. 1996; BARIL et al. 1999; MORRIS et al. 1999; COOK et al.

2000; JONES et al. 2009; WILKING et al. 2016; SAID et al. 2017), als auch der Verzehr von kurzgereiften Fleischerzeugnissen wie Rohwürste oder gepökelte und kurz gereifte Fleischerzeugnissen (BUFFOLANO et al. 1996) angesehen. Überwiegend wurde dabei rohes und nicht vollständig durchgegartes Fleisch vom Rind (BARIL et al. 1999; COOK et al. 2000; JONES et al. 2009; SAID et al. 2017; BELLUCO et al. 2018), Lamm beziehungsweise Schaf (KAPPERUD et al. 1996; BARIL et al. 1999; COOK et al. 2000; JONES et al. 2009; SAID et al. 2017) und Schwein (KAPPERUD et al. 1996; BELLUCO et al. 2018) genannt. Die Ergebnisse dieser epidemiologischen Studien werden durch serologische Untersuchungen zur Identifizierung der humanen Infektionsquellen untermauert. So konnte LIU et al. (2019) in nur 3,6 % der seropositiven Fälle eine Infektion mit Oozysten nachweisen und schlussfolgert, dass alle weiteren seropositiven Proben auf eine Infektion mit Gewebezysten zurückzuführen sind. Auch das Auftreten von Ausbrüchen, verursacht durch den Verzehr von rohem oder nicht vollständig durchgegartem Fleisch, stützt die Ergebnisse der epidemiologischen Befragungen. So beschreibt BONAMETTI et al. (1997) einen Ausbruch von akuter Toxoplasmose in 17 Fällen nach Verzehr von rohem Schaffleisch auf einer Feier. Ebenfalls nach dem Verzehr von nicht durchgegartem Schaffleisch erkrankten drei Personen in London an akuter Toxoplasmose (FERTIG et al. 1977). KEAN et al. (1969) und EDUARDO et al. (2007) beschreiben sechs bzw. fünf Fälle von akuter Toxoplasmose nach dem Verzehr von rohem Rindfleisch in Form von „Steak Tartar“ oder nicht ausreichend durchgegarten Hamburgern.

Der Verzehr von Geflügelfleisch als Risikofaktor für eine Infektion mit *T. gondii* stand bis vor einigen Jahren noch nicht im Fokus der epidemiologischen Studien. Es war bis dahin weitgehend unüblich, Geflügelfleisch und Erzeugnisse daraus im nicht durchgegarten Zustand zu verzehren, wie zum Beispiel kurzgereifte Rohwürste oder Rohschinken. Verschiedene Autoren konnten jedoch zeigen, dass vom Geflügelfleischverzehr ein Risiko einer *T. gondii-Infektion* des Menschen ausgehen kann (ALVARADO-ESQUIVEL et al. 2006, 2008; SAID et al. 2017).

2.3. Bedeutung der Toxoplasmose des Geflügels

Das Haushuhn (*Gallus gallus domesticus*) und die Pute (*Meleagris gallopavo*) sind die Hauptlieferanten von Geflügelfleisch weltweit (AVEC 2019, 2020). Der stetig steigende Geflügelfleischverzehr weltweit auf bis zu 65,1 kg pro Kopf und Jahr in den letzten zwei Jahrzehnten (OECD 2020) führt zu einem nicht zu unterschätzenden Risiko einer humanen Infektion mit *T. gondii* beim Verzehr von nicht ausreichend erhitzten Geflügelfleisch (ALVARADO-ESQUIVEL et al. 2006, 2008; SAID et al. 2017), da aviäre Spezies ebenso wie Säugetiere als Zwischenwirte für *T. gondii* dienen (MILLER et al. 1972; RUIZ und FRENKEL 1980). DUBEY (2010a) ist sogar der Auffassung, dass Geflügel, insbesondere Hühner, eine bedeutendere Rolle als Nagetiere im Lebenszyklus und somit in der Verbreitung von *T. gondii* einnimmt. Zum einen führt er die erheblich höhere Lebenserwartung

von Geflügel an, zum anderen die Resistenz dieser Zwischenwirte gegenüber klinischer Toxoplasmose.

Die Freilandhaltung und Hinterhofhaltung von Nutztieren wird unter anderem als ein bedeutender Risikofaktor für eine Infektion mit *T. gondii* angesehen (KIJLSTRA et al. 2004; ZHU et al. 2008; YAN et al. 2009; MAKSIMOV et al. 2011; ALVARADO-ESQUIVEL et al. 2012; MILLAR et al. 2012; XU et al. 2012; GUO et al. 2015a; OPSTEEGH et al. 2016; SCHARES et al. 2017a; RODRIGUES et al. 2019). Die Anwesenheit von Katzen zum Beispiel zur Schädlingsbekämpfung und auch deren Anzahl in einer Hühnerhaltung gelten ebenso als Risikofaktoren für eine *T. gondii*-Infektion (MAGALHÃES et al. 2016; SCHARES et al. 2017a). Feliden können pro Tag bis zu 360 Millionen Oozysten mit dem Kot ausscheiden (DUBEY 2002), folglich kontaminieren sie große Bereiche der Umwelt mit Oozysten, welche resistent gegen viele Umwelteinflüsse sind (DABRITZ et al. 2007; DU et al. 2012; GOTTELAND et al. 2014; LIU et al. 2017).

Hühner infizieren sich überwiegend über die Aufnahme von Oozysten (LIU et al. 2019) und gelten aufgrund ihres Fressverhaltens, dem Picken vom Boden, als Sentineltiere für eine Kontamination der Umwelt mit Oozysten von *T. gondii* (DA SILVA et al. 2003; DUBEY et al. 2005b, 2006c, 2008, 2015; LEHMANN et al. 2006; YAN et al. 2009; MORÉ et al. 2012; FENG et al. 2016; LOPES et al. 2016). Mit steigendem Alter der Tiere erhöht sich das Risiko einer Infektion durch den immer wahrscheinlicheren Kontakt zu Oozysten (ALVARADO-ESQUIVEL et al. 2012; MILLAR et al. 2012; XU et al. 2012; DUBEY et al. 2015; OPSTEEGH et al. 2016; SCHARES et al. 2017a). Aufgrund der Verwandtschaft zum Huhn und dem sehr ähnlichen Fressverhalten (EKESBO und GUNNARSSON 2018), wird bei Puten die Aufnahme von Oozysten durch Picken vom Boden ebenso als wahrscheinlichste Hauptquelle von Infektionen angenommen (LINDSAY et al. 1994).
Zudem ist eine Infektion über Gewebezysten durch die Aufnahme von anderen infizierten Zwischenwirten infolge der omnivoren Ernährungsweise bei Huhn und Pute möglich (STELZER et al. 2019). SÁ et al. (2017) sieht zum Beispiel im Vorkommen anderer Tiere insbesondere Schafen im Umfeld von Geflügel und Aufnahme von Nachgeburten durch Hühner ein Risiko für eine *T. gondii*-Infektion.

2.3.1. *T. gondii*-Infektion des Huhnes

Hühner gelten als gut geeignete Sentineltiere zur Erfassung der Kontamination der Umwelt mit Oozysten von *T. gondii*. Unzählige Studien weltweit beschäftigten sich mit der Seroprävalenz in natürlich infizierten Hühnern. In Freilandhühnern schwanken die Seroprävalenzen außerhalb Europas für *T. gondii* zwischen 0 % und 100 % (AGANGA und BELINO 1984; DEVADA et al. 1998; EL-MASSRY et al. 2000; SREEKUMAR et al. 2003; DUBEY et al. 2003, 2005c, 2006b, 2007b; ZHU et al. 2008; CHUMPOLBANCHORN et al. 2009; ALVARADO-ESQUIVEL et al. 2012; XU et al. 2012; TILAHUN et al.

2013; MATSUO et al. 2014; AYINMODE und JONES-AKINBOBOLA, 2015; LOPES et al. 2016; CHIKWETO et al. 2017; LIU et al. 2017). Zur Gewinnung eines Überblickes über die Seroprävalenzen von natürlich infizierten Hühnern in Europa und der damit verbundenen Abschätzung der Bedeutung der Toxoplasmose des Huhnes, sind in Tabelle 3 europäische Seroprävalenz-Studien zusammengefasst. Bei der Interpretation der Daten ist zu beachten, dass die ermittelten Seroprävalenzen mit unterschiedlichen Testverfahren erhoben wurden, welche sich im Laufe der Zeit zum Teil als nicht geeignet für Geflügel herausstellten (FRENKEL 1981).

Nicht nur das angewandte Testverfahren beeinflusst die Seroprävalenzen, sondern auch vielfältige Faktoren, welche Einfluss auf die Immunreaktion und damit verbundene Messgrößen der IgY-Bildung wie Zeitpunkt der Serokonversion, Höhe des Antikörperspiegels und Persistenz der Antikörper beim Huhn nehmen.
CHUMPOLBANCHORN et al. (2009) wiesen bereits eine Woche nach Infektion eine Serokonversion nach und vermuteten als Ursache das ausgewählte Infektionsmodell, das auf der intramuskulären Verabreichung von Tachyzoiten des Typ-I-Stammes RH basierte. Andererseits kann der unter natürlichen Bedingungen unwahrscheinliche Infektionsweg über Tachyzoiten zu einer schwächeren Immunantwort als bei natürlichen Infektionswegen führen (YAN et al. 2010; OPSTEEGH et al. 2016; MAKSIMOV et al. 2018). Die Serokonversion nach einer Simulation natürlicher Infektionswege mit Gewebezysten oder Oozysten ist vergleichbar häufig (OPSTEEGH et al. 2016).

Zahlreiche Publikationen belegen, dass die Anzahl verabreichter infektiöser Stadien (BIANCIFIORI et al. 1986; DUBEY et al. 1993b; SEDLÁK et al. 2000; OPSTEEGH et al. 2016) als auch der zur Infektion verwendete *T. gondii*-Stamm (DUBEY et al. 1993b) bzw. die klonale Linie des Stammes (MAKSIMOV et al. 2018) keinen Einfluss auf die Serokonversion beim Huhn hat. Dagegen zeigten HOTOP et al. (2014) einen schwachen Einfluss der inokulierten Oozystendosis auf die Serokonversion im Lineblot. Auch MAKSIMOV et al. (2018) stellten in ihren Untersuchungen den Einfluss der Infektionsdosis auf die Immunantwort, jedoch in Abhängigkeit vom verwendeten Test, heraus. Demnach führen höhere Infektionsdosen von Tachyzoiten der klonalen Linien I und III zu höheren Antikörpertitern im ELISA. Im IFAT war dieser Einfluss nicht erkennbar.

Zudem haben auch genetische Eigenschaften des Wirtes eine Wirkung auf dessen Serokonversion. SEDLÁK et al. (2000) stellten einen Einfluss der infizierten Spezies auf die Immunantwort fest, da Hühner im Vergleich zu den anderen untersuchten aviären Spezies eine spätere Serokonversion und signifikant niedrigere Antikörpertiter aufwiesen. In Untersuchungen von SCHARES et al. (2017b) traten Zuchtlinien-bedingte Unterschiede bei Legehennen in der Höhe der Antikörpertiter auf.

Tabelle 3: Seroprävalenz bei natürlich *T. gondii*-exponierten Hühnern in Europa

Literaturquelle	Land	Anzahl untersuchter Tiere	Seroprävalenz Anzahl pos. Tiere (%)	Haltungsform
BÁRTOVÁ et al. (2009)	Tschechische Republik	480	1 (0,2 %)	konventionell
DUBEY et al. (2005b)	Österreich	860	302 (36,3 %)	ökologisch
DUBEY et al. (2006c)	Portugal	225	61 (27,1 %)	Freiland
DUBEY et al. (2008)	Polen	20	6 (30 %)	Freiland
DUBEY et al. (2008)	Italien	80	11 (13,7 %)	Freiland
GRZYWIŃSKI (1967)	Polen	1200	11 (0,9 %)	nicht angegeben
HALOVÁ et al. (2013)	Irland	364	65 (18 %)	Freiland
LITERÁK und HEJLÍČEK (1993)	Tschechische Republik	3338	169 (5,1 %)	Freiland
		1120	1 (0,01 %)	konventionell
OPSTEEGH et al. (2016)	Deutschland	470	38 – 84 (8 -18 %)	Freiland/ ökologisch
RODRIGUES et al. (2019)	Portugal	170	0 (0 %)	konventionell
		178	10 (5,6 %)	Freiland
SCHARES et al. (2017a)	Deutschland	470	55 (11,7 %)	gesamt
		384	14 (3,7 %)	Freiland (> 1000 Tiere)
		87	41 (47,7 %)	Freiland (< 60 Tiere)
SROKA et al. (2010)	Polen	173	58 (33,5 %)	nicht angegeben
UMIŃSKI et al. (1961)	Polen	84	3 (3,5 %)	nicht angegeben
ZÀSTĚRA et al. (1965)	Tschechische Republik	205	11 (5,3 %)	konventionell

Die Symptome einer klinischen Toxoplasmose beim Huhn reichen von schlechten Allgemeinzustand, Apathie, Fieber, Anorexie, Myokarditis, Festliegen und Neuritis über Enzephalitis bis hin zum Tod der betroffenen Tiere (JONES et al. 1959; KINJO 1961; DUBEY et al. 1993b, 2007b; GOODWIN et al. 1994; WANG et al. 2014, 2015; VIELMO et al. 2019). Zudem treten unspezifische Symptome wie Abfall der Legeleistung auf (BIANCIFIORI et al. 1986). Jedoch werden Hühner im Allgemeinen als resistent gegenüber klinischer Toxoplasmose angesehen (SEDLÁK et al. 2000; DUBEY 2010a). Zahlreiche

Prävalenzstudien von natürlich infizierten und klinisch symptomlosen Hühnern weltweit belegen diesen Umstand (AIGNER et al. 2010; DUBEY et al. 2002, 2004, 2005a, 2005b, 2005d, 2005e, 2005f, 2006a, 2006c, 2008, 2010, 2015; SCHARES et al. 2017a), und es wurden in den vergangenen drei Jahrzehnten nur drei Ausbrüche klinischer Toxoplasmose bei Hühnern beschrieben (GOODWIN et al. 1994; DUBEY et al. 2007b; VIELMO et al. 2019).

In experimentellen Infektionen traten klinische Symptome überwiegend in den frühen Jahren der *T. gondii*-Forschung auf, als sehr hohe Infektionsdosen auf unnatürlichen parenteralen Infektionswegen, wie z.B. intrazerebrale bzw. intrakranielle oder intraperitoneale Injektion von Tachyzoiten oder Gewebezysten, verabreicht wurden (JONES et al. 1959; KINJO 1961; BICKFORD und SAUNDERS 1966). Neuere Studien mit vergleichbaren Infektionswegen bestätigen dieses Bild (WANG et al. 2014, 2015; SCHARES et al. 2017b).

Als Ursachen für die variable Ausprägung klinischer Symptome kommen einerseits in Betracht:

1. Virulenz des inokulierten *T. gondii* Stammes (DUBEY et al. 1993b; WANG et al. 2014; SCHARES et al. 2017b)
2. Art des infektiösen Stadiums (KANETO et al. 1997)
3. Höhe der inokulierten Dosis infektiöser Stadien (BIANCIFIORI et al. 1986; DUBEY et al. 1993b; KANETO et al. 1997; WANG et al. 2015)
4. genetische Variabilität zwischen z.B. Rassen- oder Zuchtlinien (SCHARES et al. 2017b)
5. Alter der Tiere zum Zeitpunkt der Infektion, wobei jüngere Tiere empfänglicher sind (KINJO 1961; WANG et al. 2014).

Andererseits beobachteten andere Autoren keinen Einfluss dieser Faktoren auf die klinische Ausprägung in experimentellen Infektionen (SEDLÁK et al. 2000; CHUMPOLBANCHORN et al. 2009; YAN et al. 2010).

Nach einer Infektion mit *T. gondii* bilden Hühner als Zwischenwirte Zysten in allen bisher untersuchten Geweben aus (Tab. 4). Prävalenzen für den Nachweis von Gewebezysten in natürlich infizierten Hühnern reichen weltweit von 2,5 % bis zu 100 % wie DUBEY (2010a) zusammenfassend zeigt. Die Herzmuskulatur gilt als wichtigste Prädilektionsstelle für *T. gondii* im Gewebe (Tab. 4). Ergebnisse einiger Autoren sehen das Gehirn als bevorzugtes Befallsorgan beim Huhn, wenn auch in diesen Untersuchungen die Herzmuskulatur nicht immer als Zielgewebe berücksichtigt wurde (JONES et al. 1959; DUBEY et al. 1993b, 2005b; KANETO et al. 1997; SCHARES et al. 2017b). Untersuchungen mittels Bioassay gehen auf Grundlage der Anzahl infizierter Mäuse von einer höheren Zystendichte im Herzmuskel im Vergleich zum Gehirn aus (DUBEY et al. 2005a, 2005d, 2005e, 2005f, 2005g, 2007b, 2015). Im Gegensatz dazu unterscheidet sich quantitativ die Menge von *T. gondii* in molekularbiologischen Untersuchungen in Herz und Gehirn nicht (AIGNER et al. 2010; OPSTEEGH et al. 2016).

Tabelle 4: Organverteilung von Zysten von *T. gondii* im Gewebe des Huhns nach natürlicher Infektionsexposition

Autor	*T. gondii* in untersuchten Organen in %				
	Gehirn	Herz	Bein-muskulatur	Brust-muskulatur	Weitere Organe
AIGNER et al. (2010)	84,6 %	80,8 %	n. u.	n. u.	n. u.
DUBEY (1981)	33,3 %	100 %	n. u.	n. u.	n. u.
DUBEY et al. (2004)	0 %	76,9 %	n. u.	0 %	n. u.
DUBEY et al. (2005a)	68,6 %	94,3 %	n. u.	5,7 %	n. u.
DUBEY et al. (2005b)	n. u.	26,7 %	n. u.	n. u.	n. u.
DUBEY et al. (2005d)	33,3 %	100 %	n. u.	33,3 %	n. u.
DUBEY et al. (2005e)	58,3 %	83,3 %	n. u.	25,0 %	n. u.
DUBEY et al. (2005f)	50 %	100 %	n. u.	37,5 %	n. u.
DUBEY et al. (2005g)	70,6 %	94,1 %	n. u.	5,9 %	n. u.
DUBEY et al. (2006a)	66,7 %	70,8 %	n. u.	n. u.	n. u.
DUBEY et al. (2006c)	54,5 %	81,8 %	57,1 %	n. u.	n. u.
DUBEY et al. (2007b)	45,5 %	100 %	72,7 %	n. u.	n. u.
DUBEY et al. (2015)	12,0 %	93,5 %	42,3 %	n. u.	n. u.
FERNANDES et al. (2016)	0 %	16,6 %	n. u.	n. u.	Lunge 16,6 % Leber 0 %
FERREIRA et al. (2018)	100 %	100 %	n. u.	n. u.	n. u.
OPSTEEGH et al. (2016)	n. u.	96,9 %	25,0 %	n. u.	n. u.
SCHARES et al. (2017a)	n. u.	42,6 %	4,9 %	n. u.	n. u.

In der Muskulatur kommen Gewebezysten unterschiedlich häufig vor, wie aus Tabelle 4 ersichtlich ist. Die Nachweisraten in der Muskulatur variieren von 0 % bis zu 72,7 %. Ebenso variabel stellt sich die Gewebezystenverteilung zwischen verschiedenen Muskelgruppen dar, wobei *T. gondii* vermehrt in der Beinmuskulatur nachgewiesen wurde (Tab. 4) (DUBEY 2010a; OPSTEEGH et al. 2016). Überdies befindet sich *T. gondii* häufiger in der Muskulatur vom Oberschenkel als im Unterschenkel bei vergleichbarer Parasitenkonzentration im Gewebe (OPSTEEGH et al. 2016).

Die Zystendichte in der Muskulatur vom Huhn ist im Vergleich zur Herzmuskulatur gering (DUBEY et al. 1993b, 2005a, 2005f, 2007b, 2015; OPSTEEGH et al. 2016). WANG et al. (2015) stellten die Hypothese auf, dass *T. gondii* in Hühnern möglicherweise keine Gewebezysten bildet oder aber die Zystendichte sehr gering ist, nachdem er in histologischen Präparaten verschiedener Organe keine Gewebezysten nachweisen konnte. Andererseits sind in Freilandhühnern ähnlich hohe Nachweisraten von Gewebezysten in Beinmuskulatur wie in Herzmuskulatur beschrieben (DUBEY et al. 2006c). Quantifizierungsdaten von AIGNER et al. (2010) und OPSTEEGH et al. (2016) stützen die Aussage, dass beim Huhn die Parasitenkonzentration in der Muskulatur signifikant niedriger als in Herz und Gehirn ist.

Der Fokus der Forschung zur Geflügeltoxoplasmose liegt auf der Untersuchung von Herz, Gehirn und Muskulatur, aber einige Autoren bezogen weitere Gewebe, wie z.B. Retina, Lunge, Milz, Leber, Ovarien, Eileiter und Muskelmagen, ein (JACOBS et al. 1962; JACOBS und MELTON 1966; DUBEY 1981; BIANCIFIORI et al. 1986; DUBEY et al. 1993b; KANETO et al. 1997; SEDLÁK et al. 2000; YAN et al. 2010; FERNANDES et al. 2016; SCHARES et al. 2017b). Auch dort konnten Gewebezysten in unterschiedlichen Größenordnungen nachgewiesen werden (Tab. 5). Zudem ist *T. gondii* bei Hühnern nicht nur in Geweben nachweisbar, sondern auch in Eiern (KINJO 1961; PANDE et al. 1961; JACOBS et al. 1962; JACOBS und MELTON 1966; KHADEMI et al. 2018).

Einflussfaktoren für die Gewebeverteilung von *T. gondii* beim Huhn sind bisher wenig untersucht. Jüngste Forschungen zeigen die Abhängigkeit der Anzahl positiver Organnachweise und der DNA-Gehalte in den Organen vom Infektionsstamm als auch von der Genetik der infizierten Hühner (SCHARES et al. 2017b). Das inokulierte Infektionsstadium beeinflusst ebenfalls die Anzahl positiver Nachweise, wobei eine Infektion mit Oozysten deutlich häufiger zu positiven Organfunden führt als eine Infektion mit Gewebezysten oder gar Tachyzoiten (KANETO et al. 1997; OPSTEEGH et al. 2016). Dahingegen beeinflusst die Höhe der Infektionsdosis die Gewebeverteilung nicht (BIANCIFIORI et al. 1986; KANETO et al. 1997; YAN et al. 2010).

Ob Gewebezysten von *T. gondii* beim Huhn persistieren, wird in der Literatur kontrovers diskutiert. Nachweise von *T. gondii* in Geweben von Hühnern reichen von drei bis 68 Tagen nach Infektion und sind in Tabelle 5 dargestellt. Nur wenige Studien, wie die von JACOBS und MELTON (1966), konnten *T. gondii* über längere Zeiträume von bis zu zehn Monate in Geweben von Hühnern nachweisen. Im Gegensatz dazu, fanden JONES et al. (1959) vier Wochen p.i. keine Gewebezysten mehr. KINJO (1961) geht ein bis zwei Monate nach Infektion sogar von einer Elimination von *T. gondii* aus Geweben von Hühnern aus.

Tabelle 5: Organverteilung von *T. gondii* im Gewebe vom Huhn nach experimenteller Infektion

Autor	Infektionsstadium Infektionsweg Studienlänge Infektionsstamm	*T. gondii* in untersuchten Organen in %			
		Gehirn	Herz	Muskulatur	Weitere Organe
BIANCIFIORI et al. (1986) *	Oozysten per os 7-40 Tage unbekannt	100 %	100 %	n. u.	Milz 0 - 66,7 % Leber 66,7 % Lunge 33,3 - 66,7 % Ovar 0 % Kropf 0 %
DUBEY et al. (1993b)	Oozysten per os 15-68 Tage GT1, ME49	100 %	60 %	Beinmuskulatur 40 % Brustmuskulatur 0 %	Leber 0 %
JACOBS und MELTON (1966)	Gewebezysten s.c. per os, i.v. 3-10 Monate H-44, C37	75,0 %	n. u.	Beinmuskulatur 71,4 % Brustmuskulatur 64,3 %	Ovar 64,3 % Eileiter 78,6 % Niere 40 % Muskelmagen 75 % Darm 64,3 %
JONES et al. (1959)	Tachyzoiten i.c. s.c. 3-27 Tage RH, 113-CE, NR-139	57,9 %	n. u.	n. u.	Leber 26,3 % Lunge 21,1 %
KANETO et al. (1997) *	Tachyzoiten i.v. 30 – 35 Tage P	16,7 %	16,7 %	Skelettmuskulatur 16,7 %	Niere 0 % Leber 16,7 % Lunge 0 % Darm 16,7 % Drüsenmagen 16,7 % Milz 33,3 % Pankreas 16,7 % Retina 0 %

Tabelle 5, ff: Organverteilung von *T. gondii* im Gewebe vom Huhn nach experimenteller Infektion

Autor	Infektionsstadium Infektionsweg Studienlänge Infektionsstamm	*T. gondii* in untersuchten Organen in %			
		Gehirn	Herz	Muskulatur	Weitere Organe
KANETO et al. (1997) *	Oozysten per os 30-35 Tage P	88,9 %	11,1 %	Skelettmuskulatur 0 %	Niere 11,1 % Leber 11,1 % Lunge 11,1 % Darm 11,1 % Drüsenmagen 22,2 % Milz 22,2 % Pankreas 44,4 % Retina 44,4 %
	Gewebezysten per os 30-35 Tage P	50,0 %	33,3 %	Skelettmuskulatur 0 %	Niere 16,7 % Leber 0 % Lunge 0 % Darm 0 % Drüsenmagen 0 % Milz 16,7 % Pankreas 0 % Retina 16,7 %
OPSTEEGH et al. (2016)	Tachyzoiten i.v. 35-70 Tage ME49, CZ-Tiger, NED	0 %	0 %	Oberschenkel-muskulatur 0 % Unterschenkel-muskulatur 0 % Brustmuskulatur 0 %	n. u.
	Oozysten per os 35 Tage ME49, CZ-Tiger, NED	79,5%	87,2 %	Oberschenkel-muskulatur 61,5% Unterschenkel-muskulatur 52,6% Brustmuskulatur 53,8 %	n. u.

Tabelle 5, ff: Organverteilung von *T. gondii* im Gewebe vom Huhn nach experimenteller Infektion

Autor	Infektionsstadium Infektionsweg Studienlänge Infektionsstamm	*T. gondii* in untersuchten Organen in %			
		Gehirn	Herz	Muskulatur	Weitere Organe
OPSTEEGH et al. (2016)	Gewebezysten per os 35 Tage ME49, CZ-Tiger, NED	56,3 %	56,3 %	Oberschenkel-muskulatur 25,0 % Unterschenkel-muskulatur 25,0 % Brustmuskulatur 25,0 %	n. u.
SCHARES et al. (2017b)	Tachyzoiten i.p. 31 Tage B6 H6	44,7 %	n. u.	n. u.	Lunge 34,2 %
	Tachyzoiten i.p. 31 Tage A7	73,0 %	n. u.	n. u.	Lunge 56,8 %
	Tachyzoiten i.p. 31 Tage 2C10	92,3 %	n. u.	n. u.	Lunge 74,4 %
SEDLÁK et al. (2000)	Oozysten per os 28 Tage K7	20,0 %	60,0 %	Beinmuskulatur 60,0 %	Milz 30 % Leber 0 %
YAN et al. (2010)	Tachyzoiten i.v. 21-28 Tage QHO	20 %	25 %	n. u.	Leber 10 % Lunge 25 % Milz 20 % Augen 20 %

*Ergebnisse entsprechen % der positiven Gruppen, da nur gruppenweise und nicht für Einzeltiere Organe untersucht wurden

2.3.2. *T. gondii*-Infektion der Pute

Natürliche Infektionen mit *T. gondii* bei Puten (*Meleagris gallopavo*) sind weit verbreitet, variieren jedoch deutlich in ihrer Häufigkeit. So wiesen HARFOUSH und TAHOON (2010) und EL-MASSRY et al. (2000) in Puten aus Ägypten Seroprävalenzen von 29,4 % bis 59,5 % nach. Serologische Untersuchungen durch KOETHE et al. (2011) von 1913 Puten aus kommerzieller Innenhaltung in Deutschland erbrachten eine Prävalenz von 18,4 %, welche bei einigen Betrieben bis zu 77,1 % betrug. GHORBANI et al. (1990) konnten bei 24 % der untersuchten Puten Antikörper gegen *T. gondii* detektieren sowie bei 16 % der Tiere *T. gondii* aus Gewebe isolieren. In einer weiteren Studie aus dem Iran waren 89,8 % der Puten seropositiv und in 61,6 % der Tiere war DNA von *T. gondii* in verschiedenen Geweben nachweisbar (SARKARI et al. 2014). In 76,6 % der serologisch untersuchten Puten im Irak fand BUTTY (2009) *T. gondii*-Antikörper. Eine ähnlich hohe Seroprävalenz (71 %) wiesen LINDSAY et al. (1994) in Wildputen in den Vereinigten Staaten nach. In 50 % der Gewebeproben gelang zudem die direkte Isolation von *T. gondii*. Im Gegensatz dazu befundeten QUIST et al. (1995) nur 10 % und CERQUEIRA-CÉZAR et al. (2019) nur 30 % der untersuchten Wildputen in den Vereinigten Staaten als seropositiv. In Israel zeigten SALANT et al. (2016) eine Seroprävalenz von 8,9 % in kommerziell gehaltenen Puten. Auch SÁ et al. (2016) wiesen ähnlich niedrige Seroprävalenzen (11 %) in Puten aus Familienbetrieben nach. Dagegen konnten weder BURRIDGE et al. (1979) in Wildputen aus Florida noch BÁRTOVÁ et al. (2009) in kommerziell gehaltenen Puten in der Tschechischen Republik Antikörper gegen *T. gondii* detektieren.

Experimentelle Studien zeigen, dass die Serokonversion der Pute durch das aufgenommene Infektionsstadium und die Infektionsdosis beeinflusst wird. Infektionen mit Oozysten rufen bei der Pute eine stärkere und belastbarere Immunantwort als eine unter natürlichen Umständen nicht vorkommende parentale Infektion mit Tachyzoiten hervor (HOTOP et al. 2014; OPSTEEGH et al. 2016). Dieser Sachverhalt spiegelt sich bei mit Tachyzoiten infizierten Tieren in frühzeitig absinkenden und nicht mehr nachweisbaren Antikörpertitern (HOTOP et al. 2014) oder in geringeren Nachweisraten wider (OPSTEEGH et al. 2016). Über den Einfluss der Infektionsdosis auf die Immunantwort herrscht in der Literatur Uneinigkeit.
Höhere Infektionsdosen führen bei MAKSIMOV et al. (2018) zu einer stärkeren Antikörperbildung, allerdings konnten Untersuchungen von OPSTEEGH et al. (2016) dies nicht bestätigen. MAKSIMOV et al. (2018) verglichen die Auswirkung der Infektion mit den klonalen Gruppen I, II und III auf die IgY-Level infizierter Tiere und stellte keine Unterschiede zwischen den Stämmen fest.

Trotz teilweise sehr hoher, nachgewiesener Seroprävalenzen gelten Puten ebenso wie Hühner als relativ resistent gegenüber klinischer Toxoplasmose (DUBEY et al. 1993a), welche bisher ausschließlich bei Wildputen beschrieben ist. So bestätigen HOWERTH und RODENROTH (1985), QUIST et al. (1995) und SCHULTE (1954) pathohistologisch das Vorliegen einer Toxoplasmose bei

verendeten Wildputen. In den wenigen derzeit dokumentierten experimentellen Infektionen von Puten mit *T. gondii* wurden auch bei sehr hohen Infektionsdosen mit bis zu 10 Millionen sporulierten Oozysten bzw. Tachyzoiten verschiedener Stämme keine klinischen Symptome beschrieben (DUBEY et al. 1993a; SEDLÁK et al. 2000; BANGOURA et al. 2013; ZÖLLER et al. 2013).

Die Zystenverteilung stellt sich bei Puten im Vergleich zu anderen Spezies variabler dar, wobei auch bei dieser Spezies eine Tendenz zu Herz, Muskulatur und Gehirn als Prädilektionsstellen von *T. gondii* Gewebezysten erkennbar ist (Tab. 6). Natürlich infizierte Wildputen wiesen in 50 % bzw. 25 % der Herzmuskelproben im Maus-Bioassay infektiöse Gewebezysten auf, wobei Gehirnproben nicht untersucht wurden (LINDSAY et al. 1994, CERQUEIRA-CÉZAR et al. 2019). DUBEY et al. (1993a) fanden bei experimentell infizierten Puten in allen untersuchten Herzproben, aber in keiner der Gehirn- und Leberproben, Gewebezysten. Zusätzlich zur Herzmuskulatur war auch Brust- (40 %) und Beinmuskulatur (80 %) positiv und es wurde daher postuliert, dass Skelettmuskulatur das Prädilektionsgewebe für *T. gondii* bei Puten ist. Im Gegensatz dazu war bei den von BANGOURA et al. (2013) und SARKARI et al. (2014) untersuchten Puten am häufigsten das Gehirn gefolgt von Herz- und Skelettmuskulatur befallen. Untersuchungen von OPSTEEGH et al. (2016) hinsichtlich der Parasitenlast im Gewebe stützen die These von Gehirn und Herz als Prädilektionsgewebe für *T. gondii* bei Puten, wobei eine signifikant höhere Parasitenlast in Gehirngewebe im Gegensatz zu Herz und anderer Muskulatur gezeigt wurde. Andere Untersuchungen kommen zu anderen Ergebnissen. So stellte sich bei ZÖLLER et al. (2013) Leber noch vor Brust-, Herzmuskel und Gehirn als Prädilektionsorgan experimentell infizierter Puten dar. HOWERTH und RODENROTH (1985) und QUIST et al. (1995) wiesen *T. gondii* in natürlich infizierten und verendeten Einzeltieren, neben den klassischen Prädilektionsstellen, auch in anderen Organen wie Milz, Lunge, Niere, Nebenniere, Speiseröhre, Pankreas und Dickdarm nach.

Die Variabilität der Organverteilung von Gewebezysten in Puten lässt den Schluss zu, dass Faktoren existieren, welche die Organverteilung beeinflussen. BANGOURA et al. (2013) und ZÖLLER et al. (2013) untersuchten verschiedene Infektionsdosen von Oozysten und Tachyzoiten, konnten aber keinen Einfluss der Dosis auf den Nachweis der *T. gondii*-DNA in verschiedenen Geweben erkennen. Andererseits beeinflusste in diesen Untersuchungen das inokulierte Entwicklungsstadium, Tachyzoiten oder Oozysten, die Verteilung in einzelnen Geweben. OPSTEEGH et al. (2016) konnten diese Abhängigkeit zwar nicht bestätigen, zeigten jedoch, dass das inokulierte Entwicklungsstadium einen Einfluss auf die Nachweishäufigkeit hat, wobei nach Infektionen mit Gewebezysten oder Oozysten in signifikant mehr Tieren und Geweben *T. gondii*-DNA im Vergleich zu Infektionen mit Tachyzoiten nachgewiesen wurde. Der Infektionsstamm hat bei Puten keinen Einfluss auf die Gewebeverteilung (BANGOURA et al. 2013).

Zur Persistenz von *T. gondii* in Putengewebe gibt es bislang wenige Studien. Sie reicht in den wenigen publizierten Untersuchungen von zwölf bis 84 Tage nach der Infektion (SIMITCH et al. 1965; DUBEY et al. 1993a; SEDLÁK et al. 2000; BANGOURA et al. 2013; ZÖLLER et al. 2013).

Tabelle 6: Organverteilung von *T. gondii* im Gewebe der Pute nach natürlicher Exposition oder experimenteller Infektion

Autor	Infektionsart Infektionsstadium Infektionsweg Studienlänge Infektionsstamm	*T. gondii* in untersuchten Organen in %			
		Gehirn	Herz	Muskulatur	Weitere Organe
CERQUEIRA-CÉZAR et al. (2019)	natürlich	n. u.	25,0 %	Skelettmuskulatur 100 % (1/1)	n. u.
LINDSAY et al. (1994)	natürlich	n. u.	50,0 %	n. u.	n. u.
SARKARI et al. (2014)	natürlich	33,3 %	n. u.	Halsmuskulatur 29,6 % Zungen-muskulatur 31,5 %	
BANGOURA et al. (2013)	experimentell Oozysten per os 84 Tage ME49, DX, Hannover 1	47,2 %	22,2 %	Oberschenkel-muskulatur 25,0 %, Unterschenkel-muskulatur 22,2 % Brustmuskulatur 8,3 %	Leber 5,6 % Drüsenmagen 16,7 % Muskelmagen 16,7 % Darm 16,7 % Lunge 13,9 % Milz 8,3 % Niere 5,6 % Hoden 5,6 % Pankreas 2,8 %
DUBEY et al. (1993a)	experimentell Oozysten per os 12-62 Tage ME49	0 %	100 %	Beinmuskulatur 80,0 % Brustmuskulatur 40,0 %	Leber 0 %

Tabelle 6, ff: Organverteilung von *T. gondii* im Gewebe der Pute nach natürlicher Exposition oder experimenteller Infektion

Autor	Infektionsart Infektionsstadium Infektionsweg Studienlänge Infektionsstamm	*T. gondii* in untersuchten Organen in %			
		Gehirn	Herz	Muskulatur	Weitere Organe
OPSTEEGH et al. (2016)	experimentell Oozysten per os 56 Tage ME49, CZ-Tiger, NED	90,0 %	85,0 %	Oberschenkel-muskulatur 77,5 % Unterschenkel-muskulatur 85,0 % Brustmuskulatur 60,0 %	n. u.
OPSTEEGH et al. (2016)	experimentell Gewebezysten per os 56 Tage ME49, CZ-Tiger, NED	94,4 %	83,3 %	Oberschenkel-muskulatur 47,1 % Unterschenkel-muskulatur 61,1 % Brustmuskulatur 22,0 %	n. u.
OPSTEEGH et al. (2016)	experimentell Tachzoiten i.v. 28 – 112 Tage ME49, CZ-Tiger, NED	4,3 %	4,2 %	Oberschenkel-muskulatur 0 % Unterschenkel-muskulatur 0 % Brustmuskulatur 0 %	n. u.
SEDLÁK et al. (2000)	experimentell Oozysten per os 28 Tage K7	60,0 %	100 %	Beinmuskulatur 40,0 %	Leber 40,0 % Milz 20,0 %

Tabelle 6, ff: Organverteilung von *T. gondii* im Gewebe der Pute nach natürlicher Exposition oder experimenteller Infektion

Autor	Infektionsart Infektionsstadium Infektionsweg Studienlänge Infektionsstamm	*T. gondii* in untersuchten Organen in %			
		Gehirn	Herz	Muskulatur	Weitere Organe
ZÖLLER et al. (2013)	experimentell Tachyzoiten i.v. i.m. 84 Tage ME49	6,7 %	20,0 %	Beinmuskulatur 16,7 % Brustmuskulatur 26,7 %	Leber 43,3 % Darm 16,7 % Niere 13,3 % Pankreas 13,3 % Muskelmagen 13,3 % Drüsenmagen 10,0 % Hoden 7,4 % Lunge 6,7 % Milz 3,3 %

3. Publikationen

3.1. Publikation 1

Persistence of *Toxoplasma gondii* tissue stages in poultry over a conventional fattening cycle.

Geuthner A-C, Koethe M, Ludewig M, Pott S, Schares G, Daugschies A, Bangoura B.
Persistence of *Toxoplasma gondii* tissue stages in poultry over a conventional fattening cycle.
Parasitology 2014; 141(11):1359-64. doi: 10.1017/S003118201400078X. Epub 2014 Jun 16.

Erklärung zum Eigenanteil

Die Versuchsplanung erfolgte in enger Zusammenarbeit mit Frau Dr. Berit Bangoura.

Die tierexperimentellen Arbeiten wurden meinerseits eigenständig durchgeführt. Darunter fallen die Gewinnung der zur Infektion der Versuchstiere benötigten Tachyzoiten mittels Zellkultur und deren Pflege, die Infektion der Versuchstiere, die Blutentnahme, Serumgewinnung, Schlachtung der Versuchstiere, Präparation und Homogenisierung der untersuchten Organe. Bei der Präparation und Homogenisierung der Organe erhielt ich Unterstützung durch die Mitarbeiter des Institutes für Parasitologie (Herr Frank Stöckel, Frau Manja Etzold, Frau Birte Zöller, Frau Juliane Hintzen, Frau Irene Malkwitz, Frau Eva-Maria Mäßig, Frau Linda Katzer, Frau Franziska Göhring, Frau Cora Delling, Frau Ira Dresely). Die Betreuung der Versuchstiere erfolgte durch die Tierpfleger (Herr Rene Schuhmacher, Frau Marion Fritsche) des Institutes für Parasitologie.
Die DNA-Extraktion und PCR-Analysen sowie deren Auswertung wurde eigenständig durchgeführt.

Die Untersuchung der Seren erfolgte durch Herrn Dr. Martin Köthe, Herrn Dr. Pavlo Maksimov und Herrn Dr. Gereon Schares.

Die statistische Auswertung der Daten führte ich eigenständig durch, ebenso die Erstellung des Manuskriptes.

Die Revision des Manuskriptes erfolgte in Zusammenarbeit aller genannten Autoren.

Persistence of *Toxoplasma gondii* tissue stages in poultry over a conventional fattening cycle

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SUMMARY

Toxoplasma gondii is a widely spread protozoon in humans, mammals and poultry. Regarding the latter, nothing is known yet about the duration of *T. gondii* persistence and distribution over a conventional fattening cycle of turkeys and chickens. Twenty-four turkeys and 12 broiler chickens were infected intravenously with 1×10^6 *T. gondii* tachyzoites (strain NED). Serum antibody levels were determined weekly by ELISA (turkeys) or immunofluorescent antibody test (chickens). Turkeys were slaughtered at 4, 8, 12 and 16 weeks post-infection (p.i.), and chickens 5 or 10 weeks p.i. ($n = 6$ per group). Sixteen different tissue samples per bird were analysed for *T. gondii* by PCR. All infected animals showed seroconversion. In turkeys, 15·9% of all samples were tested positive for *T.-gondii*-DNA. Among the edible tissues (drumstick, thigh, breast muscle, heart, liver and gizzard) 7·8% tested positive. Among poultry slaughtered after different periods of time after infection no significant differences ($P > 0{\cdot}05$) regarding the number of positive samples were observed. Only 4 out of 192 samples (2·1%) from infected chickens contained detectable *T. gondii* DNA.The PCR findings suggested that *T. gondii* may persist in poultry. Particularly in turkey it was shown that edible tissues stay infected for at least 16 weeks p.i. which indicates a potential risk for consumers of undercooked turkey meat whereas chickens appear less susceptible to *T. gondii* infection.

Key words: *Toxoplasma gondii*, chickens, turkeys, muscle, PCR.

INTRODUCTION

Toxoplasma gondii is a widespread protozoan in warm-blooded animals including humans and birds (Tenter *et al*. 2000). Felids are the definitive hosts and shed oocysts after infection while almost all warm-blooded animals and humans are intermediate hosts and develop tissue cysts in several organs, especially in neural and muscular tissue (Tenter *et al*. 2000; Dubey, 2010*a*).

The main risk factor for humans to become infected with *T. gondii* is the consumption of rare or undercooked meat (Cook *et al*. 2000). Typically, poultry meat is consumed well done. But as part of a changing lifestyle more and more poultry is consumed as raw sausages or ham. According to Dubey (1997) and Pott *et al*. (2012) tissue cysts are still viable after brief salting or curing and there is a risk of infection with *T. gondii* by consumption of fermented raw sausages.

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An average conventional fattening cycle in turkey husbandry lasts up to 16 weeks in female turkeys and 22 weeks in male turkeys (Krautwald-Junghanns *et al*. 2009). A short fattening period takes 9–12 weeks for both sexes. Usually, chickens are fattened for 35–42 days in conventional husbandry whereas organic fattening lasts up to 10 weeks. Livestock kept in free-range husbandries has a significantly increased risk of infection with *T. gondii* (Kijlstra *et al*. 2004; Maksimov *et al*. 2011) compared with livestock raised indoors. Fowl is considered to be one of the most important intermediate hosts in the life cycle of *T. gondii* (Dubey, 2010*b*), but little is known about the duration of persistence of *T. gondii* in tissue of poultry. Zöller *et al*. (2013) demonstrated a persistence of *T. gondii* in turkeys over a period of 12 weeks but to our knowledge the persistence of *T. gondii* in chicken has not been investigated. There is little information as to whether chickens are able to eliminate a *T. gondii* infection, as has been observed for some of the other intermediate host species (Beverley *et al*. 1977).

The aim of this study was to investigate the duration of persistence of *T. gondii* in turkeys and chickens and to estimate the risk of finding

T. gondii-positive meat and organs at the time of slaughtering according to the duration of conventional fattening cycles. Additionally, the distribution of *T. gondii* in tissues depending on time of slaughtering was analysed.

MATERIALS AND METHODS

Parasites

For infection of poultry the *T. gondii* type III strain NED (Howe and Sibley, 1995) was used. Tachyzoites for infection of the animals were grown in VERO cells cultivated with IMDM medium (PAA, Pasching, Austria), 5% foetal calf serum, 1% penicillin/streptomycin and 1% amphotericin B as described before (Zintl *et al*. 2009) at 37 °C, 5% CO_2. To harvest the parasites, supernatant of cell culture was centrifuged at 2000 × *g* for 5 min. The pellet was resolved in 1 mL PBS solution and tachyzoites were counted using a Neubauer chamber.

Animals and husbandry

In vivo trials were performed at the Institute of Parasitology, University Leipzig, Germany. Conventionally reared and commercially available turkeys (BUT B.I.G. 6, $n = 30$) and chickens (ISA JA 757, $n = 18$) were kept on a bedding consisting of wood shavings. Turkeys were fed *ad libitum* with poultry feed for pet birds without anticoccidials (deuka Wild-und Ziergeflügelfutter, Deutsche Tiernahrung Cremer, Duesseldorf, Germany) because no conventional starter and grower feed for turkeys without anticoccidials was available. The feed was enriched with powdered milk and supplements (Korvimin ZVT + Reptil, WDT, Garbsen, Germany) to obtain the recommended nutritional values for turkeys. Chickens received conventional starter and grower feed for chickens without anticoccidials *ad libitum*. Animals had free access to water.

Infections and study design

After a raising period of 8 weeks turkeys were divided into five groups. Four groups were infected and slaughtered at 4, 8, 12 or 16 weeks post-infection (p.i.), and one group remained uninfected as negative control. Regarding chickens, 1-week-old chicks were divided into three groups, two of which were infected and slaughtered 5 or 10 weeks p.i., to simulate the conventional and organic fattening time line, and the remaining group again served as uninfected negative control.

Animals were infected each with 1×10^6 tachyzoites of the *T. gondii* NED strain in approximately 0·5 mL of a sterile isotonic saline solution (B. Braun Melsungen AG, Melsungen, Germany) intravenously into the wing vein (*Vena cutanea ulnaris*).

Serological examinations

Blood samples were drawn from the wing vein once before and weekly after infection for *T. gondii* antibody screening (IgY fraction in both bird species) and stored at −20 °C until testing. Sera of turkeys were analysed with a kinetic ELISA (KELA) as previously described (Koethe *et al*. 2011). Briefly, microtitre plates were coated with 0·13 μg GRA7/GRA8 antigen mix (1:1) in coating buffer containing 0·1 M $NaHCO_3$ buffer (pH 9·6), 1·69 mM SDS and 1·9% 2-mercaptoethanol and subsequently frozen until use. Coated plates were thawed and washed ($4 \times 100\,\mu L\ well^{-1}$ PBS-Tween 0·05%) and blocked with $200\,\mu L\ well^{-1}$ 5% skim milk for 1 h at 37 °C. After washing, $100\,\mu L\ well^{-1}$ 1:50 diluted sera were incubated for 1 h at 37 °C and after another washing $100\,\mu L\ well^{-1}$ 1:1000 diluted detection antibody (goat-anti-turkey IgY-HRP) was incubated for 30 min at ambient temperature. After a last washing step, $100\,\mu L\ well^{-1}$ TMB substrate was added and after 105 s plates were read on an ELISA reader (SpectraMax 340PC384, Molecular Devices, Germany). Kinetic development of signal was recorded by reading every 55 s for, in total, four times. The resulting slope of increasing signal was used by the reader software (SoftMax Pro v5, Molecular Devices) for calculating KELA values. Four standard sera were included in every plate to reduce plate-to-plate variances by applying linear regression-based recalculation of obtained values.

Chicken sera were shipped to the Friedrich-Loeffler-Institut, Wusterhausen and processed by an immunofluorescent antibody test (IFAT) as described by Maksimov *et al*. (2011) but with minor modifications. Ten μL of a suspension of cell culture-derived *T. gondii* RH strain tachyzoites ($5 \times 10^6\ mL^{-1}$) in PBS were used to sensitize IFAT slide wells. Slides were air-dried and stored frozen at −20 °C until used. The slides were fixed with ice-cold acetone for 10 min and then incubated in PBS for 10 min. Chicken sera were titrated in PBS in two-fold steps starting at a dilution of 1:25. Anti-chicken IgY (H + L) produced in goat and coupled to FITC [Rockland Immunochemicals Inc., Gilbertsville, USA] diluted 1:50 in PBS, 0·2% Evans Blue was used to detect primary antibodies. The slides were examined using an Axiovert fluorescence microscope (AHBT3, Olympus, Hamburg, Germany). Only complete peripheral fluorescence of the tachyzoite was considered specific. A titre of 100 was used as the positive cut-off titre.

Sample processing

Slaughtering of the animals was performed after anaesthesia with ketamine ($25\ mg\ kg^{-1}$) and xylazine ($1\ mg\ kg^{-1}$) intramuscularly. The animals were dissected and tissue samples from 16 different

locations were taken including edible parts (heart, breast muscle, drumstick, thigh muscle, liver, gizzard) that may be purchased for human consumption and non-edible parts (glandular stomach, pancreas, spleen, kidneys, brain, retina, gonads, intestine, lung, bone marrow). Directly after slaughtering or after storage at −20 °C, samples were homogenized with commercial household blenders (La Moulinette, Tefal Groupe SEB, Offenbach, Germany) or mortar and pestles, except muscles where approximately 300 g sample material from different locations was taken. All homogenized samples were stored in microcentrifuge tubes at −20 °C until further processing.

DNA extraction was performed from 25 mg tissue per sample with the QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany) following the manufacturers instructions. After no more than three samples from infected birds at least one sample of the negative control group was handled. This order was kept up through the whole processing including PCR and gel electrophoresis as control of possible contamination. Subsequently, DNA content of samples was measured using a spectrophotometer (Nanodrop 2000*c*, Thermo Scientific, Waltham, MA, USA). 80–200 ng template DNA was used per PCR reaction. A positive control consisting of DNA from cell culture-derived ME49 tachyzoites and a negative control (aliquot of currently used DNA elution buffer) were carried along in every PCR batch. The PCR reaction was performed as a direct PCR followed by a nested PCR as described by Zöller *et al*. (2013). The mastermix for a direct PCR reaction with 25 μL contained 0·5 U GoTaq® Flexi Polymerase and 1 × GoTaq® Flexi Buffer (Promega GmbH, Mannheim, Germany), 200 μM of each dNTP (Fermentas, St. Leon Rot, Germany), 3 mM $MgCl_2$, 0·4 μM of each primer Tg1 (5′-AAA AAT GTG GGA ATG AAA GAG-3′) and Tg2 (5′-ACG AAT CAA CGG AAC TGT AAT-3′). For nested PCR the same assembled mastermix was used with the primers Tgnested1 (5′-CGC TAA TGT GTT TGC ATA GG-3′) and Tgnested2 (5′-GGC ACG TCT CTT GTT CTT CT-3′), 1·5 mM $MgCl_2$ and 2·5 μL of the direct PCR product as template. Both PCRs were performed in the iCycler® Thermal Cycler (Bio-Rad, Hercules, CA, USA) or the M × 3000P®Real-Time PCR System (Stratagene/Agilent Technologies, La Jolla, USA) under the following cycling conditions: preheating (2 min at 94 °C), amplification for 35 cycles (direct PCR) or 25 cycles (nested PCR) of 1 min at 94 °C, 40 s at 55 °C and 1 min at 72 °C and one final extension step of 5 min at 72 °C.

Approximately 10 μL of the nested PCR products were examined with gel electrophoresis on a 1·5% agarose gel, which was stained with ethidium bromide. DNA bands were visualized with UV-light in positive samples at 375 bp fragment length.

Statistics

Statistical analysis was carried out with the IBM SPSS statistics Version 20 software package (IBM, New York, USA).

The Kolmogorov–Smirnov test was performed to test data for normal distribution. All data were non-normally distributed and were therefore further analysed by Kruskal–Wallis test and Friedman test. The Spearman rank correlation test was performed to explore possible correlations. Group differences and correlations with P values of less than 0·05 were defined as statistically significant.

RESULTS

Experimental infections of turkeys

None of the turkeys showed symptoms of clinical toxoplasmosis throughout the entire experiment. Two of the 24 infected turkeys had to be euthanized due to leg and wing problems at 13 and 14 weeks p.i., respectively.

Serology. All infected animals showed a seroconversion (see Fig. 1, Table S1). Eleven turkeys already had a detectable antibody level at 1 week p.i. The remaining 13 animals seroconverted by 2 weeks p.i. At 9weeks p.i. antibody levels of the 12 remaining animals were decreasing and until slaughtering the levels of 3 animals fell below the cut-off. No seroconversion of the uninfected control was detected over the entire period of the experiment.

Direct parasite detection (PCR). Sixty-one of 384 tissue samples (15·9%) contained detectable parasite DNA. At every time of slaughtering positive samples were found. There were no significant differences between the proportions of positive findings in infected turkey slaughtered at different time points p.i. ($P > 0·05$; see Fig. 2). In 30 edible parts (7·8%) *T. gondii* was observed. Again no significant differences were observed when the proportion of positive findings in turkeys slaughtered at different time points after infection was compared ($P > 0·05$). Twenty of the 24 infected turkeys (including the two deceased animals) showed *T. gondii* DNA in at least one sampled part, 14 of these in at least one edible part. Positive findings were observed distributed evenly over the examination time points (see Fig. 3).

All parts except the kidneys tested positive at least once (see Fig. 4, Table S1). Most frequently, breast (29·2%) and thigh muscles (29·2%) tested positive followed by drumsticks, intestine, bone marrow (25% each) and brain (20·8%) though no body part was statistically significantly more often DNA positive than other parts ($P > 0·05$). Correlation of *T. gondii* detectability in different muscular tissues was tested and a statistically significant correlation between

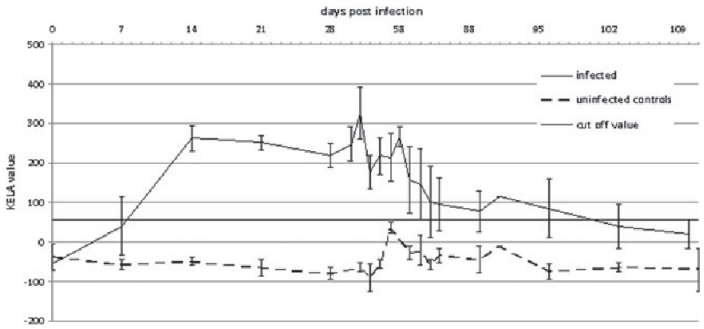

Fig. 1. Mean antibody levels of infected and uninfected turkeys determined in a kinetic ELISA (error bars indicate S.D.).

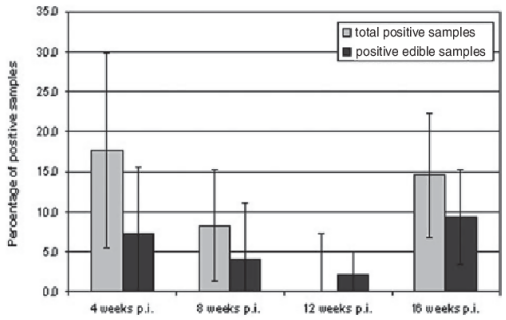

Fig. 2. Mean proportion of positive samples per turkey stratified for the length of the period between infection and slaughter (4, 8, 12 weeks p.i., $n = 96$; group 16 weeks p.i., $n = 64$, error bars indicate S.D.).

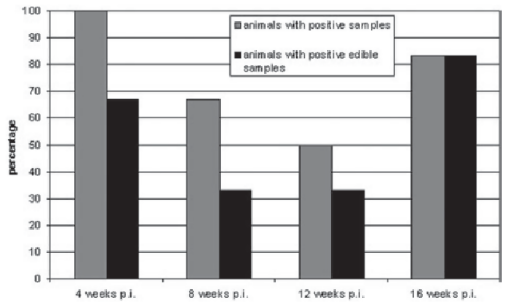

Fig. 3. Number of positive tested turkeys depending on time of slaughtering separately by animals positive in at least one organ (grey) and animals positive in at least one edible organ (black).

thigh and breast muscle positivity could be found (Spearman rho = 0·542, $P = 0·009$).

Experimental infections of chickens

None of the chickens showed signs of clinical toxoplasmosis.

Serology. Every infected chicken seroconverted during the study (see Fig. 5, Table S2). Eight chickens of the infected group showed a seroconversion at 1 week p.i. The other four chickens had a detectable antibody titre at 2 weeks p.i. Antibody titres of all chickens stayed on a high level until slaughtering, apart from one animal where the titre dropped below the cut-off at 4 weeks p.i. The uninfected negative control did not seroconvert over the entire experiment.

Direct parasite detection (PCR). Over both examination time points, a total of 4 out of 192 (2·1%) samples from infected chickens tested positive for *T. gondii* DNA by PCR (see Table S2). Two of the four positive parts were edible parts (1·0% of all organs). The positive samples were: heart, retina, pancreas and drumstick of four different positive chickens (33·3% of all infected chickens). Three positive tested parts (retina, pancreas, drumstick) were found at 5 weeks p.i. One positive part (heart) belonged to a chicken of the 10 weeks p.i. group.

DISCUSSION

None of the infected birds showed symptoms related to clinical toxoplasmosis. This is in accord with observations from previous studies in chickens and turkeys (Biancifiori *et al.* 1986; Dubey *et al.* 1993*b*; Kaneto *et al.* 1997; Sedlak *et al.* 2000; Chumpolbanchorn *et al.* 2009; Zöller *et al.* 2013) where subclinical toxoplasmosis in experimentally infected birds was seen. However, Dubey *et al.* (1993*b*) described cases of death due to acute toxoplasmosis in chickens after inoculation of a high dose (100 000 oocysts) of the *T. gondii* GT1 strain. Fatal toxoplasmosis has been described in naturally infected wild turkeys (Howerth and Rodenroth, 1985; Quist *et al.* 1995). Differences in infectivity and pathogenicity in avian toxoplasmosis could be explained by the use or occurrence of *T. gondii* strains of variable virulence and infective doses. Nonetheless, our study confirms that gallinaceous birds have a high tolerance with respect to high infection doses of *T. gondii*.

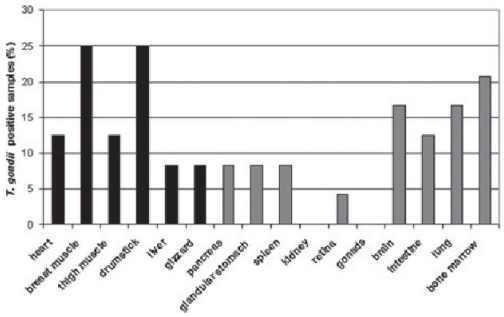

Fig. 4. Positive organ samples over all infected turkeys ($n = 24$) plotted by edible organs (black) and other organs (grey).

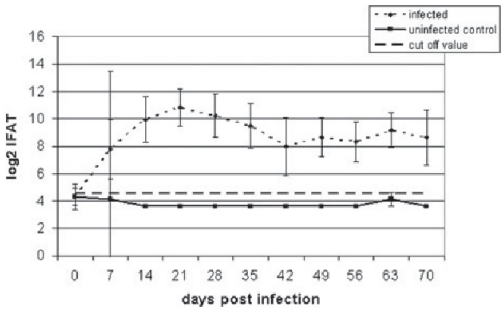

Fig. 5. Mean antibody level of infected and uninfected chickens in IFAT. Titres are provided as $\log_2$ values of the IFAT titres (error bars indicate S.D.).

We detected measurable antibody levels as soon as day 7 after infection in both species which matches several previous reports (Dubey *et al.* 1993*a*, *b*; Sedlak *et al.* 2000; Chumpolbanchorn *et al.* 2009; Zöller *et al.* 2013). Also the observed decrease of antibody levels near or beyond the cut-off was described before (Dubey *et al.* 1993*a*; Sedlak *et al.* 2000; Martinez-Carrasco *et al.* 2004; Vitaliano *et al.* 2010) but did not coincide with elimination of the parasite burden since parasite DNA was still detected in turkeys at 12 and 16 weeks p.i. at unaltered levels (seen as number of positive tested tissues) compared with earlier time points. Therefore, seroprevalence studies probably under-report the presence of *T. gondii* in turkey flocks since antibody levels may fall beyond the detection limit about 9 weeks after infection (depending on the detection method). Tissue stage persistence in animals used for meat production has been studied previously. Dubey *et al.* (1984) and Dubey (1988) found a persistence of *T. gondii* in meat of pigs at least 171–875 days after infection with oocysts. In beef cattle tissue cysts persisted up to 1191 days after infection with *T. gondii* oocysts (Dubey and Thulliez, 1993). In the present study, *T. gondii* was detected by PCR, thus the type of stages present in the tissues cannot be further described. The aim of the study was to determine the ability of the parasite to persist in a range of different tissues over a certain period of time equivalent to common fattening cycles. We demonstrated the persistence of *T. gondii* in turkeys up to 16 weeks after infection, i.e. over a whole fattening cycle, assuming infection during the first weeks of life. This fact indicates that an infection of turkeys with *T. gondii* possibly leads to *T. gondii*-positive edible tissues and a potential risk for infection of the consumer cannot be excluded. The close correlation found between *T. gondii* presence in thigh and breast muscle indicates that often several muscles are parasitized simultaneously in affected turkeys.

Though Dubey *et al.* (1993*a*) found no infectious tissue cysts in brain and liver of turkeys, we found liver and brain of the intravenously infected turkeys to be positive for *T. gondii* DNA as had been shown previously in oocysts-infected turkeys by Bangoura *et al.* (2013).

The infection of poultry with tachyzoites was practiced before in other studies (Drobeck *et al.* 1953; Kaneto *et al.* 1997) to describe effects of the parasite in an *in vivo* infection model, though this is not the natural way of infection. We have chosen this route of infection in the present trial to benefit from *in vitro* propagation of *T. gondii* instead of *in vivo* passages to gather infection material since previous studies revealed no significant differences between turkeys infected by the intravenous route or the oral route, regarding the number of positive tested (edible) tissue samples (Bangoura *et al.* 2013).

The infection of broiler chickens revealed only a small number of positive samples (4 of 192), but seroconversion of all chickens showed the validity of the infection. Our results indicate a low concentration of *T. gondii* in the organs tested. Similar results in infected chickens were found by Sedlak *et al.* (2000). We consider that the differences in positive findings between chicken and turkey are a result of the high resistance of the former to *T. gondii* (Bickford and Saunders, 1966; Biancifiori *et al.* 1986; Dubey *et al.* 1993*b*). In chickens a decrease of antibody levels was seen starting 4 weeks after infection which is in line with the findings in turkeys. Thus seroprevalence studies may in fact underestimate the true prevalence of *T. gondii* in chickens in the field.

However, in our study the detection limit of *T. gondii* DNA is high due to limited amount of sample material we could use for DNA extraction and the following PCR. Thus, in this study the number of *T. gondii*-positive samples is presumably underestimated in both host species. In further studies this problem could be avoided by using a DNA extraction method for large sample volumes as described by Opsteegh *et al.* (2010).

In turkeys, a high proportion of tissues was found positive for *T. gondii* DNA though, as stated above, methodological limitations to sensitivity were

present. This comprised also edible tissues such as breast and thigh muscle. Therefore, poultry and particularly turkey could be shown to represent potential intermediate hosts for *T. gondii* with persistence in various organs and tissues. Thus a risk of transmission of *T. gondii* by raw or undercooked poultry meat, especially turkey meat, to the consumer even after early infection at the beginning of a fattening cycle cannot be excluded by the present studies. However, the presence of *T. gondii* stages in poultry tissues as detected cannot be linked directly to infectivity by the study design chosen and has not been tested so far.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/S003118201400078X followed by article doi number.

ACKNOWLEDGEMENTS

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3.2. Publikation 2

Development of an in vivo model for *Toxoplasma gondii* infections in chickens and turkeys simulating natural routes of infection.

Geuthner A-C, Koethe M, Ludewig M, Pott S, Schares G, Maksimov P, Daugschies A, Bangoura B. Development of an in vivo model for *Toxoplasma gondii* infections in chickens and turkeys simulating natural routes of infection. Vet Parasitol. 2019; 276:108956. doi: 10.1016/j.vetpar.2019.108956. Epub 2019 Oct 13.

Erklärung zum Eigenanteil

Die Versuche plante ich in enger Zusammenarbeit mit Frau Dr. Berit Bangoura.

Der tierexperimentelle Teil der Arbeit wurde von der Autorin der vorliegenden Arbeit eigenständig durchgeführt. Dazu zählen die Herstellung der zur Infektion der Versuchstiere benötigten infektiösen Stadien (Oozysten, Gewebezysten und Tachyzoiten), die Infektionen der Versuchstiere (Mäuse, Katzen, Puten), die Blutentnahme, Serumgewinnung, Schlachtung, Präparation und Homogenisierung der untersuchten Organe. Bei der Präparation und Homogenisierung der Organe erhielt ich Unterstützung durch die Mitarbeiter des Institutes für Parasitologie (Herr Frank Stöckel, Frau Manja Etzold, Frau Juliane Hintzen, Frau Irene Malkwitz, Frau Eva-Maria Mäßig, Frau Linda Katzer, Frau Cora Delling, Frau Ira Dresely). Die Betreuung der Versuchstiere erfolgte durch die Tierpfleger (Herr Rene Schuhmacher, Frau Marion Fritsche) des Institutes für Parasitologie.

Die Autorin der vorliegenden Arbeit führte weiterhin den molekularbiologischen Teil der Arbeit eigenständig durch. Darunter fallen die DNA-Extraktion und PCR-Analysen sowie deren Auswertung. Frau Eva-Maria Mäßig unterstützte praktisch die Durchführung der DNA-Extraktion.

Die Untersuchung der Seren erfolgte durch Herrn Dr. Martin Köthe, Herrn Dr. Pavlo Maksimov und Herrn Dr. Gereon Schares.

Die statistische Auswertung der Ergebnisse und die Erstellung des Manuskriptes wurde von der Autorin der vorliegenden Arbeit eigenständig durchführt.

Die Revision des Manuskriptes erfolgte in Zusammenarbeit aller genannten Autoren.

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Research paper

Development of an *in vivo* model for *Toxoplasma gondii* infections in chickens and turkeys simulating natural routes of infection

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ABSTRACT

Turkeys and chickens were orally infected with tissue cysts (one mouse brain) or oocysts (10^3, 10^5 or 10^6 oocysts) of three *T. gondii* strains of the clonal types II and III (ME49, CZ-Tiger, NED) to investigate the influence of the applied *T. gondii* strain and infective doses on the distribution of *T. gondii* in several organs and tissues and the serologic response of chickens and turkeys. Organ samples from 16 different tissues, including heart, brain, muscles and gizzard were analyzed by PCR. Brain and heart were found most frequently positive for *T. gondii* DNA in both species, followed by gizzard. Serological analysis with kinetic ELISA for turkey samples and IFAT for chicken samples were performed once a week. In both species a dose-depending serological response was found. Turkeys seroconverted one week after infection with CZ-Tiger strain and medium and high doses of ME49 oocysts. In chickens, infection with medium and high doses of CZ-Tiger led to seroconversion one week p.i. Frequency of *T. gondii* positive organs showed a trend of a dose-effect in both species after infection with the type II strains. The NED strain showed low virulence in chickens and turkeys, demonstrated by clearly less *T. gondii* positive organs. Infection with tissue cysts of all three strains revealed *T. gondii* stages in tissues of turkeys and chickens. In conclusion, our data show a risk for human infection with *T. gondii* due to consumption of chicken and turkey meat.

1. Introduction

Toxoplasma (T.) gondii is a widespread protozoan parasite infecting warm-blooded animals including humans and birds (Tenter et al., 2000). Felids are the definitive hosts of *T. gondii* and shed oocysts after infection with one of the three infectious stages (Frenkel et al., 1970). Almost all warm-blooded animals and humans can act as intermediate hosts. The life cycle is facultative heteroxenous, i.e. intermediate hosts develop tissue cysts in several organs when infected by ingesting oocysts from fecal contamination by definitive hosts, or consuming cyst-containing tissues from another intermediate host (Dubey et al., 1998). Tissue cysts are formed especially in neural and muscular tissue (Dubey et al., 1998; Tenter et al., 2000).

Infected cats may shed up to 360 million of oocysts (Dubey, 2001, 2002, 2005) and contaminate wide areas of soil with oocysts (Gotteland et al., 2014). Thus, free-range poultry often is infected with *T. gondii* oocysts when feeding on contaminated soil. For that reason, free-range chickens are frequently used as sentinel animals to show environmental contamination with *T. gondii*, as described in numerous studies (Dubey et al., 2005, 2006, 2008, 2015). In addition, an infection of poultry via tissue cysts by feeding on infected rodents is possible (Koethe et al., 2015) enhancing infection prevalence. As *T. gondii* causes zoonotic infections, humans most often acquire *T. gondii* infections by ingestion of tissue cysts through consumption of raw or undercooked meat (Cook et al., 2000; Wilking et al., 2016).

Consumption of poultry meat has expanded over the last 20 years all over the world (OECD, 2019). Chickens and turkeys are the most important source of meat worldwide and in North America and the second important source in Europe following pork (OECD, 2019). Usually, poultry meat is consumed well-cooked, rendering tissue cysts no longer

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infective (Dubey et al., 1990). Nonetheless, in many countries, an increasing number of raw poultry meat products is placed onto the market, for example, dry-cured ham and short-matured raw sausages. The processing of these products is not always sufficient to inactivate *T. gondii* tissue cysts (Dubey, 1997; Pott et al., 2013). Hence, such products and undercooked poultry meat can pose an infection risk for consumers.

Worldwide, there are different *T. gondii* strains causing infections in animals and humans. In Europe and North America, the species *T. gondii* is currently assumed to contain four major clonal lineages (Howe and Sibley, 1995; Khan et al., 2011) and atypical strains. Across Europe, type II strains are clearly dominating in humans and animals (Aubert et al., 2010; Herrmann et al., 2012; Nowakowska et al., 2006; Schares et al., 2008; Shwab et al., 2014). However, additionally type III strains were found in Europe (De Sousa et al., 2006; Dubey et al., 2006; Messaritakis et al., 2008). In contrast, evidence of type I strains in animals in Europe is rare in the literature (Ajzenberg, 2010).

For mammalian host species, many data are available on *T. gondii* strain- and dose-dependent cyst formation patterns (Dubey, 1988; Dubey et al., 2016; Esteban-Redondo et al., 1999; Jungersen et al., 1999; Opsteegh et al., 2010). Regarding poultry, previous experimental infection studies use a variety of *T. gondii* strains in different gallinaceous bird species (Bangoura et al., 2013; Dubey et al., 1993a; Kaneto et al., 1997; Koethe et al., 2015; Schares et al., 2018; Sedlak and Franti, 2000) leading to inconsistent results. In addition, many field studies refer to the *T. gondii* positivity of the presumed predilection organs heart and brain though they do not play a significant role in human consumption (Dubey, 2010; Schares et al., 2017).

Evidence of T. gondii cyst organ distribution differences between turkeys and chickens was already shown by our group (Geuthner et al., 2014; Koethe et al., 2015; Schares et al., 2018), although intravenous infection with tachyzoites was utilized which limits applicability of the study results to field infections. Thus, there is a need for research into the influence of different strains and infection doses on the organ distribution of *T. gondii* in turkeys and chickens including edible organs, for both natural routes of infection, i.e. oocyst or cyst ingestion.

Comparative infections with representative strains of different clonal lineages are a prerequisite to judge the potential for *T. gondii* transmission by poultry meat. Accordingly, the aim of the study was to develop a model to simulate two naturally possible ways of infection of *T. gondii* in chickens and turkeys. For that reason, we infected chickens and turkeys with two well-described laboratory strains and one field strain of *T. gondii* belonging to two distinct clonal lineages (type II and III). The aim of the study was to investigate the influence of these three representative *T. gondii* strains, applied in different infective doses, on the distribution of *T. gondii* in several organs and tissues of chickens and turkeys. Additionally, the impact of the varied parameters on the seroconversion of infected animals was examined.

2. Material and methods

2.1. Parasites

In this study, three different strains belonging to two of the four major clonal lineages of *T. gondii* were used for animal infection, in particular the *T. gondii* strains ME49 (type II; Lunde and Jacobs, 1983; ToxoDB RFLP-Genotype 1), a field strain from the Czech Republic (type II strain CZ-Tiger; Juránková et al., 2013; ToxoDB RFLP-Genotype 3), and NED (type III; Dardé et al., 1992; ToxoDB RFLP-Genotype 2).

2.1.1. Tachyzoites

For infection of mice, ME49 and NED tachyzoites were cultured in VERO cells cultivated with Iscove's Modified Dulbecco's Medium (IMDM, PAA, Pasching, Austria), 5% fetal calf serum, 1% penicillin/streptomycin and 1% amphotericin B as described before (Geuthner et al., 2014) at 37 °C, 5% CO_2. For both ME49 and NED strains, the number of cell culture passages before mouse infection has not been tracked, both are long-term used laboratory strains. The supernatant of cell culture was centrifuged at 2000 × *g* for 5 min to harvest the parasites. The pellet was resolved in 1 mL PBS solution and tachyzoites were counted using a Neubauer chamber. For intraperitoneal infection of mice, tachyzoites were diluted in 100 µL sterile isotonic 0.9% sodium chloride solution (B. Braun Melsungen AG, Melsungen, Germany).

2.1.2. Oocysts

Six cats (ME49, CZ-Tiger, NED, n = 2 per strain) were orally infected with *T. gondii* tissue cysts from 1 to 2 mice brains per animal as described by Schares et al. (2018). Feces of experimentally infected cats were collected starting at day 3 after infection and *T. gondii* oocysts were obtained by flotation in saturated saline solution (specific gravity 1.20 g/mL). After sporulation at room temperature, oocysts were stored in 2% potassium dichromate at 4 °C. For infection dose preparation, sporulated oocysts were washed twice with faucet water before dosing.

2.1.3. Tissue cysts

For generating tissue cysts, Balb/c and CD1 mice were infected with 1000–2000 tachyzoites intraperitoneally (strains ME49 and NED), or 10–100 oocysts or tissue cysts orally (strain CZ-Tiger), respectively. Mice passage was done for all three *T. gondii* strains separately, with the actual infection dose depending on the amount of infectious material available. At least four weeks *post infectionem* (p.i.), mice were killed by cervical dislocation. Brains were dissected, and a squash preparation of every brain was examined for tissue cysts under light microscope.

Per chicken or turkey, the tissue amount of one positive mouse brain was used for infection, simulating the natural ingestion amount in case of preying on infective rodents. Therefore, for the infection of a group of six chickens or turkeys, respectively, six positive mouse brains and isotonic 0.9% sodium chloride solution (B. Braun Melsungen AG, Melsungen, Germany) were mixed to yield 6 mL of a brain homogenate. 1 mL of the homogenate was applied to each bird of the group to ensure a similar infection dose within each group.

2.2. Animals and husbandry

All animal trials were conducted at the Institute of Parasitology, Leipzig University, Germany and were approved by the responsible authority (Landesdirektion Leipzig, Germany, trial no. TVV 29/10).

2.2.1. Poultry

Commercially available turkeys (BUT B.I.G. 6, n = 78) and chickens (ISA JA 757, n = 78) were purchased as day-old poults. Chickens and turkeys were reared in separate groups for one week (chickens) or three weeks (turkeys) before experimental infection. The birds were kept on litter comprised of wood shavings. Chickens obtained conventional starter and grower feed for chickens without anticoccidials *ad libitum*. Because conventional starter and grower feed for turkeys without anticoccidials were not available, turkeys were receiving poultry feed for pet poultry without anticoccidials (deuka Wild-und Ziergeflügelfutter, Deutsche Tiernahrung Cremer, Duesseldorf, Germany) *ad libitum*. The feed was enriched with powdered milk and supplements (Korvimin ZVT + Reptil, WDT, Garbsen, Germany) to match the recommended nutritional values for growing turkeys. Animals had free access to water.

2.2.2. Cats

Specific pathogen-free cats were housed in disinfected, tiled rooms and were fed with commercially available canned cat food and dry food. Water was available *ad libitum*.

2.2.3. Mice

For all experiments, Balb/c and CD1 mice were acquired from the experimental center (Medizinisch-Experimentelles Zentrum), Leipzig

Table 1
Study design for chicken and turkey experiments (identical groups were formed for both poultry species, i.e. there was each of the given groups for chickens and for turkeys, respectively. NC, uninfected negative control).

Study group (n = 6 per group)	*T. gondii* strain	Infective stage	Infection dose
Cy-M	ME49	cysts	1 mouse brain equivalent
O-M1		oocysts	1×10^3
O-M2			1×10^5
O-M3			1×10^6
NC-M	–	–	–
Cy-T	CZ-Tiger	cysts	1 mouse brain equivalent
O-T1		oocysts	1×10^3
O-T2			1×10^5
O-T3			1×10^6
NC-T	–	–	–
Cy-N	NED	cysts	1 mouse brain equivalent
NC-N		–	–
O-N1	NED	oocysts	1×10^3
NC-N	–	–	–

University, Germany. They were kept on wood shavings and autoclaved hay. Commercially available rodent feed and water were provided *ad libitum*.

2.3. Infection and study design

2.3.1. Infection of poultry

78 chickens and 78 turkeys, raised for 9–10 days (chickens) or 21–26 days (turkeys), respectively, were included into the study.

For each of the type II strains ME49 and CZ-Tiger, 24 chickens and 24 turkeys were infected (see Table 1). Chickens and turkeys were divided into 4 groups each (n = 6). Per animal, either 1 mL of mouse brain homogenate, or 1 mL of water containing 1×10^3, 1×10^5, or 1×10^6 oocysts were orally administered by using an animal feeding needle into the crop.

For infection with NED strain, 12 chickens and turkeys were allocated into 2 groups (n = 6) each (see Table 1). They were infected with 1 mL mouse brain homogenate or water containing 10^3 oocysts as described above.

The remaining 18 chickens and 18 turkeys stayed uninfected as a negative control group (n = 6 per strain). All chickens were slaughtered at 5 weeks p.i. All turkeys were slaughtered at 8 weeks p.i. The time point of slaughtering was determined on the basis of previously published data (Geuthner et al., 2014).

2.3.2. Serological examinations

For screening of *T. gondii*-specific antibody concentrations, blood samples were drawn from the wing vein prior to infection and weekly after infection until slaughtering. Blood was centrifuged at 2500 × *g* for 15 min and serum samples were stored at – 20 °C until analyzing. Two different assays were performed to assess the serum antibody concentration; for turkey sera, a kinetic ELISA (KELA) was employed as previously described (Geuthner et al., 2014; Koethe et al., 2011), while an immunofluorescent antibody test (IFAT) was performed for chicken sera as described by Maksimov et al. (2011).

2.3.3. Sample processing

Animals were slaughtered after intramuscularly applied anesthesia with ketamine (25 mg/kg) and xylazine (1 mg/kg). During dissection, tissues samples were taken from 16 different organs/locations, including edible parts (heart, breast muscle, drumstick, thigh muscle, liver, gizzard) that may be purchased for human consumption, as well as non-edible parts (glandular stomach, pancreas, spleen, kidneys, brain, retina, gonads, intestine, lung, bone marrow).

Tissues were homogenized immediately after slaughter using commercial household blenders (La Moulinette, Tefal Groupe SEB, Offenbach, Germany) or mortar and pestles for brain, gonads, and bone marrow. The whole organ homogenate was stored at − 20 °C, except for muscles; for this tissue up to 300 g sample material per bird was taken from the different locations, and stored at − 20 °C.

25 mg tissue per sample or 10 mg spleen tissue were used for DNA extraction using the commercially available QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany) following the manufacturer's instructions. As control of possible DNA contamination during extraction, after no more than three samples from infected birds, at least one sample of the negative control group was handled and carried along during the whole processing including PCR and gel electrophoresis. DNA concentration of samples was measured using a spectrophotometer (Nanodrop 2000c, Thermo Scientific, Waltham, MA, USA). For PCR, DNA from cell culture-derived ME49 tachyzoites served as positive control. Positive and negative controls (aliquot of currently used DNA elution buffer) were carried along in every PCR batch. PCR reactions were carried out as a direct PCR and followed by a nested PCR as described by Zöller et al. (2013) with minor modifications. For direct PCR, each reaction contained 1 U DreamTaq Green DNA Polymerase®, 10X DreamTaq Green Buffer® (Thermo Scientific, Mannheim, Germany), 200 µM of each dNTP (Fermentas, St. Leon Rot, Germany), 1.5 mM $MgCl_2$, 0.4 µM of each primer Tg1 (5′-AAA AAT GTG GGA ATG AAA GAG-3′) and Tg2 (5′-ACG AAT CAA CGG AAC TGT AAT-3′). DNA extract was added to obtain 80–200 ng template DNA, and reaction volume was adjusted to 25 µL by adding DNase-free water. For the subsequent nested PCR, PCR reactions were assembled as described above using primers Tgnested1 (5′-CGC TAATGT GTT TGC ATA GG-3′) and Tgnested2 (5′-GGC ACG TCT CTT GTT CTT CT-3′), 3 mM $MgCl_2$ and 2.5 µL of the direct PCR product as template. Both PCRs were performed in a peqSTAR thermocycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany) or a M × 3000P®Real-Time PCR System (Stratagene/Agilent Technologies, La Jolla, USA) under the following cycling conditions: preheating (2 min at 94 °C), amplification for 35 cycles (direct PCR) or 25 cycles (nested PCR) of 1 min at 94 °C, 40 s at 55 °C and 1 min at 72 °C and one final extension step of 5 min at 72 °C. The nested PCR products were analyzed by gel electrophoresis. Therefore, approximately 10 µL of the nested PCR products were applied on a 1.5% agarose gel, which was stained with ethidium bromide. Visualization of DNA bands took place with UV-light in positive samples at 375 bp fragment length.

2.3.4. Statistics

IBM SPSS Statistics Version 22 software package (IBM, New York, USA) was used for statistical analysis. All data were non-normally distributed and were therefore further analyzed by Kruskal–Wallis test followed by Mann-Whitney U test (infection group differences in number of animals positive per organ, sum of positive organs and seroconversion) and Friedman test (determination if any organs are more frequently positive than others). The Spearman rank correlation test was performed to explore possible correlations between the positivity status of individual organs. Group differences and correlations with P values of less than 0.05 were defined as statistically significant.

3. Results

3.1. Serology

3.1.1. Chickens

All chickens included into the study were tested negative for *T. gondii* antibodies on the day of infection (day 0). Each infected chicken was seroconverting not later than week four p.i. (see Table 2 for detailed seroconversion data by infection mode), except for one chicken of group Cy-N. Interestingly, chickens infected with the CZ-Tiger strain seroconverted significantly earlier than chickens of the two other

Table 2
Seroconversion of chickens over the complete time of study (Seropositive animals /infected animals (relative number of seropositive animals in %).

Infection mode[a]	Number and proportion of seroconverted animals stratified for time after infection					
	0 dpi	7 dpi	14 dpi[b]	21 dpi	28 dpi	35 dpi
Cy-M	0/6 (0%)	0/6 (0%)	0/6 (0%)[c]	5/6 (83.3%)	5/5 (100%)	4/5 (80.0%)
O-M1	0/6 (0%)	0/6 (0%)	0/6 (0%)[c]	5/6 (83.3%)	6/6 (100%)	6/6 (100%)
O-M2	0/6 (0%)	0/6 (0%)	1/6 (16.7%)[c, g]	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-M3	0/6 (0%)	0/4 (0%)	4/4 (100%)[d]	4/4 (100%)	4/4 (100%)	4/4 (100%)
M-total	0/24 (0%)	0/22 (0%)	5/22 (22.7%)[E]	20/22 (90.9%)	21/21 (100%)	20/21 (95.2%)
Cy-T	0/6 (0%)	0/6 (0%)	4/6 (66.7%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-T1	0/6 (0%)	0/6 (0%)	4/6 (66.7%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-T2	0/6 (0%)	1/6 (16.7%)	6/6 (100%)[h]	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-T3	0/6 (0%)	3/6 (50.0%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
T-total	0/24 (0%)	4/24 (16.7%)	20/24 (83.3%)[F]	24/24 (100%)	24/24 (100%)	24/24 (100%)
Cy-N	0/6 (0%)	0/6 (0%)	3/6 (50.0%)	3/5 (60.0%)	4/5 (80.0%)	4/5 (80.0%)
O-N1	0/6 (0%)	0/6 (0%)	0/5 (0%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
N-total	0/12 (0%)	0/12 (0%)	3/11 (27.3%)[E]	8/10 (80%)	9/10 (90%)	9/10 (90%)

[a] Abbreviatons of infection modes are illustrated in Table 1.
[b] Statistically significant differences between infection groups are indicated by a superscript letter (c–h), Mann-Whitney-U-test ($P < 0.05$)\. Different letters indicate statistically significant differences in the respective comparisons. [c,d] differences between ME49 infection modes; [E,F] differences between ME49, CZ-tiger and NED total seroconverted chickens; [g, h] differences between chickens infected with 10^5 oocysts of ME49, CZ-tiger and NED.

groups ($P = 0.008$ compared with strain NED, $P < 0.001$ compared with strain ME49), with 83.3% of the birds testing IFAT positive already 14 days after infection. For strains ME49 and CZ-Tiger, high-dose oocyst infections (groups O-M3, O-T3) yielded positive IFAT titers more often (100% of the chickens) at 14 days p.i. compared with the other infection modes of the respective strain. Seroconversion indicated a valid infection with *T. gondii*. Maximum reciprocal IFAT titers observed ranged from 800 to 51,200. *T. gondii*-specific antibodies were detectable over the complete period of the experiment. Only in one chicken (group Cy-M), an IFAT titer had decreased below the cut-off value at the point of slaughtering. Chickens of the negative control groups remained seronegative for the entire study.

3.1.2. Turkeys

All turkeys included in the study were tested negative for *T. gondii*-specific antibodies on the day of infection except for two turkeys with antibody levels just above the cut-off value.

Seroconversion was first observed one week p.i., when it was seen in 50% of ME49-infected, 58.3% of CZ-Tiger infected, and 33% of NED-infected turkeys, respectively. All infected animals showed a seroconversion not later than two weeks p.i. (see Table 3). The serum-antibody concentrations remained above the cut-off over the entire experiment in all animals except for one turkey of group O-T1 that showed concentrations below the cut-off in week five p.i. (see Table 3). The *T. gondii* strain did not significantly influence the time point of seroconversion of turkeys, this was true for both infective stages (cysts and oocysts). In contrast, 83.3% of moderate-dose oocyst infected turkeys (groups O-M2, O-T2), and 91.7% of high-dose oocyst infected turkeys (groups O-M3, O-T3) seroconverted 1 week p.i. irrespective of the strain. With 22% (of the groups Cy-M, Cy-T, Cy-N) for tissue cyst infection ($P = 0.001$ compared with moderate-dose and high-dose oocyst infections) and 27.8% (of the groups O-M1, O-T1, O-N1) for low-dose oocyst infection ($P = 0.003$ compared with moderate-dose and high-dose oocyst infections), respectively, significantly fewer animals revealed a seroconversion after or at 1 week p.i. in these groups. Interestingly, all turkeys of groups Cy-M and O-M1 seroconverted in week 2 p.i. only, i.e. significantly ($P = 0.002$) later than O-M2 and O-M3 animals. For CZ-Tiger and NED strains, the infection mode (infective stage and dose) did not clearly influence the point of seroconversion.

3.2. Animal health

3.2.1. Chickens

Five animals died before the end of the experiment. Two chickens of

Table 3
Seroconversion of turkeys the complete time of study.

Infection mode[a]	Number and proportion of seroconverted animals stratified for time after infection								
	0 dpi	7 dpi[b]	14 dpi	21 dpi	28 dpi	35 dpi	42 dpi	49 dpi	56 dpi
Cy-M	0/6 (0%)	0/6 (0%)[c]	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-M1	0/6 (0%)	0/6 (0%)[c]	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-M2	0/6 (0%)	6/6 (100%)[d]	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-M3	0/6 (0%)	6/6 (100%)[d]	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)
M-total	0/24 (0%)	12/24 (50%)	22/22 (100%)	22/22 (100%)	22/22 (100%)	22/22 (100%)	22/22 (100%)	22/22 (100%)	22/22 (100%)
Cy-T	0/6 (0%)	2/6 (33.3%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-T1	0/6 (0%)	3/6 (50%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	5/6 (83.3%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-T2	0/6 (0%)	4/6 (66.7%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-T3	1/6 (16.7%)	5/6 (83.3%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
T-total	1/24 (4.2%)	14/24 (58.3%)	24/24 (100%)	24/24 (100%)	24/24 (100%)	23/24 (95.8%)	24/24 (100%)	24/24 (100%)	24/24 (100%)
Cy-N	1/6 (16.7%)	2/6 (33.3%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
O-N1	0/6 (0%)	2/6 (33.3%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
N-total	1/12 (8.3%)	4/12 (33.3%)	12/12 (100%)	12/12 (100%)	12/12 (100%)	12/12 (100%)	11/11 (100%)	11/11 (100%)	11/11 (100%)

[a] Abbreviatons of infection modes are illustrated in Table 1.
[b] Statistically significant differences between infection groups are indicated by a superscript letter ([c, d]), Mann-Whitney-U-test ($P < 0.05$). Different letters indicate statistically significant differences.

group O-M3, one chicken of group Cy-N, and one chicken of group O-N1 died during the study. However, in all four chickens, the pathological findings did not provide evidence for acute toxoplasmosis as the cause of death. In one chicken of group Cy-M, which died three weeks p.i., histopathological results suggested an acute heart failure as the cause of death due to a non-purulent myocarditis, most probably caused by *T. gondii*.

The remaining animals appeared clinically healthy throughout the study.

3.2.2. Turkeys

Five animals died during the study due to several reasons; all of them seemingly unrelated to the experimental *T. gondii* infection. Two turkeys of group O-M3 deceased of *E. coli* septicemia seven and ten days p.i., respectively. Because of cannibalism or skeletal problems, respectively, two turkeys of group NC-T and one turkey infected of group O-N1 had to be euthanized during the study.

All remaining turkeys seemed clinically healthy until the end of the experiment.

3.3. PCR

3.3.1. Chickens

Regarding type II strains, *T. gondii* DNA was detected in 90.5% (19/21) of all ME49-infected and 95.8% (23/24) of all CZ-Tiger-infected animals (Table 4). Over all infection modes, 76.2% of the ME49-infected animals and 87.5% of the CZ-Tiger-infected animals tested positive for *T. gondii* DNA in at least one edible organ. *T. gondii* DNA was detectable in 30.0% (3/10) of all NED infected chickens. The NED strain induced a significantly lower number of total positive organs (1.9%) ($P < 0.001$) and positive edible organs (1.3%) ($P = 0.005$ compared with strain ME49, $P < 0.001$ compared with strain CZ) compared with both type II strains

There were no marked differences in the type of positive organs between the different type II strain infected groups. However, the total number of positive organs overall infection groups was notably ($P = 0.016$) higher in CZ-Tiger infected groups (27.9%) than in ME49 group (16.4%). Besides, the infection mode (parasite stage and dose) influenced the total number of positive organs per group significantly (see Table 4).

Looking at the frequency of T. gondii positivity for different organ type (see Table 5), in ME49-infected chickens, *T. gondii* DNA was most commonly detected in hearts (38.1%) and brains (23.8%), thigh muscles (23.8%), drumsticks (23.8%), glandular stomach (23.8%) and lungs (23.8%). In the CZ-Tiger groups, most frequently heart (70.8%), brain (54.2%), gonads (45.8%) and gizzard (41.7%) were tested positive, whereby heart was notably ($P = 0.009$) more often affected than all other analyzed organs except the brain (Fig. 1).

For each of the three PCR-positive NED-infected chickens, only one organ tested positive. In group Cy-N, one animal's liver and another animal's bone marrow contained *T. gondii* DNA. In group O-N1, *T. gondii* DNA was found in one heart.

3.3.2. Turkeys

T. gondii DNA positive animals were found for all three infection strains used. Regarding ME49 infection, 95.5% (21/22) of the animals featured *T. gondii* DNA positive organs. Every organ type tested positive from at least one turkey in the ME49 infected groups except for kidney. For CZ-Tiger infected groups, 87.5% (21/24) were PCR positive. Infection with the NED strain resulted in 36.4% *T. gondii* DNA positive animals (5/11). Edible organs were often tested positive in all three strains, with 54.6% of all ME49 infected birds, 62.5% of all CZ-Tiger infected animals, and 36.4% of all NED infected turkeys, respectively (see Table 4).

Regarding individual organs, brain was tested *T. gondii* positive most frequently for all three infection strains with 68.2% of all brains in ME49 infected, 62.5% in CZ-Tiger infected, and 27.3% in NED infected groups.

Total frequencies of positive tested organs for all strains are shown in Fig. 1. In general, comparable to our chicken experiments, turkeys infected with ME49 as well as with CZ-Tiger showed distinctly higher proportions of positive organs (18.8% and 11.7%, respectively, of all analyzed organ samples) than turkeys infected with NED (5.7%).

The numbers of edible organs tested positive after infection with tissue cysts showed comparable results over all strains (ME49, 6.3%; CZ-Tiger, 4.2%; NED, 3.1%) (see Fig. 1, Table 5).

3.3.3. Comparison of results chicken and turkeys

T. gondii DNA was found in none of the uninfected animals of both host species.

ME49 infection in turkeys led to a lower variation in the tissue distribution of *T. gondii* DNA over all analyzed organs compared with the two other infection strains. As shown in Fig. 1, this phenomenon could not be observed in chickens.

In both tested host species, hearts were more often positive after infection with the CZ-Tiger strain than after ME49 infection (i.e., in chickens 70.8% vs. 38.1% or in turkeys 37.5% vs. 13.6%, respectively) (see Table 5), while NED strain induced positive results for only one chicken heart (1/10) and no turkey heart.

For chicken brain analysis, markedly more positive findings

Table 4
T. gondii DNA positive findings depending on parasite stage and dose used for infection. Overall infection groups for each strain. Listed as positive animals /infected animals, in brackets: (positive organs per infection mode /positive edible organs per infection mode).

	Infection strain	Infection mode				
		Tissue cysts[a]	1×10^3 oocysts[a]	1×10^5 oocysts[a]	1×10^6 oocysts[a]	Total[a]
Turkeys	CZ-Tiger	4/6 (6.3%^{b,d}/4.2%)	5/6 (8.3%^{f}/4.2%)	6/6 (15.6%/10.4%^{h})	6/6 (16.7%^{c}/6.3%)	21^{l}/24 (11.7%^{j}/16.7%)
	ME49	5/6 (24.0%^{e}/6.3%)	6/6 (24.0%^{g}/11.5%)	6/6 (12.5%/2.1%^{i})	4/4* (24.0%/4.7%)	21^{l}/22 (18.8%^{j}/16.7%)
	NED	3/6 (6.3%^{d}/3.1%)	2/5* (5.0%^{f}/1.3%)	NA	NA	5^{m}/11 (5.7%^{k}/6.1%)
Chicken	CZ-Tiger	6/6 (26.0%^{T}/8.3%^{N,V})	5/6 (17.7%^{X}/5.2%^{N})	6/6 (25.0%/8.3%^{N})	6/6 (42.7%/18.8%^{O})	23^{Z}/24 (27.8%^{Z}/27.1%^{Z})
	ME49	4/5* (6.3% ^{R,U}/2.5%^{P})	5/6 (13.5%^{X}/7.3%)	6/6 (20.8%/7.3%)	4/4* (26.6% ^{S}/12.5%^{Q})	19^{Z}/21 (16.4%$^{\$}$/7.1%$^{\$}$)
	NED	2/5* (2.5%^{U}/1.3%^{W})	1/5* (1.3% ^{Y}/1.3%)	NA	NA	3$^{\$}$/10 (1.9%$^{\$}$/1.3%$^{\$}$)

NA = not applicable.

* Smaller group sizes due to deceased animals during the study period.

[a] Statistically significant differences between infection groups are indicated by a superscript letter ($^{b-\ z,\ \S,\$,\ \#}$), Mann-Whitney-U-test ($P < 0.05$). Different letters indicate statistically significant differences in the respective row/column. $^{b,\ c}$ differences between turkeys infected with CZ-Tiger; $^{d,\ e}$ differences between tissue cyst infected turkeys overall strains; $^{f,\ g}$ differences between 10^3 oocyst infected turkeys overall strains; $^{h,\ i}$ differences between 10^5 oocyst infected turkeys overall strains; $^{j,\ k,\ l,\ m}$ differences between turkeys overall infection modes and strains; $^{N,\ O}$ differences between chickens infected with CZ-Tiger; $^{P,\ Q,\ R,\ S}$ differences between chickens infected with ME49; $^{T,\ U,\ V,\ W}$ differences between tissue cyst infected chickens overall strains; $^{X,\ Y}$ differences between 10^3 oocyst infected chickens overall strains; $^{Z,\ \S,\ \$}$ differences between chickens overall infection modes and strains.

Table 5
T. gondii DNA positive organs for type II strains depending on parasite stage and dose used for infection.

	Tested organ	Infection mode				Total
		Cy-M/Cy-T[a]	O-M1/O-T1[a]	O-M2/O-T2[a]	O-M3/O-T3[a]	M/T[a]
Chickens	Heart	4. 8 %/ 16.7 %	4.8 %/ 8.3 %	19.0 %/ 20.8 %	9.5 %/ 25.0 %	38.1 %/ 70.8 %*
	Breast muscle	4.8 %/ -	4.8 %/ 8.3 %	4.8 %/ -	4.8 %/ 4.2 %	19.0 %/ 12.5 %
	Thigh muscle	- /4.2 %	14.3 %/ -	- / -	9.5 %/ 16.7 %	23.8 %/ 20.8 %
	Drumstick	- / -	4.8 %/ -	4.8 %/ -	14.3 %/ -	23.8 %/ -*
	Liver	- / 8.3 %	- / -	4.8 %/ -	- / 8.3 %	4.8 %/ 16.7 %
	Gizzard	- /4.2 %	4.8 %/ 4.2 %	- / 12.5 %	- / 20.8 %*	4.8 %/ 41.7 %*
	Glandular stomach	9.5 %/ 8.3 %	- /4.2 %	4.8 %/ 4.2 %	9.5 %/ 12.5 %	23.8 %/ 29.2 %
	Pancreas	- / 8.3 %	4.8 %/ 4.2 %	4.8 %/ 4.2 %	- / -	9.5 %/ 16.7 %
	Spleen	- / 8.3 %	4.8 %/ 8.3 %	4.8 %/ 8.3 %	4.8 %/ 8.3 %	14.3 %/ 33.3 %
	Kidney	- / -	4.8 %/ -	- / 4.2 %	9.5 %/ 4.2 %	14.3 %/ 8.3 %
	Brain	- / 12.5 %	4.8 %/ 12.5 %	14.3 %/ 8.3 %	4.8 %/ 20.8 %	23.8 %/ 54.2 %*
	Retina	- / 8.3 %	- /4.2 %	4.8 %/ 12.5 %	4.8 %/ 4.2 %	9.5 %/ 29.2 %
	Gonads	4.8 %/ 12.5 %	- / 8.3 %	- / 8.3 %	- / 16.7 %	4.8 %/ 45.8 %*
	Intestine	- / -	4.8 %/ -	14.3 %/ 16.7 %	- / 12.5 %	19.0 %/ 29.2 %
	Lung	- /4.2 %	4.8 %/ 4.2 %	14.3 %/ -	4.8 %/ 8.3 %	23.8 %/ 16.7 %
	Bone marrow	- / 8.3 %	- / 4.2 %	- / -	4.8 %/ 8.3 %	4.8 %/ 20.8 %
Turkeys	Heart	- / 4.2 %	9.1 %/ 4.2 %	- / 20.8 %*	4.5 %/ 8.3 %	13.6 %/ 37.5 %
	Breast muscle	- / 4.2 %	13.6 %/ 4.2 %	- / -	- / -	13.6 %/ 8.3 %
	Thigh muscle	4.5 %/ -	9.1 %/ 4.2 %	- / 8.3 %	9.1 %/ -	22.7 %/ 12.5 %
	Drumstick	9.1 %/ 4.2 %	9.1 %/ -	- / 4.2 %	- / -	18.2 %/ 8.3 %
	Liver	4.5 %/ -	9.1 %/ -	4.5 %/ -	- / -	18.2 %/ -*
	Gizzard	9.1 %/ 4.2 %	- / 4.2 %	4.5 %/ 8.3 %	- / 16.7 %	13.6 %/ 33.3 %
	Glandular stomach	9.1 %/ -	- / -	9.1 %/ -	- / 4.2 %	18.2 %/ 4.2 %
	Pancreas	9.1 %/ -	4.5 %/ -	- / -	- / -	13.6 %/ -
	Spleen	4.5 %/ -	4.5 %/ -	4.5 %/ -	- / -	13.6 %/ -
	Kidney	- / -	- / -	- / -	- / -	- / -
	Brain	13.6 %/ 8.3 %	13.6 %/ 12.5 %	22.7 %/ 20.8 %	18.2 %/ 20.8%	68.2 %/ 62.5 %
	Retina	- / -	4.5 %/ -	- / -	4.5 %/ -	9.1 %/ -
	Gonads	13.6 %/ -	4.5 %/ -	- / -	- / 4.2 %	18.2 %/ 4.2 %
	Intestine	- / -	9.1 %/ -	4.5 %/ -	- / 4.2 %	13.6 %/ 4.2 %
	Lung	9.1 %/ -	4.5 %/ 4.2 %	4.5 %/ -	- / 8.3 %	18.2 %/ 12.5 %
	Bone marrow	18.2 %/ -	9.1 %/ -	- / -	4.5 %/ 8.3 %	27.3 %/ -*

[a] Abrreviatons of infection modes are illustrated in Table 1.
* Indicates statistically significant differences between the amounts of positive organs of the respective infection group Mann-Whitney-U-test ($P < 0.05$).

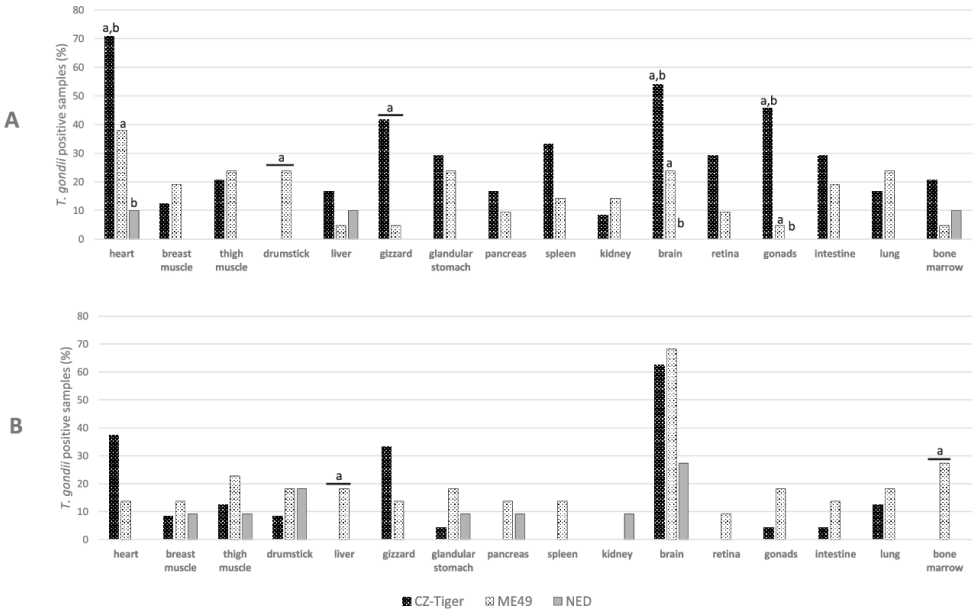

Fig. 1. Frequencies of organs tested T. gondii positive, over all infectious modes, by infection strain. (A) chickens (B) turkeys.a,b identical indices indicate statistically significant differences between the respective groups Mann-Whitney-U-test ($P < 0.05$).

occurred in CZ-Tiger infections (54.2%) than in ME49 infections (23.8%); while NED infection did not result in detection of *T. gondii* DNA in any of the chicken brains. In turkeys, the percentages of positive brains were similar between both type II strains (68.2% for ME49, and 62.5% for CZ-Tiger). A lower proportion of the brains were tested positive in NED infected turkeys (27.3%) but still clearly more than in chickens.

Gizzard was one of the most frequently *T. gondii* positive organs except heart and brain for CZ-Tiger infected chickens (41.7%) as well as turkeys (33.3%). Distribution of the single organs depending on the infection mode and tested bird species showed no significant strain-related differences. No differences in positive rates detected in the different tested organs (see Table 5) except in heart for medium level CZ-Tiger oocyst infected turkeys (CZ-Tiger 20.8%; ME49 0%) and in gizzard for high level CZ-Tiger oocyst strain infected chickens (CZ-Tiger 20.8%; ME49 0%).

The infection with ME49 revealed similar percentages of total positive samples in turkeys (18.8%) and chickens (16.4%). In chickens, but not in turkeys infected with ME49 or CZ-Tiger, the total numbers of positive samples as well as of positive edible samples increased with an increasing oocyst infection dose (see Table 4). However, there was no consistent tendency of higher numbers of positive samples in chickens or turkeys, when ME49 infection groups were compared.

4. Discussion

The aim of the present study was to investigate the influence of three different *T. gondii* strains of two clonal lineages and different infection doses and routes on the distribution of *T. gondii* in several organs and tissues of chickens and turkeys. Additionally, the impact of the infection strain on the seroconversion of infected animals should be examined. The organs were screened for *T. gondii* stages by PCR, which is not able to prove viability of the pathogen but is suitable to check a high amount of tissue samples for the presence and distribution of *T. gondii* and compare the different poultry groups. The study results could show an influence of the infection strain and dose on the seroconversion of the infected animals. However, the serologic data from the two bird species cannot be compared directly because different analysis methods were used for detection of *T. gondii* antibodies in the different species which was reported to result in varying outcome even for a single species (Dubey, 2010; Dubey et al., 1993a, 1993b; Schares et al., 2018), but a tendency is still noticeable.

Chickens are described as resistant hosts to clinical toxoplasmosis in general (Dubey, 2010). In the present study one chicken showed clinical symptoms after infection with ME49 tissue cysts. Clinical toxoplasmosis in chickens was previously described after infection with oocysts of the highly virulent type I GT1 strain (Dubey et al., 1993b), but to our knowledge there are no descriptions of clinical toxoplasmosis in chickens after infection with type II or III strains (Dubey, 2010). On the other hand, none of the infected turkeys showed clinical symptoms associated with toxoplasmosis during the study. This observation is in accord complying with former studies in turkeys (Bangoura et al., 2013; Dubey et al., 1993a; Geuthner et al., 2014; Sedlak and Franti, 2000; Zöller et al., 2013) although fatal toxoplasmosis in wild turkeys has been described previously (Howerth and Rodenroth, 1985; Quist et al., 1995).

One week after infection with ME49 or NED, no chicken tested seropositive though some chickens seroconverted as early as one week p.i. in the medium- and high-dose oocyst infected CZ-Tiger strain groups. Different authors (Biancifiori et al., 1986; Sedlak and Franti, 2000) described similar findings of seroconversion as late as two weeks p.i. in chickens after infection with oocysts of various amounts of oocysts of a type I or type III strain. To our knowledge, this is the first study investigating serological data of turkeys after infection with different *T. gondii* strains and infection modes. Our results show that turkeys reliably develop a measurable serologic response to type II and type III infections, irrespective of infection dose and route, and that antibody concentrations may drop several weeks after infection. Turkeys showed similar dose-depending serological responses as previously described for other non-avian (Forbes et al., 2012) and avian species after infection with various *T. gondii* strains, e.g. OV51/95 in partridges (Martínez-Carrasco et al., 2004), K21 in ducks (Bartova et al., 2004), RH in chickens (Maksimov et al., 2018). Seroconversion in turkeys infected with tissue cysts or oocysts of the CZ-Tiger strain and medium and high doses of ME49 oocysts took place one week after infection already. Results of previous studies (Bangoura et al., 2013; Sedlak and Franti, 2000) investigating infection of turkeys with oocysts of different strains support these findings.

To our knowledge, there is only one experiment (Dubey et al., 1993a) comparable to our study using ME49 oocyst infections in turkeys, describinga seroconversion on day 14 p.i. which was the earliest investigated time point.

One turkey of the Cy-N and O-T3 group, respectively, showed a seroconversion as early as the day of infection. The KELA-values of the two animals barely exceeded the cut-off and stayed on this low level until 14 dpi or even dropped under the cut-off. Koethe et al. (2011) described similar findings when infecting turkeys with *Eimeria* spp. and *Hammondia hammondi* where 10% of the infected animals showed low KELA-values above the cut-off in the *T. gondii* assay. Contact of turkeys in example in the hatchery with other apicomplexan protozoa than *T. gondii* cannot be ruled out definitively but are deemed highly unlikely since the animals were stabled as day-old poults. Two weeks after infection an intense immune response with strong increases of the KELA-values was observed in both turkeys similar to the 0 dpi seronegative turkeys. Therefore, both turkeys were kept in the study.

Infection with tissue cysts of NED did not yield seroconversion in one chicken over the entire time of the study. Schares et al. (2018) additionally analyzed the serum with MAT and TgSAG1-ELISA$_{SH}$, detecting a seroconversion. Furthermore, the bone marrow of the respecting chicken was found positive for *T. gondii* DNA, thus a valid infection of this animal can be assumed. Nonetheless, these findings in single turkeys and chickens indicates that the used methods are sensitive and specific, but performance of multiple tests may be advisable to explain disagreement between adverse findings. In birds, the availability of serological tests is much more restricted than in mice or humans, thus the tests that proved to be highly reliable in previous studies were chosen (Geuthner et al., 2014; Koethe et al., 2011, 2015; Schares et al., 2018).

In our hands, type III NED strain infections yielded a lower percentage of positive birds and positive individual organs in turkeys as well as in chickens compared to both utilized type II strains, regardless of the infection mode. This fact is in accordance with results from own previous NED tachyzoite infection of chickens (Geuthner et al., 2014), which also confirmed low *T. gondii* tissue stage quantities in positive organs after tachyzoite infection (Koethe et al., 2015; Schares et al., 2018). These reproducible findings underline the low virulence of the NED strain in chickens and turkeys. However, during passage in mice to obtain the tissue cysts for poultry infections, the NED strain showed high virulence in mice in our laboratory contrary to descriptions by Dardé (1996) (data not shown).

Infection with ME49 did not reveal a consistent tendency of higher numbers of positive samples following application of higher infection doses in chickens as well as turkeys, whereas such trend was noticeable after infections with CZ-Tiger in both host species. A possible explanation for this observation is the often noted altered pathogenicity of *T. gondii* strains after variable passages in cell culture (Saraf et al., 2017). The standard laboratory ME49 strain used in this study was maintained in cell culture with an unknown number of passages before used in mouse infections. In contrast, strain CZ-Tiger was conserved and applied as oocysts for infections of mice and cats. Therefore, it is hypothesized that ME49 showed a decreased pathogenicity by cell culture attenuation in contrast to the CZ-Tiger strain in this study.

The trend of a dose-dependent effect after oocyst infection with CZ-Tiger appears much clearer in chickens than in turkeys. It can be assumed that this is related to the distinct organ size differences, especially for muscles, between the two poultry species upon examinations. Accordingly, the concentration of tissue cysts in the same amount of sample material should be lower in turkeys given the same infection dose. Thus, the probability of finding *T. gondii* using PCR in a restricted portion of the homogenized tissue might be lower in turkeys than in chickens. This may have caused a more variable dose-effect relation curve considering the limited animal numbers analyzed per group. However, the general trend of a positive dose-effect correlation was visible in our experiments.

Brain and heart are described as the predilection sites for *T. gondii* in many species (Bartova et al., 2004; Esteban-Redondo et al., 1999; Gisbert Algaba et al., 2018; Juránková et al., 2014; Koethe et al., 2015; Schares et al., 2018). In general, *T. gondii* DNA was found most frequently in heart and brain compared with all tested organs of both studied host species, with brain being the most targeted organ in turkeys, and heart in chickens respectively, regardless of the infection strain. These observations are partly differing from previous studies (Biancifiori et al., 1986; Dubey et al., 1993b; Kaneto et al., 1997; Koethe et al., 2015; Sedlak and Franti, 2000) that are employing a variety of analytical methods. Our results are based on sample analysis by conventional PCR, utilizing a small amount of homogenized tissue for DNA extraction. The comparison of our data with results from Koethe et al. (2015) and Schares et al. (2018), who partly analyzed up to 100 g of the current sample material with MC-PCR and pepsin-digestion RT PCR on acidic pepsin digested chicken tissue (PD-RT PCR), show a much higher proportion of *T. gondii* positive tissues than found in the present study. This outcome indicates that in this study the number of positive organs in both host species is clearly underestimated due to methodical limitations in sample size used for DNA extraction. Nonetheless, considering the high amount of analyzed samples to obtain comprehensive systemic distribution data on the bird organisms, the PCR used was a pragmatic approach allowing to screen all samples.

As of today, little is known on the distribution of *T. gondii* in chickens and especially in turkeys after infection with tissue cysts. The current study could show that the infection with tissue cysts compared with low and medium dose oocyst infections are resulting in similar abundance of *T. gondii*-positive tissues in chickens and turkeys. However, the observations of group differences regarding the number of positive organs in animals infected with tissue cysts have to be interpreted with caution. Tissue cysts for infection were not enumerated before use, and 6 total mice brains per 6 chickens or turkeys were pooled and applied evenly to the birds of each group. The rationale behind was that poultry as omnivores are likely to catch mice on farms and then consume a whole brain, so could the selected infection regimen simulated the natural infection process. Hence, the amount of fed tissue cysts between the study groups possibly varied. Nevertheless, infections with tissue cyst numbers resembling natural conditions caused detectable *T. gondii* infections in tissues of turkeys and chickens. Consequently, a risk for human infection with *T. gondii* due to consumption of chicken and turkey meat can be anticipated. This is emphasized by our finding that at least half of the type II strain infected animals featured *T. gondii* PCR positive edible organs. Similar or even higher rates of detection were described in turkeys before (Bangoura et al., 2013; Geuthner et al., 2014; Zöller et al., 2013).

In terms of human *T. gondii* infection risk related to consumption of undercooked poultry products, it should be noted that gizzard – which is consumed regularly by humans – was the third most often PCR positive organ after heart and brain in CZ-Tiger infected animals in this experiment. Gizzard was not tested in other experimental infections of poultry except in previous studies of our working group (Bangoura et al., 2013; Geuthner et al., 2014; Zöller et al., 2013). Our currently detected high proportion of *T. gondii* positive gizzards in 41.7% of the chickens and 33.3% of the turkeys may be credited to the morphology of the organ since a gizzard is a well-circulated muscle, and indicates a potential consumers' risk. Nonetheless, it should be mentioned, that the risk is maybe decreased in conventionally raised chickens and turkeys in Europe, due to the common practice of feeding anticoccidials. *T. gondii* DNA was detected in a considerable proportion of the infected animals, including a high number of their gonads. Though literature is divided on a potential vertical transmission of *T. gondii* in birds (Biancifiori et al., 1986; Khademi et al., 2018; Pande et al., 1961), our current and previous data (Bangoura et al., 2013) indicate that there may be a need for further investigation.

5. Conclusion

In conclusion, our study demonstrated the impact of the infection strain and doses on seroconversion as well as on the amount of *T. gondii* positive organs in chickens and turkeys. We could show lesser virulence of the type III strain NED in poultry in contrast to type II strains. Nevertheless, the risk for human infection with *T. gondii* after consumption of products from type III infected animals cannot be excluded. Our data show clear evidence that heart and brain are possibly not the only predilection sites for *T. gondii* in turkeys and chickens, but also organs like gizzard that pose a risk for human *T. gondii* infection. Furthermore, the study could give further evidence that the second natural route of infection with tissue cysts will lead to *T. gondii* in different organs in poultry.

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3.3. Publikation 3

Quantitative detection of *Toxoplasma gondii* in tissue of experimental infected turkeys and in retail turkey products by magnetic-capture PCR.

Koethe M, Straubinger RK, Pott S, Bangoura B, Geuthner A-C, Daugschies A, Ludewig M. Quantitative detection of *Toxoplasma gondii* in tissue of experimental infected turkeys and in retail turkey products by magnetic-capture PCR. Food Microbiol. 2015; 52:11-7. doi: 10.1016/j.fm.2015.06.005. Epub 2015 Jun 20.

Erklärung zum Eigenanteil

Die Versuchsplanung wurde von Herrn Dr. Martin Köthe vorgenommen.

Meinerseits erfolgte die Herstellung der zur Infektion der Hühner genutzten Oozysten durch Passage in Katzen inkl. Aufreinigung des Katzenkotes. Ebenfalls generierte ich Gewebezysten durch Infektion von Mäusen, Tötung und Präparation der Gehirne und Tachyzoiten durch Zellkulturpassagen. Eigenverantwortlich führte ich die Infektionen der Versuchstiere (Mäuse, Katzen, Puten), die Blutentnahme, Serumgewinnung, Schlachtung, Präparation und Homogenisierung der in der Publikation untersuchten Organe durch. Bei der Präparation und Homogenisierung der Organe erhielt ich Unterstützung durch die Mitarbeiter des Institutes für Parasitologie (Herr Frank Stöckel, Frau Manja Etzold, Frau Juliane Hintzen, Frau Irene Malkwitz, Frau Eva-Maria Mäßig, Frau Linda Katzer, Frau Cora Delling, Frau Ira Dresely). Die Betreuung der Versuchstiere erfolgte durch die Tierpfleger (Herr Rene Schuhmacher, Frau Marion Fritsche) des Institutes für Parasitologie.

Die *magnetic-capture real-time* PCR inklusive der zugehörigen DNA-Extraktion der untersuchten Organe wurde von Herrn Dr. Martin Köthe unter meiner Beteiligung durchgeführt.

Die Auswertung der Ergebnisse und die Erstellung des Manuskriptes wurde von Herrn Dr. Martin Köthe durchführt.

Die Revision des Manuskriptes erfolgte in Zusammenarbeit aller genannten Autoren.

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Quantitative detection of *Toxoplasma gondii* in tissues of experimentally infected turkeys and in retail turkey products by magnetic-capture PCR

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ABSTRACT

Magnetic-capture PCR was applied for the quantitative detection of *Toxoplasma gondii* in tissues of experimentally infected turkeys and retail turkey meat products. For experimental infection, three *T. gondii* strains (ME49, CZ-Tiger, NED), varying infectious doses in different matrices (organisms in single mouse brains or 10^3, 10^5, or 10^6 oocysts in buffer) were used. From all animals, breast, thigh, and drumstick muscle tissues and for CZ-Tiger-infected animals additionally brains and hearts were analyzed. Using the magnetic-capture PCR large volumes of up to 100 g were examined. Our results show that most *T. gondii* parasites are present in brain and heart tissue. Of the three skeletal muscle types, drumsticks were affected at the highest and breast at the lowest level. Type III strain (NED) seems to be less efficient in infecting turkeys compared to type II strains, because only few tissues of NED infected animals contained *T. gondii* DNA. Furthermore, the number of detected parasitic stages increased with the level of infectious dose. Infection mode by either oocyst or tissue cyst stage did not have an effect on the amount of *T. gondii* present in tissues. In retail turkey meat products *T. gondii* DNA was not detectable although a contact with the parasite was inferred by serology.

1. Introduction

Toxoplasma gondii is one of the most common protozoan parasites worldwide. It is assumed to be able to infect all warm-blooded species while felids represent its definitive host. Infection of humans can remain asymptomatic, particularly in immunocompetent hosts, but can also lead to severe toxoplasmosis in immunocompromised patients. Especially at risk are seronegative pregnant women, because an infection with the parasite can severely damage the unborn child (Weiss and Dubey, 2009). Humans mainly get infected by the consumption of raw or undercooked meat from infected animals, in which the parasites persist within tissue cysts in tissues such as muscles and brain. Turkey meat is very popular in some regions of the world. Most turkey meat is consumed in the USA with 7.4 kg/capita in 2012, followed by Austria (6.1 kg), Germany (5.7 kg), France (5.3 kg), and Italy (4.8 kg) (AVEC, 2013). Turkey meat has been identified as a risk factor for *T. gondii* infection (Alvarado-Esquivel et al., 2006, 2008). However, there is only little information available about the presence of *T. gondii* in turkeys. After a fatal toxoplasmosis was reported in a wild turkey (Howerth and Rodenroth, 1985), Dubey et al. (1993a) experimentally infected 14 turkeys with *T. gondii* strain ME49 for the first time and bioassayed brain, breast muscle, leg muscle, liver, and heart. Bioassays were positive for heart and muscles. Sedlák et al. (2000) also performed experimental infection of five turkeys resulting in positive bioassays of pooled brain, liver, spleen, heart, and leg muscles. Several serological studies have been published showing seroprevalences from 10.0 % to 76.6 % in wild or private turkeys in Iran, USA, Egypt, and Iraq (Butty, 2009; El-Massry et al., 2000; Ghorbani et al., 1990; Harfoush and Tahoon, 2010; Lindsay et al., 1994; Quist et al., 1995) while in the Czech Republic no seropositive turkeys were found (Bártová et al., 2009; Literák and Hejlícek, 1993). We have previously published results on fattening turkeys in Germany intended for human consumption.

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Average seroprevalence in these conventionally indoor raised animals was 18.4% but in individual fattening cycles it was as high as 77.1% indicating intensive contact of the turkeys with the parasite (Koethe et al., 2011). Furthermore, we could show by nested PCR that many tissues were parasitized after experimental, oral or parenteral *T. gondii* infection with oocysts or tachyzoites, respectively (Bangoura et al., 2013; Zöller et al., 2013). Previous results are all based on conventional DNA extraction methods, where only as little as about 25 mg of tissue could be used as starting material. Having in mind that *T. gondii* accumulates in tissue cysts that are not equally distributed within tissues it is obvious that with conventional DNA extraction methods *T. gondii* detection and quantification may be flawed. In this context, Opsteegh et al. (2010) described a DNA extraction method by which *T. gondii* DNA sequences specifically are captured using magnetic beads and thereby making it possible to examine large samples of up to 100 g. Accurate and reliable quantification of *T. gondii* equivalents in the samples is possible by subsequent quantitative real-time PCR that has been performed on pig meat and sheep heart samples (Opsteegh et al., 2010). This method was further applied to pig, goat, and mice tissues and organs (Juránková et al., 2014, 2013a, 2013c) as well as for quantitative detection of *T. gondii* in Serrano ham (Gomez-Samblas et al., 2015) and has been confirmed to be a highly sensitive and specific method.

The aim of our study was to apply the sequence-specific magnetic-capture PCR method to quantify the amount of *T. gondii* equivalents in different tissues and organs of experimentally infected turkeys and to examine whether or not there are differences regarding the parasite strain or infection dose or material. Either brains of infected mice or oocysts shed by infected cats were used for infection to mimic natural infection routes. We mainly focused on different muscle tissues that are relevant for human consumption but particularly also examined brains and hearts for comparison. Furthermore, retail turkey meat and meat products were analyzed to assess a potential risk of infection by the consumption of such products.

2. Materials and methods

2.1. Parasites

Three different *T. gondii* strains were used in the study: CZ-Tiger (Juránková et al., 2013c, oocysts kindly provided by Walter Basso, Institute of Parasitology, University of Zurich, Switzerland) and ME49 (Lunde and Jacobs, 1983) as type II strains; NED (Howe and Sibley, 1995) as type III strain. Tachyzoites of ME49 and NED strains were maintained in VERO cell culture in IMDM medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphotericin B at 37 °C and 5% CO_2 as previously described (Zintl et al., 2009). Cell culture supernatants were centrifuged at 2000 × *g* for 5 min, the resulting pellet was resolved in 1 ml PBS and tachyzoites were counted in a Neubauer chamber. Six CD1 mice were intraperitoneally infected with either 2000 ME49 tachyzoites (four mice) or 1500 NED tachyzoites (two mice). Two CD1 mice were infected with 100 CZ-Tiger oocysts orally. Cats were fed with infected mouse brains to passage the parasites and yield sufficient amounts of oocysts. In detail, two cats each were fed with one brain of a mouse infected with the CZ-Tiger strain, another two cats each were fed with one NED-infected mouse brain and two other cats each received two ME49-infected mouse brains. To obtain brain tissue cysts for turkey infections, each six CD1 mice were infected with 10–100 oocysts orally or 100–2000 tachyzoites intraperitoneally of the respective strains. Mouse infections were verified by light microscopically examination of brain squashes and by serology. Infections of cats and mice met legal requirements and were granted by the competent authority (Landesdirektion Leipzig, Germany, trial no. V09/12).

2.2. Animals

BUT B.I.G. 6 turkeys were stabled as one-day-old chicks and raised at the Institute of Parasitology, University of Leipzig where they were kept on a bedding of wood shavings and underwent daily clinical observations. A total of 48 infected and 6 uninfected turkeys were included in the study. Turkeys were fed with poultry feed for pet birds without anticoccidials (deuka Wild-und Ziergeflügelfutter, Deutsche Tiernahrung Cremer, Germany) which was supplemented with vitamins, minerals (Korvimin ZVT + Reptil, WDT, Germany), and powdered milk to obtain recommended nutritional values for turkeys. Feed and water were available *ad libitum*.

2.3. Experimental study design

Turkeys were divided into nine groups of six animals each. Eight groups were infected with different doses (10^3–10^6 oocysts or tissue cysts within one mouse brain), strains and stages while one group remained uninfected to serve as negative control group as outlined in Table 1. Oocysts or mouse brains were applied orally into the crop of the turkeys. Eight weeks after the day of infection, animals were sacrificed and tissue samples were collected and stored frozen at −20 °C for examination. Infection experiments complied with legal regulations and were approved by the responsible authority (Landesdirektion Leipzig, Germany, trial no. TVV 29/10).

2.4. Serology

Sera of experimentally infected turkeys were examined for seroconversion to confirm infection or absence of infection in control group animals, respectively. Blood was drawn from the wing vein (*Vena cutanea ulnaris*) prior to infection and in weekly intervals thereafter. After blood clotting, sera were separated by centrifugation at 2600 × *g* for 15 min at room temperature and collected thereafter. Serum samples were stored frozen at −20 °C until used for testing. Kinetic ELISA (KELA) was performed on turkey sera as described previously (Koethe et al., 2011). All infected animals seroconverted within the first two weeks after infection while all uninfected turkeys remained seronegative throughout the study.

From animals killed at the abattoir, blood was collected at the bleeding station into a sterile 50 ml tube. During transportation to the Institute of Food Hygiene, blood clotted and subsequently serum was obtained and frozen the same day. Afterward, sera were also examined by the above mentioned KELA.

From wholesale breast fillets, meat fluid was used for serologically examination instead of serum. KELA with meat fluid was performed accordingly as described previously (Koethe et al., 2011).

2.5. Samples

Focusing mainly on meat, breast muscles, thigh muscles, and lower leg (drumstick) muscles from all experimentally infected turkeys were examined. For comparison, brains and hearts from CZ-Tiger infected turkeys were examined additionally. Of the muscles, 100 g were cut into pieces with edges of approximately 2 cm in length. Complete brains were homogenized with a mortar and pestles and complete hearts were ground with a commercial household blender (La Moulinette, Tefal Groupe SEB, Germany). Fractions of the homogenates (mean weights were 1.8 g for brains and 17.4 g for hearts) were subjected to DNA capture.

Table 1
Study design.

Group (n = 6)[a]	*T. gondii* infection		Samples
	Strain	Dose	
A1	ME49	10^3 oocysts	Breast, thigh, drumstick muscles
B1	CZ-Tiger	10^3 oocysts	Breast, thigh, drumstick muscles, brain, heart
B2	CZ-Tiger	10^5 oocysts	Breast, thigh, drumstick muscles, brain, heart
B3	CZ-Tiger	10^6 oocysts	Breast, thigh, drumstick muscles, brain, heart
C1	NED	10^3 oocysts	Breast, thigh, drumstick muscles
D1	ME49	1 mouse brain	Breast, thigh, drumstick muscles
D2	CZ-Tiger	1 mouse brain	Breast, thigh, drumstick muscles, brain, heart
D3	NED	1 mouse brain	Breast, thigh, drumstick muscles
NC	Uninfected		Breast, thigh, drumstick muscles, brain, heart

[a] One animal of group C1 and two NC animals died from reasons other than toxoplasmosis. No samples could be examined from the two NC animals, which died prematurely.

Furthermore, 23 turkey meat products were purchased at supermarkets and subjected to examination. These comprised 7× meat cutlets, 6× minced meat, 4× cured, smoked breast fillet, 2× German cured, slightly fermented, spreadable sausage ("Zwiebelmettwurst"), 2× stripe-cut meat, 1× sliced, air-dried, cured ham, and 1× breast fillet. Further, 15× breast fillet were purchased at wholesale level.

Finally, a total of 479 turkeys were sampled at the abattoir. In one batch, 241 turkeys were sampled, i.e. breast muscles and blood were taken. Out of these animals, 21 were serologically positive and three showed equivocal antibody levels. The respective breast muscle samples of those 24 animals as well as of seven seronegative animals were subjected to the DNA capture method. In another batch, 238 birds were sampled for breast muscle, blood, brain, and heart. To obtain brains, total heads were collected and transported cooled to the Institute where brains were dissected from the heads. From 46 seropositive turkeys of this batch 44 breast muscles, 44 brains (mean weight: 6.2 g), and 45 hearts (mean weight: 42.2 g) were available. These as well as all three samples from two uninfected turkeys were further examined for *T. gondii* DNA. Examined retail, wholesale and abattoir samples are listed in Table 2.

2.6. DNA capture and real-time PCR

Samples were prepared as described above and further processed according to the protocol provided by Opsteegh et al. (2010) with minor modifications. For each muscle tissue sample, a new, sterile scalpel was used to cut it into pieces instead of reusing cleaned knives. Brains of abattoir animals were squeezed with a clean, small plastic spoon and hearts were ground with a professional lab blender (Grindomix GM 200, Retsch GmbH, Germany). An alignment of the two available sequences for the repetitive 529 bp fragment (AF146527 and AF487550) revealed a one-base variability of A and G within the binding sequence of primer Tox11R. Therefore, sequence of that primer was modified (Tox-SC revers, 5′-GCGTCGTCTCGTCTRGAT-3′). Additionally, the difference in primer melting temperature of originally 8.7 °C was lowered down to 4.7 °C by redesigning the forward primer (Tox-SC forward, 5′-GAGGGGGTGGCGTGGTT-3′). The competitive internal amplification control (CIAC) was not included in the PCR assay which was performed on a StepOnePlus cycler (Applied Biosystems, Germany) in 96-well plates. A 27.5-µl reaction mixture contained PCR grade water, 2.5 µl DNA, 5.5 mM $MgCl_2$, 1× Gold Buffer, 900 nM of each primer, 200 nm Tox-TP1 probe, 0.2 mM dATP, dCTP, and dGTP, 0.4 mM dUTP, 0.01 U of uracil-N-glycosylase (UNG) per µl, and 0.025 U/µl Gold Taq polymerase (Life Technologies, Germany). The cycling program consisted of 10 min UNG action at 50 °C, 10 min initial denaturation at 95 °C followed by 45 cycles of denaturation (15 s at 95 °C), annealing (30 s at 50 °C), and elongation (15 s at 72 °C). For quantification a standard series was developed by adding serially diluted tachyzoites (10^6 to 10^2) to each 100 g breast muscle meat. The capture-extracted DNA of these standards was included into every PCR plate to calculate the amount of *T. gondii* in the examined samples. The 10^3 standard certainly gave reproducible results and at times also the 10^2 standard was detected. Therefore, the sensitivity of our assay is between 10^2 and 10^3 which is the amount of single stages within a tissue cyst. This means if there is a tissue cyst present in 100 g of sample material it will be

Table 2
Magnetic capture PCR results from retail, wholesale, and abattoir turkey samples for the presence of *T. gondii* DNA.

Sample	Origin	MC-PCR result[a]	Antibody detection[b]
Turkey breast fillet	Retail	0/1	na
Turkey meat cutlets	Retail	0/7	na
Turkey meat strips	Retail	0/2	na
Turkey minced meat	Retail	0/6	na
Smoked, cured turkey breast fillet	Retail	0/4	na
Sliced, air-dried, cured turkey ham	Retail	0/1	na
Turkey "Zwiebelmettwurst"[c]	Retail	0/2	na
Turkey breast meat	Wholesale	0/11	Meat fluid: negative
Turkey breast meat	Wholesale	0/4	nd
Turkey breast meat	Abattoir	0/65	Serum: positive
Turkey breast meat	Abattoir	0/12	Serum: questionable or negative
Turkey heart	Abattoir	0/45	Serum: positive
Turkey heart	Abattoir	0/2	Serum: negative
Turkey brain	Abattoir	0/44	Serum: positive
Turkey brain	Abattoir	0/2	Serum: negative

[a] No. positive/No. examined.
[b] na – not applicable, nd – not done.
[c] Cured, slightly fermented, spreadable sausage.

detected in most cases. A positive (10^3 tachyzoites in 100 g breast muscle) and a negative (100 g breast muscle without tachyzoites) extraction control were also included.

2.7. Statistics

Prism for Windows 4.00 software (GraphPad Inc., USA) was used for statistical analysis of results. Gaussian distribution of data was assessed by Kolmogorov–Smirnov test. Logarithmic transformation resulted in normally distributed data. One-way ANOVA was performed to reveal global differences. Where applicable, Bonferroni test was subsequently applied to check pairs of groups for differences. When only two groups were compared, t-test was applied instead of ANOVA. Differences were considered statistically significant at an error level of $p < 0.05$.

3. Results

3.1. Experimental infection

3.1.1. Impact of strains

All samples of the NC group animals were negative. To check whether or not the choice of *T. gondii* strain had an impact on the number of parasites in different tissues groups A1, B1, and C1 were compared. Animals of these groups had been infected with 10^3 oocysts of strain ME49, CZ-Tiger, and NED, respectively. Qualitatively, differences between strains were revealed. While the type II strains ME49 and CZ-Tiger were similarly often positive for *T. gondii*, only one of the NED infected animals showed parasite DNA in the examined muscle tissues. In Fig. 1 the quantitative results are shown. Four ME49-infected and three CZ-Tiger-infected animals had *T. gondii* DNA in their breast muscles (Fig. 1A) at a $\log_{10}$ quantity of 2.25 and 2.57 per sample, respectively. The quantity in the breast muscle of the single NED infected turkey was 4.27 $\log_{10}$. Since statistical analysis is not reasonable and not possible to compute when there is a group with only one value, t-test was applied to compare only ME49 and CZ-Tiger. No statistically significant differences were observed ($p = 0.71$). However, the single NED value was considerably higher than all others. Comparable results were obtained for thigh and drumstick muscles (Fig. 1B and C). There were no statistically significant differences between ME49 and CZ-Tiger while the single NED value was higher in every case.

When turkeys had been infected with tissue cysts by administration of a whole brain of an infected mouse, qualitatively, ME49 parasitized the examined muscle tissues most often (4 times breast, 3 times thigh, 5 times drumstick muscles). Although no CZ-Tiger strain *T. gondii* DNA was found in breast muscles, identical numbers of positive tissues were obtained in the other tissues compared to ME49. Type III strain NED again was detected in the fewest numbers of tissues (1, 3, 2 times, respectively). Quantitative results are presented in Fig. 2. For breast muscles, statistical analysis was not computable since one group had zero and one group only one result. However, the ME49 group showed more parasites per sample (2.61 $\log_{10}$) than NED (0.34 $\log_{10}$). In thigh muscles there were no statistically significant differences between all groups as determined by ANOVA ($p = 0.62$). The mean $\log_{10}$ quantities were 2.74, 3.28, and 2.15 for ME49, CZ-Tiger, and NED, respectively. Analogous but higher results were obtained in drumstick muscles. No statistically significant differences were found (ANOVA, $p = 0.17$) and mean $\log_{10}$ quantities were 3.29, 4.74, and 3.52, respectively.

3.1.2. Impact of infection dose

Experimentally infections with CZ-Tiger oocysts were performed with three different doses, namely 10^3, 10^5, and 10^6. As shown in Fig. 3, animals infected with the low dose of 10^3 oocysts harbored the lowest number of parasites in all examined tissues. However, the differences were not statistically significant for breast, thigh, and drumstick muscle tissues (ANOVA; $p = 0.20$, 0.27, and 0.19, respectively). In hearts, there were significantly fewer parasites per sample in 10^3 group (4.90 $\log_{10}$) compared to both others (5.55 and 5.44 $\log_{10}$, respectively; $p < 0.05$ each). In brains, 10^6 group (5.89 $\log_{10}$) showed significantly higher results compared to 10^5 (5.06 $\log_{10}$; $p < 0.05$) and to 10^3 (4.52 $\log_{10}$; $p < 0.01$). However, 10^3 and 10^5 results did not differ statistically.

3.1.3. Impact of infection material

It was compared if the type of infection material influences the quantitative result of *T. gondii* detection in different muscle tissues. Data are illustrated in Fig. 4. On a qualitative level it became obvious that NED-infected turkeys showed *T. gondii* DNA in muscle tissues clearly less often than ME49-and CZ-Tiger-infected animals (see figures above columns in Fig. 4). Because of the low number of positive tissues in NED-infected animals no statistical analysis was computed for these groups. Furthermore, the breast muscle groups of CZ-Tiger have not been analyzed because of zero positive samples from tissue cyst-infected turkeys. All other groups were compared pairwise by t-test to check for significant differences between oocysts- and tissue cyst-infected animals. No differences were revealed at all and this is also true for heart and brain of CZ-Tiger-infected turkeys (data not shown).

3.1.4. Parasite distribution in sample tissues

To reveal potential differences in tissue distribution of *T. gondii*, results were grouped by tissue and compared statistically. Results of animals infected with 10^3 oocysts are depicted in Fig. 5. Heart and brain could only have been examined from CZ-Tiger infected animals. There, these both tissues were quantitatively most

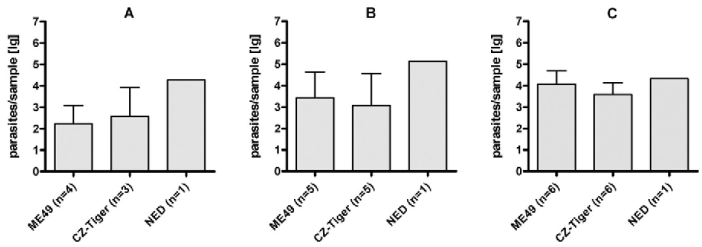

Fig. 1. Quantitative detection of *T. gondii* DNA in breast (A), thigh (B), and drumstick (C) muscle tissues of turkeys experimentally infected with 10^3 oocysts of the ME49, CZ-Tiger, or NED strain. Results are expressed as $\log_{10}$ values.

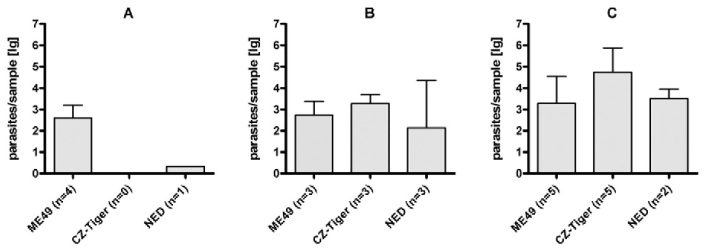

Fig. 2. Quantitative detection of *T. gondii* DNA in breast (A), thigh (B), and drumstick (C) muscle tissues of turkeys experimentally infected with one brain of a mouse infected with ME49, CZ-Tiger, or NED strain. Results are expressed as $\log_{10}$ values.

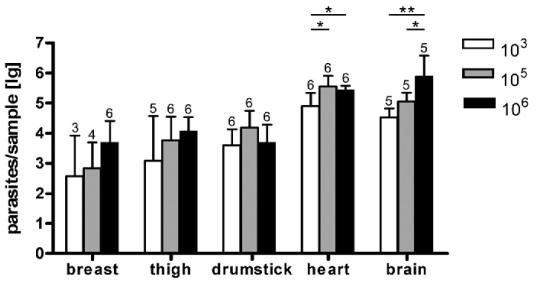

Fig. 3. Quantitative detection of *T. gondii* DNA in breast, thigh, drumstick muscle, heart, and brain tissues of turkeys experimentally infected with 10^3 (white), 10^5 (gray), or 10^6 oocysts (black) of CZ-Tiger strain. Results are expressed as $\log_{10}$ values. Figures above columns indicate number of positive samples. Asterisks indicates statistically significant differences (* = $p < 0.05$; ** = $p < 0.01$).

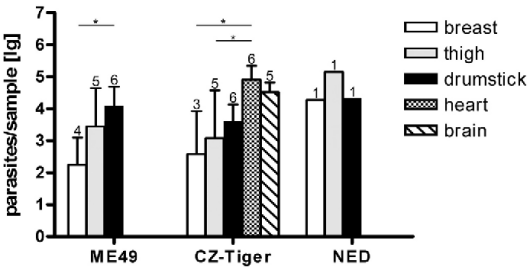

Fig. 5. Quantitative detection of *T. gondii* DNA in breast, thigh, and drumstick muscle, heart, and brain tissues of turkeys experimentally infected with 10^3 oocysts of ME49, CZ-Tiger, or NED strain. Results are expressed as $\log_{10}$ values. Figures above columns indicate number of positive samples. Asterisks indicate statistically significant differences ($p < 0.05$).

affected. Results in hearts (4.90 $\log_{10}$) were significantly higher than in breast (2.57 $\log_{10}$) and thigh (3.08 $\log_{10}$) muscles (ANOVA; $p < 0.05$). At higher infectious doses (10^5 and 10^6) heart results were always statistically higher than all muscle tissues and brain results were at least statistically higher than breast and thigh muscle results (data not shown). Besides heart and brain, drumstick muscles showed the highest extent of parasitization. In ME49-infected animals, results in drumstick muscles were significantly higher than in breast muscles (4.08 $\log_{10}$ vs. 2.25 $\log_{10}$; see Fig. 5; ANOVA; $p < 0.05$). Out of the six examined NED-infected animals only one presented parasites in the analyzed muscle tissues. Statistical comparisons were therefore not performed with those results.

3.2. Natural infection

3.2.1. DNA detection in products

At retail level, none of the 23 examined products (meat cutlets, minced meat, cured and smoked breast fillet, "Zwiebelmettwurst", stripe-cut meat, sliced, air-dried and cured ham, and breast fillet) contained detectable *T. gondii* DNA. At wholesale level, 15 breast fillets were examined, 11 of which have been tested negative for the presence of anti-*T. gondii* antibodies in meat fluid. Again, in none of those samples *T. gondii* DNA was detected. Finally, breast muscles, hearts and brains were directly collected at an abattoir and those tissues were tested from all seropositive and a few seronegative animals. *T. gondii* DNA was neither detected from seronegative nor from seropositive animals in any tissue. Detailed results are listed in Table 2.

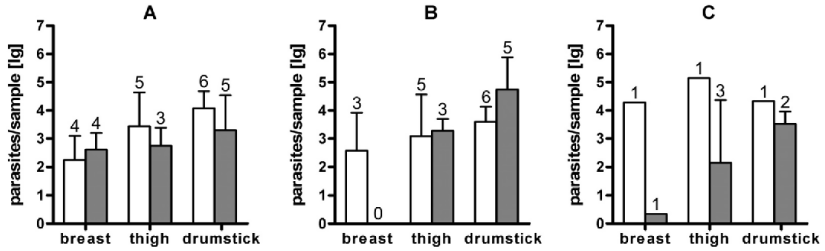

Fig. 4. Quantitative detection of *T. gondii* DNA in breast, thigh, and drumstick muscle tissues of turkeys experimentally infected with 10^3 oocysts (white) or with the brain of a mouse (gray) infected with ME49 (A), CZ-Tiger (B), or NED (C) strain. Results are expressed as $\log_{10}$ values. Figures above columns indicate number of positive samples.

4. Discussion

As previously reported by others (Dubey et al., 1993a; Sedlák et al., 2000) and recently found by our working group (Bangoura et al., 2013; Geuthner et al., 2014; Zöller et al., 2013) we could show that turkeys can be easily infected with *T. gondii* and seroconverted within 7–14 days post infection. Furthermore, it became obvious that there are differences in the frequency of infested tissues depending on the strain used for infection. In turkeys infected with type II strains the analyzed tissues were more often parasitized than in type III strain-infected animals. The effect of different *T. gondii* strains on the quantitative burden in different tissues has not been examined thoroughly to the best of our knowledge so far. Most publications describe infection experiments using only one strain or strains from the same type whereas especially type III strains have been used rarely. Suzuki and Joh (1994) compared two type II and one type III strains regarding the number of cysts in brains of infected mice. Their results support our findings since the type III strain presented significantly less cysts in the brain than one of the type II strains. However, the cyst amount did not differ between type III and the other type II strain. Dubey (1988) reported presence of *T. gondii* in tissues of pigs which were comparatively infected with four different strains. However, it could only be shown that an avirulent strain (genotype not determined) was least infective compared to another avirulent strain and each one type I and II strain but no further comparisons between the strains have been made.

Regardless of the deployed *T. gondii* strains and infectious material used in our study, breast muscles were qualitatively and quantitatively least affected of all tissues tested. This is in accordance with our recently published results on oocysts-infected turkeys, where only three out of 36 animals infected with different type II strains showed parasite DNA in breast muscles as examined by nested PCR (Bangoura et al., 2013). In five experimentally infected chickens, Dubey et al. (1993b) did also not detect infective *T. gondii* in breast muscles.

Dubey et al. (1993a) reported that they found hearts from turkeys infected with oocysts of ME49 strain most frequently affected, followed by drumstick muscles. None of the examined brains have been positive in their study. Our results are in agreement with their results, since from the three muscle tissues that we analyzed from ME49 infected animals, drumstick muscles were affected most often and with highest parasites density. However, although we did not analyze brains from ME49 infected animals, the CZ-tiger infected turkeys showed high amounts of up to 10^6 parasites in brains. This complies with a report from chickens where Dubey et al. (1993b) demonstrated infective brain cysts by bioassay in all of five infected animals. However, previous experimental infections in turkeys gave contradictory results since Dubey et al. (1993a) did not find *T. gondii* in the brain of ME49 infected turkeys at all. This may be attributed to variable strain specific predilection sites or general variation found in relatively low case numbers as well in the referenced study as in our own trial. In general, hearts and brains are often found to be positive or even predilection site for *T. gondii* in many species (Dubey, 2010; Jurànková et al., 2013a). This may be due to a higher extent of blood circulation in those tissues and, therefore, a higher extent of dissemination during parasitemia.

The number of *T. gondii* in brains and hearts were partly significantly higher in turkeys exposed to higher doses compared to those infected with lower doses of oocysts. Although there is no quantitative data for comparison of different infectious doses available, Esteban-Redondo et al. (1999) reported that sheep infected with 10^5 oocysts had *T. gondii*-positive brains more frequently than those infected with only 10^3 oocysts. Dubey et al. (1993b) infected chickens with 10^3 or 10^4 oocysts of ME49 strain. They did not report significant differences in the number of infected animals as analyzed by mouse bioassay of pools of breast, leg, heart, and brain tissues. Maybe the addition of 9000 oocysts is not large enough to infect tissues more frequently, while the difference between 10^3 and 10^5 means an application of additional 99,000 oocysts which may have led to an increased infection rate as we could show in the case of hearts of CZ-Tiger infected turkeys. However, in another study using the type III strain VEG to experimentally infect pigs positive tissues were found more frequently from animals infected with 10 oocysts compared to those infected with only 1 oocyst (Dubey et al., 1996). A semi-quantitative approach was done in rats. They were inoculated with 10^1 to 10^6 oocysts of the type III VEG strain. The number of tissue cysts in brains of the infected animals was calculated based on counts of a small brain smear. In general, it was shown that there were more tissues cysts in the brains of animals that were infected with higher doses (Dubey, 1996).

None of the examined turkey products contained detectable *T. gondii* parasites. For the retail products this may be explained by the use of meat from uninfected animals for production of those products. However, for the samples from abattoir this was surprising since also none of pre-selected seropositive animals were *T. gondii* DNA-positive in brains, hearts, or breast muscles. The fact that breast muscles were negative could be explained by the experimental data where this tissue was also least affected. Brains and hearts, however, were expected to be positive. A possible explanation could be the husbandry especially regarding the feeding of conventionally raised turkeys in Germany. They are fed conventional feed that contains anticoccidials. Although these medicals are tested to be effective *in vivo* against *Eimeria* one can assume that they could also affect *Toxoplasma*. Currently, three substances are approved to be used as feed additives for turkeys by Regulation (EC) No 1831/2003: Monensin-Sodium, Lasalocid-Sodium and Diclazuril. All have been demonstrated to be effective against *T. gondii in vitro* (Lindsay and Blagburn, 1994; Melton and Sheffield, 1975; Ricketts and Pfefferkorn, 1993). When turkeys fed with these additives get infected with *T. gondii* the immune system could react against the parasite and detectable antibodies could be produced. That immune reaction occurs in presence of anticoccidials is supported by investigations of Lee et al. (2012) who reported specific antibodies in serum against challenging *Eimeria* in chickens treated with different anticoccidials. However these treatments were not able to totally inhibit *Eimeria* oocyst shedding. *T. gondii* is not adapted on host species to the extent *Eimeria* species are. So if anticoccidials better prevent further *T. gondii* dissemination and infection of cells in the turkeys, tissue cysts may not develop. This would be an explanation of our observed results in retail products.

5. Conclusions

Magnetic-capture PCR provides a powerful tool for quantification of *T. gondii* parasites in tissues. The main advantage is the use of large amounts of samples up to 100 g compared to about 25 mg that can be examined by conventional DNA extraction methods. The method was first applied to isolate *T. gondii* DNA from experimentally infected pigs and naturally infected sheep by Opsteegh et al. (Opsteegh et al., 2010). Recently it was compared to conventional DNA extraction (Jurànková et al., 2014) and used for analysis of goat and pig infections (Jurànková et al., 2013a, 2013c). As described there, we can also confirm that the method is applicable for sensitive and specific detection of *T. gondii* in turkey tissues. However, because DNA is detected by this method no conclusion regarding viability or infectivity of the present parasites can be drawn. Based on our results from experimental infections there is

clear evidence that turkeys develop tissue cysts in muscle tissues, which are relevant for human consumption. Although *T. gondii* seems not to be present in conventionally raised turkeys in Germany despite these animals have had contact to the pathogen as revealed by serologic examination, a definitive absence of potential infection risk from turkey meat and turkey meat products (especially when they are raw or undercooked) cannot be stated.

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3.4. Publikation 4

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

Schares G, Koethe M, Bangoura B, Geuthner A-C, Randau F, Ludewig M, Maksimov P, Sens M, Bärwald A, Conraths FJ, Villena I, Aubert D, Opsteegh M, Van der Giessen J.
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Erklärung zum Eigenanteil

Die Versuchsplanung wurde von Herrn Dr. Gereon Schares vorgenommen.

Von mir wurden die zur Infektion der Hühner genutzten Oozysten durch Passage in Katzen inkl. Aufreinigung des Katzenkotes gewonnen. Ebenfalls generierte ich Gewebezysten durch Infektion von Mäusen, Tötung und Präparation der Gehirne und Tachyzoiten durch Zellkulturpassagen. Eigenverantwortlich führte ich die Infektionen der Versuchstiere (Mäuse, Katzen, Hühner), die Blutentnahme, Serumgewinnung, Schlachtung der experimentell infizierten Hühner, sowie die Präparation der Organe der experimentell infizierten Tiere durch. Bei der Schlachtung und Präparation der Organe erhielt ich Unterstützung durch die Mitarbeiter des Institutes für Parasitologie (Herr Dr. Frank Stöckel, Frau Dr. Manja Etzold, Frau Dr. Juliane Hintzen, Frau Dr. Irene Malkwitz, Frau Eva-Maria Mäßig, Frau Linda Katzer, Frau Dr. Cora Delling, Frau Ira Dresely). Die Betreuung der Versuchstiere erfolgte durch die Tierpfleger (Herr Rene Schuhmacher, Frau Marion Fritsche) des Institutes für Parasitologie.

Die *magnetic-capture real-time* PCR inclusive der zugehörigen DNA-Extraktion der untersuchten Organe und Auswertung der Ergebnisse wurde von Herrn Dr. Martin Köthe durchführt.

Die Durchführung des Bioassays, der serologischen Blut- sowie Fleischsaft-Untersuchungen und die statistische Auswertung der Daten erfolgte am Friedrich-Löffler-Institut. Ebenso die Erstellung des Manuskriptes.

Die Revision des Manuskriptes erfolgte in Zusammenarbeit aller genannten Autoren.

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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

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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

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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 2000g for 5 min, the resulting pellet was resolved in PBS and the

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,	CZ-Tiger	–	6	6
	1 mouse brain	ME49	–	5	5
	per bird	NED	–	5	5
Inf-3	Tachyzoites, 1×10^6	NED	6	6	12
Total	–	–	12	79	91

Inf, infection.

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

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, $P < 0.05$).

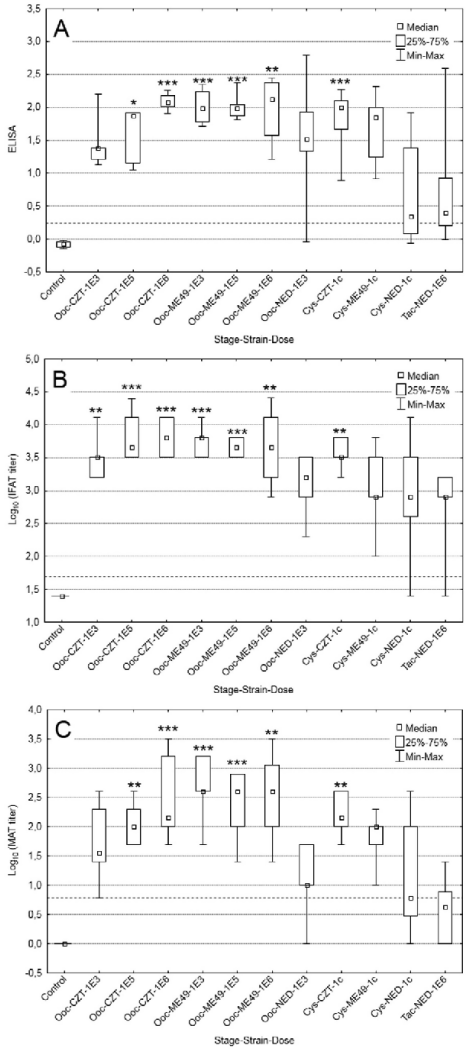

Fig. 1. Serological responses of chickens (median, 25–75% percentiles, minimum and maximum values) inoculated with *Toxoplasma gondii* oocysts of the CZ-Tiger strain 5 weeks after inoculation (Ooc-CZT-1E3, Ooc-CZT-1E5, Ooc-CZT-1E6: inoculation with 10^3, 10^5 or 10^6 oocysts), oocysts of the ME49 strain (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.

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.

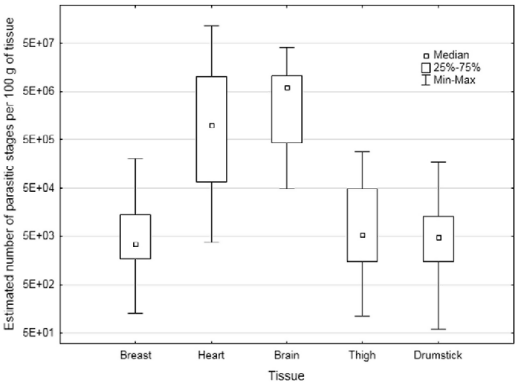

Fig. 2. Magnetic-Capture real-time PCR (MC-RT PCR) results for samples of chickens experimentally infected with *Toxoplasma gondii* oocysts or tissue cysts, 5 weeks after inoculation (median, 25–75% percentiles, minimum and maximum values). The parasite genome equivalents in MC-RT PCR-positive samples 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

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

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 ≥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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4. Diskussion

T. gondii infiziert zahlreiche warmblütige Tierarten und den Menschen. Dabei kann eine Infektion zu schwerwiegenden Erkrankungen bis hin zu Enzephalitis, Pneumonie und septischem Schock führen (MONTOYA und LIESENFELD 2004; DUBEY 2010b; DEMAR et al. 2012; HALONEN und WEISS 2013; SOBANSKI et al. 2013; ABBASI FARD et al. 2020).
Die Hauptinfektionsquelle des Menschen ist mit Gewebezysten kontaminiertes Fleisch (BUFFOLANO et al. 1996; KAPPERUD et al. 1996; BARIL et al. 1999; MORRIS et al. 1999; COOK et al. 2000; JONES et al. 2009 WILKING et al. 2016; SAID et al. 2017). Im Mittelpunkt der Übertragung von *T. gondii* stand bis vor einigen Jahren das Schwein als einer der wichtigsten Fleischlieferanten, welches durch Geflügel 2011 abgelöst wurde (OECD 2020). Der stetig steigende Bedarf für Geflügelfleisch wird überwiegend durch die Produktion von Hühner- und Putenfleisch gedeckt (AVEC 2019, 2020). In Studien zur Risikobetrachtung von humanen *T. gondii*-Infektionen rückte der Verzehr von Geflügelfleisch nach und nach in den Fokus (ALVARADO-ESQUIVEL et al. 2006, 2008; SAID et al. 2017). Ziel dieser Arbeit war es daher, ein Infektionsmodell für *T. gondii* bei Huhn und Pute unter Verwendung verschiedener *T. gondii*- Stämme und Infektionswege zu etablieren, sowie Einflussfaktoren auf die Gewebeverteilung und Antikörperantwort dieser Spezies zu prüfen. Da bisher kaum Erkenntnisse zur Persistenz von *T. gondii* im Gewebe von Geflügel vorlagen, wurde zuvor die Persistenz von *T. gondii* aus Geweben bei Huhn und Pute untersucht.

4.1. Klinische Beobachtungen

Hühner und Puten gelten als weitgehend resistent gegenüber klinischer Toxoplasmose (DUBEY et al. 1993a; SEDLÁK et al. 2000; DUBEY 2010a). Unter Nutzung verschiedener Stämme, Infektionsdosen und -stadien trat bei 84 Puten und 72 Hühnern, welche experimentell mit Infektionsdosen von bis zu 1 Millionen *T. gondii*-Oozysten infiziert wurden, der Verdacht auf klinische Toxoplasmose nur bei einem Huhn auf (Publikation 2). Bei den Puten wurde keine klinische Toxoplasmose beobachtet. Somit kann keine Aussage über den Einfluss der in dieser Arbeit untersuchten Faktoren auf das Auftreten einer klinischen Toxoplasmose bei Huhn und Pute getroffen werden. Diese Ergebnisse bestätigen jedoch die klinische Resistenz von Huhn und Pute und dass, wie in der Literatur beschrieben, Fälle klinischer Toxoplasmose die Ausnahme darstellen (JONES et al. 1959; KINJO 1961; BIANCIFIORI et al. 1986; DUBEY et al. 1993b, 2007b; GOODWIN et al. 1994; WANG et al. 2014, 2015; VIELMO et al. 2019).

4.2. Serologische Untersuchungen

Serologische Untersuchungen werden häufig für Prävalenzstudien zum Screening von natürlich infiziertem Geflügel auf *T. gondii*-Infektionen genutzt wie in Tabelle 3 und aus den Studien

verschiedener Autoren ersichtlich ist (AGANGA und BELINO 1984; DEVADA et al. 1998; EL-MASSRY et al. 2000; DUBEY et al. 2003, 2005c, 2006b, 2007b; SREEKUMAR et al. 2003; ZHU et al. 2008; CHUMPOLBANCHORN et al. 2009; ALVARADO-ESQUIVEL et al. 2012; XU et al. 2012; TILAHUN et al. 2013; MATSUO et al. 2014; AYINMODE und JONES-AKINBOBOLA 2015; LOPES et al. 2016; CHIKWETO et al. 2017; LIU et al. 2017). Diese Verfahren sind weit weniger aufwendig als direkte Nachweise wie Bioassay oder PCR-Verfahren. Sie erlauben zudem einen höheren Durchsatz, sind kostengünstiger und die Ergebnisse liegen in kurzer Zeit vor.

4.2.1. Serokonversion und Infektionsstadium

Die Antikörperantwort bei Huhn und Pute wird durch das Infektionsstadium stark beeinflusst. So führt die in der Natur eher unwahrscheinliche Infektion mit Tachyzoiten bei beiden Spezies zwar zu einer messbaren Antikörperantwort für einen kurzen Zeitraum nach der Infektion, welche aber bei einem Teil der Tiere bereits vor Schlachtung unter den *cut-off* Wert der angewandten Tests absinkt (Publikation 1) und bei den simulierten natürlichen Infektionswegen in dieser Arbeit nicht auftritt (Publikation 2). Einen vergleichbaren Verlauf der Antikörperspiegel beobachteten MAKSIMOV et al. (2018) bei Puten und Hühnern nach Infektionen mit Tachyzoiten der Stämme RH, ME49 sowie dem in dieser Arbeit eingesetzten Stamm NED als Vertreter der klonalen Linien I, II und III. Absinkende Antikörperspiegel werden in der Literatur bei Tachyzoiten auch unabhängig vom Infektionsstamm, -dosis und eingesetztem Testverfahren bei Hühnern beschrieben (CHUMPOLBANCHORN et al. 2009; YAN et al. 2010). Enten zeigen bei parenteralen Tachyzoiteninfektionen dem Huhn entsprechend sinkende Antikörperspiegel, während bei Gänsen keine relevante Antikörperantwort erfolgt (MAKSIMOV et al. 2011). Das Absinken der Antikörperspiegel im Laufe einer Infektion mit Tachyzoiten führt unter anderem zum Unterschätzen der Seroprävalenz bei späten Untersuchungszeitpunkten wie in Publikation 4 angesprochen und auch zu der Schlussfolgerung, dass die angewendeten Testverfahren eine niedrigere Sensitivität bei Tachyzoiteninfektionen aufweisen (OPSTEEGH et al. 2016). Zudem führten in den eigenen Studien Infektionen mit Tachyzoiten sowie Gewebezysten von NED und ME49 bei Hühnern im Vergleich zu Infektionen mit Gewebezysten des Feldstammes (CZ-Tiger) oder Oozysten aller Stämme zu niedrigeren Antikörperspiegeln, welche sich nicht signifikant von den Antikörperspiegeln der uninfizierten Kontrolltiere unterscheiden (Publikation 4).

Aufgrund der von MAKSIMOV et al. (2018) gezeigten analogen Antikörperantwort von Huhn und Pute bei identischen Versuchsbedingungen, ist bei Puten von vergleichbar niedrigen Antikörperspiegeln nach Tachyzoiteninfektionen im Vergleich zu Oozysteninfektionen auszugehen. Zur Bestätigung dieser Hypothese sind weitere Untersuchungen nötig.
Bei beiden Spezies unterscheidet sich der Beginn der messbaren Antikörperantwort zwischen Gewebezysteninfektionen und der Infektion mit niedrigen Oozystendosen nicht (Publikation 2).

Jedoch erreichen die Antikörpertiter beim Huhn nach Gewebezysteninfektionen, mit Ausnahme von Infektionen mit Zysten des Feldstammes (CZ-Tiger), das gleiche Niveau wie nach Infektion mit Tachyzoiten (Publikation 4). Diese Beobachtung kann möglicherweise durch die niedrigere Empfänglichkeit von Zwischenwirten für Infektionen mit Gewebezysten im Vergleich zu Oozysteninfektionen erklärt werden (DUBEY 2001).

OPSTEEGH et al. (2016) untersuchten Seren, die von Puten der vorliegenden Untersuchung stammten, und stellten fest, dass sich die Antikörperspiegel bei Puten zum Schlachtzeitpunkt acht Wochen nach Gewebezysteninfektion auf höherem Niveau als beim Huhn bewegen. Infektionen von Geflügel mit Gewebezysten standen in der Vergangenheit nur sehr selten im Fokus von Untersuchungen. KANETO et al. (1997) führten zwar Gewebezysteninfektionen bei Hühnern durch, machten jedoch keine Aussage zum zeitlichen Verlauf der mittels IFAT festgestellten Serotiter. Ein systematischer Vergleich der Antikörperantwort nach Gewebezysteninfektionen bei Huhn und Pute ist nach bestem eigenem Wissen außer von OPSTEEGH et al. (2016), die Seren aus der vorliegenden Studie untersuchten, zuvor noch nicht publiziert worden. Offensichtlich gibt es also Unterschiede in der Antikörperantwort verschiedener Geflügelspezies, ebenso wie zwischen verschiedenen Zuchtlinien innerhalb einer Spezies, wie von SCHARES et al. (2017b) für das Huhn gezeigt.

Unterschiede in der Antikörperantwort zwischen Huhn und Pute wurden in der eigenen Arbeit vor allem zu Beginn der Antikörperbildung beobachtet (Publikation 2). So war bei Hühnern die Serokonversion unabhängig vom gewählten *Toxoplasma*-Infektionsstadium früher messbar als bei Puten. Allerdings wurden die serologischen Daten für das Huhn mittels IFAT und bei Puten mittels KELA erhoben, so dass beobachtete Unterschiede in der Antikörperdynamik der Spezies durchaus auf der Verwendung verschiedener Testverfahren beruhen können, wie in Publikation 4 und bei anderen Autoren zuvor besprochen (DUBEY et al. 1993a, 1993b, 1993c, 1994a, 1994b, 1995; MARTÍNEZ-CARRASCO et al. 2004; YAN et al. 2010; CASARTELLI-ALVES et al. 2014; OPSTEEGH et al. 2016). Auch die Ergebnisse von OPSTEEGH et al. (2016) legen nahe, dass festgestellte Unterschiede zwischen Puten und Hühnern auf den verschiedenen Testverfahren beruhen. Insbesondere die mittels KELA erhobenen Daten müssen daher noch im Vergleich zu den Ergebnissen anderer serologischer Tests kritisch geprüft werden. Vor dem Hintergrund variabler Ergebnisse in Abhängigkeit vom jeweiligen Testverfahren sind für vergleichende seroepidemiologische Studien Verlaufsuntersuchungen, so wie in der eigenen Arbeit realisiert (Publikation 1 und 2), mit identischen Nachweisverfahren durchzuführen.
Mit dem KELA generierte Daten für zum Zeitpunkt der Schlachtung gewonnenen Seren von Puten, die mit Gewebezysten oder Oozysten infiziert wurden, ergaben in den eigenen Untersuchungen (Publikation 2) ähnliche Ergebnisse wie die von OPSTEEGH et al. (2016) dokumentierten.

4.2.2. Serokonversion und Infektionsdosis

Die Antikörperantwort von Huhn und Pute als auch von anderen aviären Spezies wird in früheren Studien durch die Infektionsdosis beeinflusst (KAJEROVÁ et al. 2003; BARTOVA et al. 2004; MARTÍNEZ-CARRASCO et al. 2004; HOTOP et al. 2014; MAKSIMOV et al. 2018). Die Ergebnisse der eigenen Arbeit stützen diese Aussage, da Infektionen von Hühnern und Puten mit unterschiedlichen Dosen von ME49-Oozysten zu einem dosisabhängigen Beginn der Antikörperantwort führten (Publikation 2). Auch nach Infektion mit Oozysten des verwendeten Feldstamms (CZ-Tiger) ließ sich bei Puten wie bei Hühnern eine Tendenz zu einer früher einsetzenden Antikörperantwort bei höherer Dosis erkennen (Publikation 2).
Die Serokonversion wäre vermutlich noch früher und deutlicher erkennbar gewesen, wenn in kürzeren als wöchentlichen Untersuchungsintervallen beprobt worden wäre. Dies war aber aufgrund des niedrigen Alters der Tiere zum Infektionszeitpunkt und der benötigten Blutmenge zur Serumgewinnung aus Tierschutzaspekten nicht möglich.
Bei Hühnern konnten zudem in Publikation 4 dosisabhängige Unterschiede in der Höhe der Antikörperspiegel zum Schlachtzeitpunkt 5 Wochen nach Infektion mit Oozysten des Feldstammes (CZ-Tiger) gezeigt werden.

Zu anderen Ergebnissen kamen BIANCIFIORI et al. (1986) und DUBEY et al. (1993a, 1993b), die für Hühner, Puten und Tauben keine Dosisabhängigkeit hinsichtlich des Beginns der Antikörperantwort oder des Verlaufes der Antikörperspiegel nach Infektionen mit unterschiedlichen Dosen von Oozysten feststellen konnten. Als Ursache für die widersprüchlichen Befunde können die unterschiedlichen verwendeten Infektionsstämme, die uneinheitlichen serologischen Testverfahren sowie Infektionszeiträume vermutet werden.

In der Höhe der Antikörperantwort zum Schlachtzeitpunkt (acht Wochen p.i.) war bei mit Oozysten des Feldstamms (CZ-Tiger) infizierten Hühnern eine Dosisabhängigkeit sicher nachweisbar (Publikation 4). Im ELISA und MAT trat dieser Effekt deutlicher hervor als im IFAT. Hier zeigt sich erneut der Einfluss der Nachweismethode auf die serologischen Ergebnisse (DUBEY et al. 1993a, 1993b, 1994b; CASARTELLI-ALVES et al. 2014). Ein Vergleich von mit unterschiedlichen serologischen Methoden generierten Daten muss daher stets kritisch betrachtet werden. Auf Speziesunterschiede kann aus den serologischen Ergebnissen in dieser Arbeit nicht geschlossen werden, weil die Serologie für Puten mittels KELA und für Hühner mittels IFAT erfolgte.

OPSTEEGH et al. (2016) konnten mit den aus dieser Arbeit generierten Seren von Huhn und Pute zum Schlachtzeitpunkt (fünf bzw. acht Wochen p.i.) keinen Einfluss der Infektionsdosis auf Testsensitivitäten beobachten. Es ist anzunehmen, dass dies aufgrund der gleichmäßig hohen Antikörperspiegel im Verlauf der Infektion mit Oozysten der Fall ist (Abb. 2, Abb. 3). Infolge

abfallender Antikörperspiegel im Verlauf der Infektion mit Tachyzoiten sowohl bei Hühnern als auch bei Puten (Publikation 1) sind im Gegensatz zu den Oozysteninfektionen die Testsensitivitäten bei Tachyzoiteninfektionen deutlich geringer (OPSTEEGH et al. 2016).

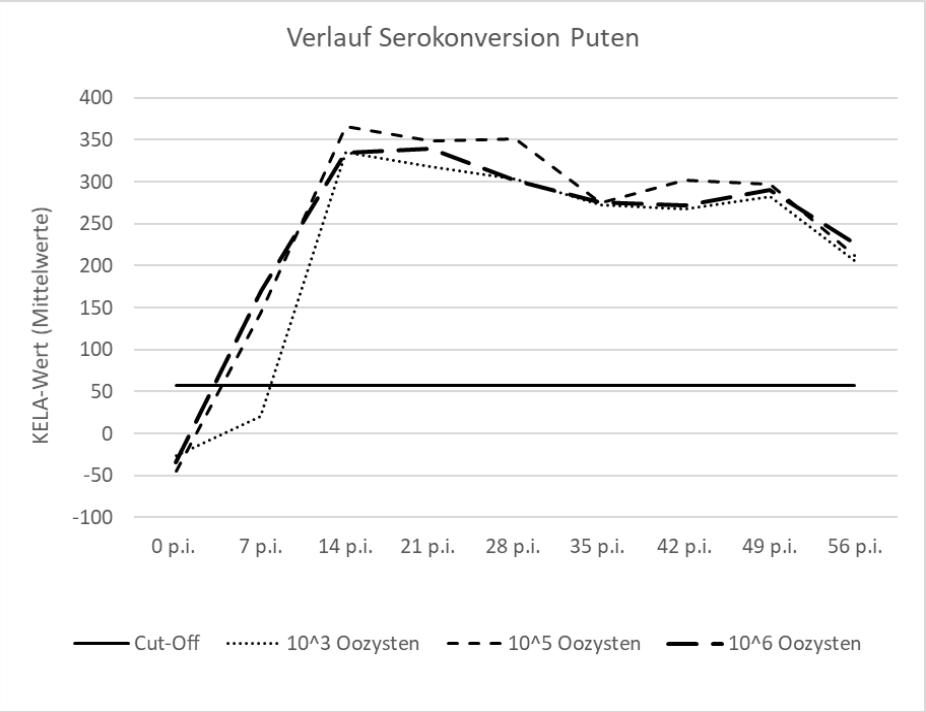

Abbildung 2: Verlauf Serokonversion bei Puten nach Infektion mit Oozysten

Daten gemittelt über alle Infektionsstämme einer Infektionsdosis (10^3, 10^5 und 10^6 Oozysten)

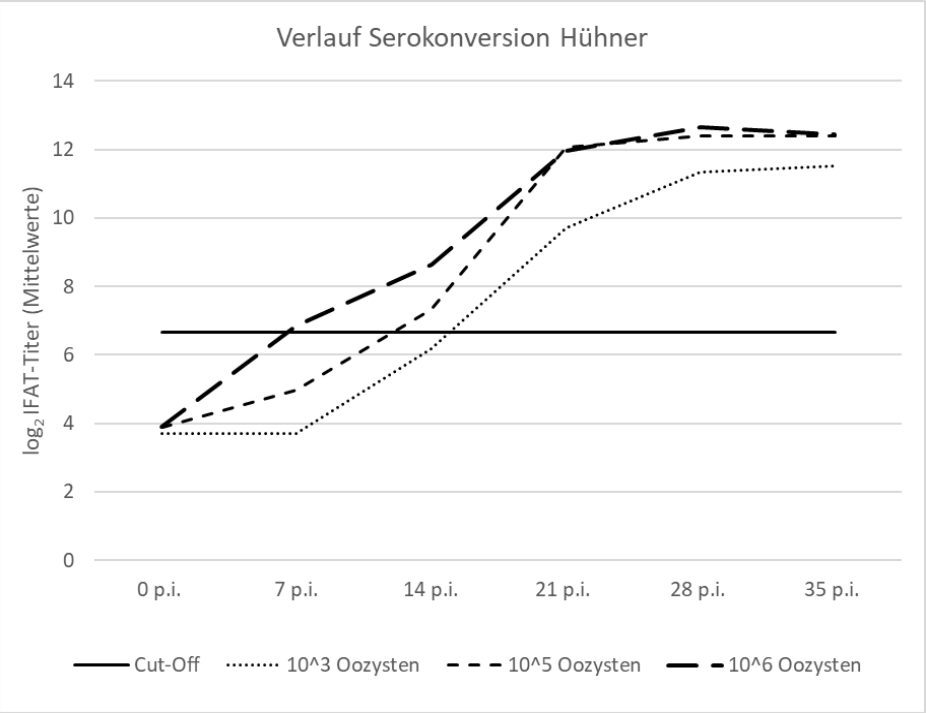

Abbildung 3: Verlauf Serokonversion bei Hühnern nach Infektion mit Oozysten

Daten gemittelt über alle Infektionsstämme einer Infektionsdosis (10^3, 10^5 und 10^6 Oozysten)

4.2.3. Serokonversion und Infektionsstamm

Der Infektionsstamm beeinflusst die Serokonversion beim Huhn deutlich. In allen Untersuchungen dieser Arbeit hebt sich die Antikörperantwort nach Infektionen mit dem Feldstamm (CZ-Tiger) deutlich im Beginn der messbaren Antikörperspiegel als auch in deren Höhe von ME49 und NED infizierten Hühnern ab (Publikation 2 und 4). Bei Puten konnten keine Hinweise auf einen Einfluss des Infektionsstammes auf den Beginn der Serokonversion nachgewiesen werden. In keiner mir bekannten Studie wurde bei Hühnern, Puten und anderen aviären Spezies ein Stammeinfluss auf den Zeitpunkt der Serokonversion beobachtet (DUBEY et al. 1993b, 1993c, 1994a, 1994b; MAKSIMOV et al. 2018).

Als Ursache für diese Diskrepanz zwischen den eigenen Ergebnissen und der Literatur wird die jeweilige *T. gondii*-Stammauswahl vermutet. Die Autoren der genannten Studien verwendeten ausschließlich lang etablierte Laborstämme für die Infektionsversuche. Eine Aussage zur Passagehäufigkeit und -art wurde nicht getroffen. Da bekannt ist, dass Eigenschaften wie die Pathogenität von *T. gondii*-Stämmen sich bei häufiger Passage als Tachyzoiten (*in vivo* oder in der Zellkultur) verändern (FRENKEL et al. 1976; LINDSAY et al. 1991; DUBEY et al. 1999), kann die Nutzung von in der Regel häufig passagierten Laborstämmen möglicherweise als Ursache für den publizierten fehlenden Stammeinfluss (DUBEY et al. 1993b, 1993c, 1994a, 1994b; MAKSIMOV et al. 2018) gesehen werden. Diese Hypothese wird durch die Beobachtung gestützt, dass keine Unterschiede im Beginn der Antikörperantwort bei Hühnern zwischen den in dieser Arbeit verwendeten Laborstämmen ME49 und NED nachweisbar waren. Dass die Immunantwort bei Puten den bei Hühnern gezeigten Stammeinfluss nicht aufwies, kann einerseits durch die unterschiedlichen Untersuchungsverfahren, wie weiter oben bereits beschrieben, bedingt sein. Andererseits ist ein tatsächlicher Speziesunterschied nicht auszuschließen und mit weiteren Untersuchungen abzuklären.

4.3. Gewebeverteilung

4.3.1. Persistenz von *T. gondii* im Gewebe von Geflügel

Gewebezysten von *T. gondii* persistieren mitunter lebenslang im Zwischenwirt (TENTER et al. 2000), so wurde eine Persistenz in Geweben von Rindern, Schweinen und Pferden von bis zu 1191 Tage nach einer Infektion beobachtet (DUBEY et al. 1984; DUBEY 1985, 1988; DUBEY und THULLIEZ 1993). Für das Geflügel wurde eine Persistenz von mindestens 10 Monaten bei Hühnern (JONES et al. 1959; JACOBS und MELTON, 1966; BIANCIFIORI et al. 1986; DUBEY et al. 1993b; KANETO et al. 1997; SEDLÁK et al. 2000; YAN et al. 2010; SCHARES et al. 2017b) sowie von mindestens 84 Tagen bei Puten (DUBEY et al. 1993a; SEDLÁK et al. 2000; BANGOURA et al. 2013; ZÖLLER et al. 2013) berichtet. Im ersten Teil der vorliegenden Arbeit (Publikation 1) wurde eine Persistenz von *T. gondii* im Gewebe von Puten nach Infektion mit Tachyzoiten von 16 Wochen nachgewiesen. Die Persistenz könnte

tatsächlich noch wesentlich länger sein, aber spätere Zeitpunkte konnten aufgrund des festgelegten Zeitpunktes der Euthanasie nicht untersucht und beurteilt werden. Zur Persistenz von *T. gondii* bei Hühnern nach parenteraler Tachyzoiteninfektionen ist anhand der geringen Anzahl von vier (2,1 %) DNA-Nachweisen keine Aussage möglich.

Auf Grundlage der im vorherigen Absatz zitierten Literatur zur Persistenz und der eigenen Daten (Publikation 1) wurde der Studienzeitraum im zweiten Teil der eigenen Arbeit (Publikation 2, 3 und 4) auf acht Wochen bei Puten und fünf Wochen bei Hühnern festgelegt, so dass in jedem Fall zum Studienende Zysten zu erwarten waren. In der Tat konnte hier gezeigt werden, dass unabhängig vom gewählten natürlichen Infektionsweg (perorale Gabe von Oozysten oder Zysten) *T. gondii*-DNA über den gesamten Studienzeitraum sowohl bei Puten wie auch Hühnern in verschiedenen Geweben nachweisbar waren. Bei Hühnern konnte somit unter Simulation natürlicher Infektionswege eine Persistenzdauer von fünf Wochen gezeigt werden. Eine Persistenz von über fünf Wochen beim Huhn ist jedoch aufgrund der Ergebnisse anderer Studien (JACOBS und MELTON, 1966; BIANCIFIORI et al. 1986; DUBEY et al. 1993b) wahrscheinlich.

4.3.2. Prädilektionsstellen bei Hühnern und Puten

T. gondii besitzt eine hohe Affinität zu Nerven- und Muskelgewebe (DUBEY et al. 1998; TENTER et al. 2000; DUBEY 2010b). Beim Huhn gelten Herz und Gehirn als Prädilektionsstelle für Gewebezysten (JONES et al. 1959; JACOBS und MELTON 1966; DUBEY 1981; Aigner et al. 2010; DUBEY et al. 1993b, 2004, 2005a, 2005d, 2005e, 2005f, 2005g, 2006a, 2006c, 2007b, 2015; KANETO et al. 1997; YAN et al. 2010; FERNANDES et al. 2016; OPSTEEGH et al. 2016; SCHARES et al. 2017b; FERREIRA et al. 2018). Zur Verteilung von *T. gondii*-Gewebezysten bei Puten lagen bislang nur wenige Daten vor (DUBEY et al. 1993a; LINDSAY et al. 1994; SEDLÁK et al. 2000; BANGOURA et al. 2013; ZÖLLER et al. 2013; SARKARI et al. 2014; OPSTEEGH et al. 2016; CERQUEIRA-CÉZAR et al. 2019). Einheitliche Prädilektionsorgane lassen die Daten aus der Literatur bei der Pute, anders als beim Huhn, nicht erkennen.

In der Literatur zur Bildung von Gewebezysten (zu Literatur, siehe Tabellen 4, 5 und 6) wurden überwiegend Herz und Gehirn berücksichtigt, nur gelegentlich die Skelettmuskulatur und selten andere Organe. Insbesondere in Untersuchungen von natürlich infizierten Tieren wurden andere Lokalisationen als Hirn und Herz vernachlässigt, so dass die Kenntnis anderer Ansiedlungsorte von Zysten gering ist.

Die Ergebnisse der vorliegenden Arbeit (Publikation 1, 2, 3 und 4) beinhalten Daten aus der Untersuchung von 16 verschiedenen Organen und Geweben und sollten beitragen, diese Wissenslücke zu schließen. Gehirn und Herz stellten sich unter Simulation natürlicher Infektionswege

wie bereits in der Literatur (Tabellen 4, 5 und 6) beschrieben, sowohl qualitativ als auch quantitativ hinsichtlich der Parasitenlast bei beiden Spezies als Hauptansiedlungsorte für *T. gondii* heraus. Die im Vergleich dazu niedrigeren Nachweisraten und Befallsstärken in der Skelettmuskulatur bestätigen ebenfalls bisherige Studien zum Huhn (DUBEY et al. 2015; OPSTEEGH et al. 2016). Unerwartet wurde dagegen in Hühnern und Puten der Muskelmagen als ein weiteres Prädilektionsorgan für *T. gondii* erkannt, und auch die Gonaden von Hühnern waren ein von *T. gondii* bevorzugtes Organ. Vergleichbar hohe Nachweisraten in Muskelmagen und Ovarien von Hühnern berichteten JACOBS und MELTON (1966), neuere Daten hierzu konnten aber in der recherchierten Literatur nicht gefunden werden, ebenso fehlen Befunde für diese Organe bei natürlich infizierten Tieren.

4.3.3. Einflussfaktoren auf die Organverteilung

Die Verteilung von *T. gondii* in den Geweben nach parenteraler Tachyzoiteninfektion erfolgte bei Puten disseminiert, so dass kein konkretes Prädilektionsorgan festgestellt werden konnte (Publikation 1). Da dies im Widerspruch zu den Befunden nach peroraler Infektion mit Zysten oder Oozysten steht, ist ein Einfluss des Infektionsstadiums auf die Gewebeverteilung bei der Pute offensichtlich. Als Ursache wird die intravenöse Infektion mit Tachyzoiten und die damit verbundene, unmittelbare hämatogene Streuung von *T. gondii* in alle Organe durch Umgehung der im Darmepithel stattfindenden Endodyogenie angenommen. Einen ähnlichen Einfluss des Infektionsstadiums auf die Organverteilung lassen die Untersuchungen von (KANETO et al. 1997; YAN et al. 2010; BANGOURA et al. 2013; ZÖLLER et al. 2013) erkennen. Ob es einen Stammeinfluss auf die Organverteilung gibt, ist derzeit nicht klar festzustellen. Da in den zitierten früheren Studien andere Stämme verwendet wurden, die Ergebnisse aber ähnlich waren, erscheint ein solcher Einfluss aber unwahrscheinlich oder zumindest gering.

Beim Huhn konnte in Hinblick auf die geringe Positivrate der untersuchten Gewebe im Vergleich zur Pute nach Tachyzoiteninfektion sowohl mit konventioneller PCR als auch mit *magnetic-capture* PCR die Gewebeverteilung nicht beurteilt werden (Publikation 1 und 4). Einen vergleichbaren Unterschied in den Nachweisraten von *T. gondii* nach Tachyzoiteninfektion beschrieben SCHARES et al. (2017b) bereits zwischen verschiedenen Hühner-Zuchtlinien, daher ist eine derart unterschiedliche Nachweisrate auch zwischen verschiedenen Spezies, wie Pute und Huhn, wahrscheinlich. Da frühere Tachyzoiteninfektionen von Hühnern mit anderen Stämmen zu deutlich höheren Nachweisraten führten (JONES et al. 1959; KANETO et al. 1997; YAN et al. 2010; SCHARES et al. 2017b) als in den eigenen Arbeiten gezeigt, kann auch ein Stammeinfluss für die niedrige Positivrate ursächlich sein.

4.3.4. Einfluss der Methodik

In der vorliegenden Arbeit wurden zwei PCR-Methoden mit unterschiedlichen DNA-Extraktionen zur Untersuchung der Gewebeverteilung von *T. gondii* angewandt. Dabei handelte es sich um eine konventionelle PCR des B1-Gens mit Silica-Säulen basierter DNA-Extraktion sowie eine real-time PCR des „529 bp *repeat elements*" mit *magnetic-capture* DNA-Extraktion. Grundsätzlich erbrachten beide Methoden eine im Verhältnis übereinstimmende Organverteilung von *T. gondii* beim Huhn als auch bei der Pute. Jedoch wird in Publikation 2, 3 und 4 dieser Arbeit deutlich, dass die konventionelle PCR die Anteile an positiven Organen bei Huhn und Pute im Vergleich zur real-time PCR unterschätzt. Ursächlich hierfür dürfte der Einsatz einer bis zu 4000-fach höheren Probenmenge von 100 g Gewebe in die *magnetic-capture* DNA-Extraktion sein. Bedenkt man, dass beim Lebensmittel-liefernden Tier die Zystendichte mit weniger als einer Zyste pro 50 g Gewebe in der Regel niedrig ist (DUBEY et al. 1996) und sich Zysten zudem inhomogen verteilen (ESTEBAN-REDONDO et al. 1999; JURÁNKOVÁ et al. 2014a; RANI et al. 2019), ist es offensichtlich, dass die *magnetic-capture* DNA-Extraktion mit nachfolgender real-time PCR deutlich sensitiver ist als die konventionelle PCR mit einer Silica-Säulen basierten DNA-Extraktion.

Dieser Vorteil der *magnetic-capture* DNA-Extraktion in Kombination mit der real-time PCR wird insbesondere bei der Analyse von großvolumigen Organen mit niedriger Zystendichte deutlich, wie Brust-, Oberschenkel- oder Unterschenkelmuskulatur und verstärkt sich zusätzlich mit zunehmender Größe der untersuchten Spezies und der damit steigenden Organgröße (Publikation 2, 3 und 4). Aber auch bei den zystenreichen Organen Gehirn und Herzmuskulatur ist die Nachweisrate durch die konventionelle PCR im Vergleich zur *magnetic-capture* real-time PCR bis zu 50 % niedriger. Es ist wahrscheinlich, dass die Untererfassung der Nachweise durch die konventionelle PCR Ursache für die schwache Ausprägung der erwarteten Dosiseffekte bei der Pute ist, anders als bei der kleineren Geflügelart Huhn (Publikation 2 und 3).

Ein weiterer Grund für die höheren Nachweisraten mit der *magnetic-capture* real-time PCR ist das verwendete Zielgen der real-time PCR, das „529 bp *repeat element*". Aufgrund seines bis zu 300-fachen Vorkommens im *T. gondii*-Genom, ist die PCR sensitiver als die PCR des B1-Gens (STERKERS et al. 2010), welches nur 30-fach im Genom auftritt (BURG et al. 1989). Entsprechend lässt sich schlussfolgern, dass die weniger aufwendige und kostengünstigere konventionelle DNA-Extraktion mit anschließender PCR als erste Screening-Methode durchaus anwendbar ist, um ein Mindestmaß an *T. gondii*-DNA im Untersuchungspool zu detektieren. Zur Erhöhung der Sensitivität der PCR bei Verwendung kleiner Probenmengen ist der Einsatz einer auf dem „529 bp *repeat element*" basierenden real-time PCR dagegen vorteilhaft (JURÁNKOVÁ et al. 2014b). Ein für die Sensitivität der Methode entscheidender Faktor ist aber die zu analysierende Probenmenge, die für eine erfolgreiche Detektion möglichst groß sein sollte (ESTEBAN-REDONDO et al. 1999; DUBEY et al. 2015). Insofern ist

die DNA-Extraktion mittels des *magnetic-capture* Verfahrens in Verbindung mit einer hochsensitiven PCR unerlässlich, um belastbare und mit dem Bioassay vergleichbare Ergebnisse zur Zystenverteilung in Organen und Geweben zu generieren.

4.4. Weitere Einflussfaktoren auf die Untersuchungen

Die in der vorliegenden Arbeit beobachteten Unterschiede zwischen Huhn und Pute in Immunantwort und Verteilung von *T. gondii* im Gewebe können auch von anderen Faktoren als der Spezies beeinflusst sein, da Hühner und Puten aufgrund begrenzter Stallkapazität in allen Versuchen nacheinander infiziert wurden. Somit sind als Einflussfaktoren die unterschiedlichen Infektionszeitpunkte zu betrachten, welche zu einer Variabilität im für die Infektion der Hühner und Puten verwendeten Materials zum Infektionszeitpunkt wie z.B. Alter des Infektionsmaterials, Passagehäufigkeit und, bei Gewebezysteninfektionen die Anzahl an pro Tier applizierten infektiösen Zysten geführt haben können. Bereits FRENKEL et al. (1976) konnten den Verlust von Erregereigenschaften, in diesem Fall geringeres Vermögen zur Bildung von Oozysten nach häufiger und ausschließlicher Passage von Tachyzoiten in Mäusen, zeigen. Häufige Passagen in der Zellkultur können zum Verlust der Pathogenität führen (LINDSAY et al. 1991; DUBEY et al. 1999; KHAN et al. 2009). Ebenso sinkt die Infektiosität von Oozysten durch längere Lagerung (CHRISTIE 1977). Um diese Faktoren vollständig ausschließen zu können, muss bei zukünftigen Versuchen zum direkten Vergleich verschiedener aviärer Wirtsspezies – welches hier nicht der Hauptfokus war – eine zeitgleiche Infektion aller Versuchstiere angestrebt werden.

4.5. Zusammenfassung der Diskussion

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass Geflügel auf verschiedenen Infektionswegen erfolgreich mit *T. gondii* infiziert werden kann. Unabhängig vom Infektionsweg und appliziertem Erregerstadium wurde *T. gondii*-DNA beim Huhn bis zu fünf Wochen und bei der Pute bis zu 16 Wochen nach der Infektion in allen untersuchten Organen, unter anderem auch in zum menschlichen Verzehr geeigneten Geweben, detektiert. Aufgrund der übereinstimmenden Ergebnisse der *magnetic-capture* real-time PCR mit dem Bioassay (OPSTEEGH et al. 2016) (Publikation 4) wird davon ausgegangen, dass die nachgewiesene DNA (Publikation 1 und 2) aus infektiösen Gewebezysten stammt. Lichtmikroskopisch nachgewiesene Gewebezysten in experimentell infizierten Puten (ZÖLLER et al. 2013) stützen diese These. Das Risiko einer Infektion des Verbrauchers mit *T. gondii* beim Verzehr von oder Umgang mit nicht ausreichend gegartem Geflügelfleisch oder Geflügelfleischerzeugnissen kann demzufolge keinesfalls ausgeschlossen werden. Eine endgültige Risikobewertung sollte jedoch erst nach Prüfung der Infektiosität nachgewiesener Gewebestadien mittels Bioassay oder alternativer Verfahren (OPSTEEGH et al. 2020) erfolgen.

5. Zusammenfassung

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In vivo-Modelle der *Toxoplasma gondii*-Infektion von Huhn und Pute

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81 Seiten, 3 Abbildungen, 6 Tabellen, 313 Literaturangaben

Schlüsselwörter: *T. gondii*, Geflügel, Toxoplasmose, Gewebeverteilung, Serologie

Einleitung: *Toxoplasma* (*T.*) *gondii* ist ein weltweit verbreitetes Protozoon warmblütiger Tiere und des Menschen. Einer der größten Risikofaktoren einer Infektion des Menschen mit *T. gondii* ist der Verzehr von rohem oder nicht ausreichend gegartem Fleisch und Fleischerzeugnissen. Geflügel wird als ein bedeutender Zwischenwirt im Lebenszyklus von *T. gondii* angesehen. Gegenwärtig gibt es aber nur wenige Untersuchungen zur Persistenz und Gewebeverteilung von für den humanen Konsum relevanten Organen und Geweben von *T. gondii* der Pute (*Meleagris gallopavo*) und des Haushuhns (*Gallus gallus domesticus*).

Ziel der Untersuchung: Ziel im ersten Teil der vorliegenden Arbeit war es, die Persistenz von *T. gondii* in verschiedenen Geweben von Huhn und Pute unter Berücksichtigung der üblichen Mastperioden zu ermitteln. Im zweiten Teil der Arbeit wurde unter Simulation der natürlich auftretenden Infektionswege ein Infektionsmodell bei Huhn und Pute unter Nutzung von drei verschiedenen *T. gondii*-Stämmen etabliert. In diesem Modell wurde der Einfluss der Parameter Infektionsdosis, -stadium und -stamm auf die Verteilung von *T. gondii* im Gewebe von Huhn und Pute sowie die Serokonversion in den Zielspezies evaluiert.

Tiere, Material und Methoden: 108 Puten und 96 Hühner wurden als Eintagsküken eingestallt. Die zur Infektion der Hühner und Puten benötigten Entwicklungsstadien von *T. gondii* wurden durch Passagen in der Zellkultur (Tachyzoiten), Infektion von Mäusen (Zysten) und durch Infektion von Hauskatzen (Oozysten) gewonnen. Die Evaluierung der Persistenz erfolgte nach intravenöser Gabe von 10^6 Tachyzoiten des Typ III-Stammes (NED) vier, acht, zwölf und 16 Wochen *post infectionem* (p.i.) bei Puten und fünf und zehn Wochen p.i. bei Hühnern. Die natürlichen Infektionswege wurden mit der oralen Verabreichung von Gewebezysten (ein Mausgehirn je Tier) oder Oozysten (10^3, 10^5 oder 10^6) eines *T. gondii*-Stammes der klonalen Linien der Typen II (ME49, Feldstamm CZ-Tiger) und III (NED) über eine Versuchsdauer von acht Wochen bei Puten und fünf Wochen beim Huhn simuliert (n = 6). Zum Versuchsende wurden die Tiere tierschutz-gerecht narkotisiert und durch Dekapitation getötet. Es wurden 16 verschiedene Gewebe (u.a. Gehirn, Herz, Muskulatur, Muskelmagen) entnommen und die Gewebeverteilung der Zysten von *T. gondii* mittels konventioneller Polymerase-Kettenreaktion (PCR) und *magnetic-capture real-time* PCR untersucht. Für die serologische

Untersuchung von Blutproben wurden ein kinetischer *enzyme linked immunosorbent assay* (ELISA) und ein Immunfluoreszenzantikörpertest (IFAT) verwendet. Die Blutentnahmen erfolgten am Tag der Infektion und anschließend wöchentlich bis Versuchsende. Die Daten wurden mit dem Kruskal-Wallis-Test und Mann-Whitney-U-Test (Unterschiede Infektionsgruppen in Serokonversion und Gewebeverteilung) und dem Friedman-Test (Organverteilung) analysiert. Signifikante Unterschiede wurden bei einer Irrtumswahrscheinlichkeit von $p < 0,05$ angenommen.

Ergebnisse: In allen 16 untersuchten Geweben von Pute und Huhn konnte nach Infektion mit Tachyzoiten, Zysten oder Oozysten *T. gondii*-DNA detektiert werden. Nach Tachyzoiteninfektion waren bei der Pute insgesamt 15,9 % der Gewebeproben positiv für *T. gondii*-DNA, wobei es sich bei 7,8 % der Proben um essbare Gewebe (Oberschenkel-, Unterschenkel-, Brustmuskulatur, Herz, Leber, Muskelmagen) handelte. Zwischen den vier Untersuchungszeitpunkten waren bei der Pute keine signifikanten Unterschiede in der Nachweisrate feststellbar. Bei Hühnern führten die Infektionen mit Tachyzoiten zu vier Nachweisen (2,1 %) von *T. gondii*, davon in drei Proben fünf Wochen p.i. (Pankreas, Unterschenkelmuskulatur, Retina) und in einer Probe zehn Wochen p.i. (Herz). Ein statistischer Vergleich der Untersuchungszeitpunkte war aufgrund der wenigen positiven Befunde nicht möglich.

Die Infektionen mit Zysten oder Oozysten ergaben hohe Nachweisraten in Gehirn (Pute 44,4 % - 64,1 %, Huhn 18,8 % - 38,5 %) und Herz (Pute 5,6 % - 28,2 %, Huhn 31,3 % - 53,8 %). sowie Muskelmägen (Pute 16,7 % - 20,5 %, Huhn 6,3 % - 25,6 %). Bei Infektionen mit Oozysten der Typ II-Stämme stiegen mit der Infektionsdosis auch die Positivraten bei Huhn und Pute. Infektionen mit dem Typ III-NED-Stamm führten zu signifikant niedrigeren Nachweisraten bei Huhn (1,9 %) und Pute (5,7 %) als solche mit den Typ II-Stämmen ME49 (Huhn: 16,4 %, Pute: 18,8 %) und CZ-Tiger (Huhn: 27,8 %; Pute: 11,7 %).

Eine Serokonversion trat bei Huhn und Pute ab einer Woche p.i. auf. Mit steigender Dosis von Typ II-Stamm Oozysten war bei beiden Spezies eine frühere Serokonversion nachweisbar.

Schlussfolgerungen: Das in der vorliegenden Arbeit etablierte In vivo-Modell ist geeignet, Hühner und Puten auf verschiedenen Wegen mit *T. gondii* zu infizieren. Die Nachweise von *T. gondii*-DNA in Organen und Geweben, auch in zum menschlichen Verzehr genutzten, bei Hühnern bis zu fünf Wochen p.i. und Puten bis zu 16 Wochen p.i. zeigen eine Persistenz von *T. gondii* bis mindestens zur Schlachtreife an. Das Risiko einer Infektion des Verbrauchers mit *T. gondii* beim Verzehr von nicht ausreichend gegartem Geflügelfleisch oder Geflügelfleischerzeugnissen oder dem Umgang mit rohem Fleisch oder sonstigem Gewebe von Geflügel ist demzufolge grundsätzlich gegeben. Die Rolle von Geflügel oder anderen Vögeln in der Epidemiologie der humanen Toxoplasmose ist aber noch nicht gänzlich klar und verdient weitere Aufmerksamkeit.

6. Summary

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In vivo-models for *Toxoplasma gondii* infections in chicken and turkey

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Submitted in February 2022

81 pages, 3 illustration, 6 tables, 313 references

Keywords: *T. gondii*, poultry, toxoplasmosis, tissue distribution, serology

Introduction: *Toxoplasma* (*T.*) *gondii* is a widespread protozoon of warm-blooded animals and humans. One risk factor for a human infection with *T. gondii* is the consumption of raw or insufficiently cooked meat and products thereof. Poultry is considered as one of the most important intermediated hosts in the life cycle of *T. gondii*. Currently, there do exist only a small number of studies about the persistence and tissue distribution of *T. gondii* in tissues and organs from turkeys (*Meleagris gallopavo*) and chickens (*Gallus gallus domesticus*) relevant for human consumption.

Objective: In the first part of this study, the aim was to evaluate the persistence of *T. gondii* in different tissues of chicken and turkey considering the duration of conventional fattening cycles. In the second part of this work an infection model for chicken and turkey was established simulating the natural ways of infection with three various *T. gondii* strains. The influence of infective dose, developmental stage and strain on the distribution of *T. gondii* in tissue from chickens and turkeys and seroconversion of the infected species was investigated in this model.

Animals, Material and Methods: 108 turkey and 96 chicken day-old chicks were stabled. The developmental stages of *T. gondii* for infection of chickens and turkeys were obtained by passage in cell culture (tachyzoites), infection of mice (tissue cysts) and infection of domestic cats (oocysts). The evaluation of persistence was done four, eight, twelve and 16 weeks for turkeys and five and ten weeks p.i. for chickens after infection with 10^6 tachyzoites of the type III strain NED. The natural ways of infection were simulated by orally administering tissue cysts (one mouse brain) or oocysts (10^3, 10^5 or 10^6) of a *T. gondii* strain belonging to the clonal lineage II (ME49, field strain CZ-Tiger) and III (NED) (six animals per group). The trial was determined eight weeks *post infectionem* (p.i.) for turkeys and five weeks p.i. for chickens. At the end of each experiment the animals were narcotized in accordance with animal welfare and sacrificed through decapitation. 16 different tissues and organs (for example brain, heart, musculature, gizzard) were taken. Subsequently, the tissue cyst distribution of *T. gondii* were examined by conventional polymerase chain reaction (PCR) and magnetic-capture real-time PCR. Serological investigation of blood samples was done with a kinetic *enzyme linked immune sorbent assay* (ELISA) and an immunofluorescent antibody test (IFAT). Blood samples were drawn on the day of infection and afterwards in a weekly interval including the day of euthanasia. The statistical analysis was performed with the Kruskal-Wallis test and Mann-Whitney-U

test (differences in infection groups for seroconversion and tissue distribution) and Friedman test (organ distribution). Differences and correlations with P values of less than 0.05 were defined as statistically significant.

Results: *T. gondii* DNA was detected in all 16 analyzed tissues and organs of turkeys and chickens after infection with tachyzoites, tissue cysts or oocysts. Regarding the tachyzoite infections of turkeys, 15,9 % of the analyzed samples were positive for *T. gondii* DNA. In 7,8 % of the samples of edible organs (musculature of drumstick, thigh, breast, heart, liver, gizzard) *T. gondii* DNA was found, too. There were no significant differences between the proportions of positive findings in infected turkeys slaughtered at different time points p.i. In chickens, infections with tachyzoites led to *T. gondii* DNA detection in four organs (2,1 %), including three samples five weeks p.i. (pancreas, drumstick muscle, retina) and one sample ten weeks p.i. (heart). Therefore, statistical comparison of the different time points of slaughtering was not possible in chicken samples.

Infections with tissue cysts or oocysts indicated high detection rates of *T. gondii* in brain (turkeys 44,4 % - 64,1 %, chickens 18,8 % - 38,5 %), heart (turkeys 5,6 % - 28,2 %, chickens 31,3 % - 53,8 %) and gizzard (turkeys 16,7 % - 20,5 %, chickens 6,3 % - 25,6 %). Increasing positive rates from chickens and turkeys were detected after infection with increasing doses of type II-strain oocysts. Infections with the type III strain NED resulted in significant lower positive rates in chickens (1,9 %) and turkeys (5,7 %) compared to type II strain infections with ME49 (chicken: 16,4 %, turkey: 18,8 %) and CZ tiger (chicken: 27,8 %, turkey 11,7 %).

In all experiments, seroconversion in chickens and turkeys was observed starting earliest one-week p.i.. The seroconversion in both species was earlier detectable with increasing infective doses of type II strain oocysts.

Conclusions: The in-vivo-model, established in this work, is applicable to infect chickens and turkeys with *T. gondii* in various ways. The positive findings of *T. gondii* DNA in organs and tissues, as well in tissues used for human consumption, in chickens up to five weeks p.i. and turkeys up to 16 weeks p.i. demonstrate a persistence of *T. gondii* in the two species at least up to slaughter age. The risk for the consumer of an infection with *T. gondii* after consumption of raw or undercooked poultry meat, products thereof or contact with raw meat or other tissues can be assumed. The role of poultry or other birds in the epidemiology of human toxoplasmosis is not completely distinct yet and deserves additional attention.

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