PROTOZOOLOGY - ORIGINAL PAPER

Toxoplasma gondii in water buffaloes (*Bubalus bubalis*) from Romania: what is the importance for public health?

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Abstract

The purpose of our study was to evaluate the prevalence of *Toxoplasma gondii* infection in autochthonous Carpathian buffaloes from northwestern Romania by serology, PCR techniques, and mouse bioassay. Agreement between MAT and ELISA, correlation between indirect and direct detection methods, and risk factors were evaluated. The apparent overall seroprevalence of *T. gondii* was 8.1% by MAT and 6.6% by ELISA. The agreement between ELISA and MAT was fair. The apparent seroprevalence was significantly higher in adult buffaloes (12.5%) compared to calves (0.0%) and juveniles (1.9%) by MAT. Most of the positive adult buffaloes detected by MAT had antibodies at a low sera dilution and the highest dilution was 1:768 in a juvenile female (30 months). No viable *T. gondii* was detected by mouse bioassay, as no *T. gondii* cyst or DNA was found in the brain of mice and they did not seroconvert. However, *T. gondii* DNA was detected in two buffaloes: in a 30-month-old male buffalo by qPCR on the diaphragm digest and in a 252-month-old female buffalo by RE nPCR on the mesenteric lymph node. Both animals were negative in MAT and ELISA. The total prevalence of *T. gondii* by direct detection methods was 2.7%. There was no correlation between indirect and direct detection methods. Since no viable *T. gondii* was detected in buffaloes, the risk of human infection from buffalo meat is minimal. Buffaloes' biological response to a *T. gondii* infection appears to be very similar to the response of cattle.

Keywords *Toxoplasma gondii* · Buffaloes · Seroprevalence · PCR · ELISA · MAT

Introduction

Toxoplasma gondii is one of the most well-studied parasites because of its medical and veterinary importance. *T. gondii* is a coccidian parasite with felines as the definitive host and warm-blooded animals as intermediate hosts. It is an unusual parasite because of its extremely broad host range, from birds to mammals and with only one species in the genus (Dubey

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2010). It presents three infectious stages: the oocysts, the bradyzoites, and the tachyzoites that are linked in a complex life cycle. Toxoplasmosis is a widespread food-borne zoonosis with an important impact on public health. *T. gondii* ranked fourth in a global ranking of food-borne parasites and second in Europe based on a multicriteria ranking tool for scoring parasites (FAO/WHO 2014; Bouwknegt et al. 2018).

The buffalo (*Bubalus bubalis*) industry represents an important economic, mainly food-borne resource, based on their milk and meat production (Borghese 2005). In Europe, there are around 460,000 heads (0.25% of the global population) mainly in Italy and the Balkan countries (Romania, Bulgaria, Macedonia, Greece, Albania, Serbia) and a very low population (< 2500 breeding females/country) in other countries (UK, Germany, Hungary, Ukraine, The Netherlands, Switzerland) (Borghese 2013).

Romania, in spite of a constant decrease of buffalo's population in the last 40 years, has the 2nd largest population in Europe, after Italy (Borghese 2005). They are still a common finding on the pastures, especially in Transylvania (North-West), where there have been historically recorded for the first time in the seventeenth century (Kos 1975). Buffaloes are raised mainly on small private farms in backyard system (1–5 animals) for milk and meat production, as subsistence farms. Intensive farming is limited to a national center for breeding research of buffaloes established in 1981 and 2–3 farms of around 100 animals. Calves are fattened and home slaughtered for familial consumption. The animals are kept indoor during the winter due to the unfavorable weather conditions and fed mainly with hay, and grazing on pasture in the warm season.

The epidemiological data concerning *T. gondii* infection in buffaloes are important from a public health point of view due to the human consumption of buffalo's milk, dairy products (*mozzarella*, etc.), and meat. Human *T. gondii* transmission by ruminant's milk is unlikely since only few reports have found *T. gondii* in milk. Generally, milk is pasteurized or boiled (Dubey 2010; Dubey et al. 2014). In Romania, even most of the quantity of buffalo milk is used for familial consumption, it is boiled prior to consumption, or pasteurized in the dairy industry.

On contrary, the consumption of raw/undercooked meat is considered an important risk factor for human infection with *T. gondii* in Europe and worldwide (Belluco et al. 2017). Buffalo meat in comparison with beef has a lower energy yield (131 kcal vs 289 kcal every 100 g of cooked meat) and a higher concentration of protein (26.8 g vs 24.1 g). Also, it presents a lower concentration of lipid (1.8 g vs 20.7 g), especially saturated fatty acids (0.6 g vs 8.1 g), and similar iron content (2.1 mg vs 2.4 mg) (Naveena and Kiran 2014). Therefore, it is considered that buffalo meat has a strong potential for increased per capita consumption worldwide (Kondaiah and Anjaneyulu 2003). Moreover, consumption of buffalo meat seems associated with several beneficial effects on cardiovascular risk profile, including lower carotid atherosclerotic burden and susceptibility to oxidative stress (Giordano et al. 2010).

Buffaloes (*Bubalus bubalis*) like cattle (*Bos taurus*) belong to the same family and subfamily of Bovidae and Bovinae respectively. *T. gondii* antibodies are less prevalent in cattle and buffaloes comparing with other members of the Bovidae family (e.g., sheep, goat) and isolation of viable parasite from naturally infected animals was not yet reported, suggesting that they are more resistant species to *T. gondii* (Dubey and Thulliez 1993; Dubey 2010).

The purpose of our study was to evaluate the prevalence of *T. gondii* infection in buffaloes by direct (PCR and mouse bioassay) and indirect (serology) techniques in order to answer to the following questions: how is the prevalence of *T. gondii* in buffaloes? Do buffalo meat consumption represent a risk of toxoplasmosis for humans? Also, the correlation between direct and indirect methods of *T. gondii* detection was analyzed.

Material and methods

Buffaloes

In Romania, the buffalo population (Mediterranean Carpathian breed) was estimated at around 300,000 animals in 1985 and decreased gradually since, to around 20,000 animals in 2016 (Borghese 2005; National Institute of Statistics 2017).

Our study focused in the northwestern Romania, collecting samples in 5 counties: Sălaj, Cluj, Bihor, Maramureş, and Bistriţa-Năsăud (Fig. 1, Table 1). The estimated sample size for a population of 20,000 animals with 5% margin of error, 95% confidence level, and a 10% response distribution was 139 (Sergeant 2017). The animals were sampled in small private farms (1–5 buffaloes) in backyard system or in the slaughterhouse. In backyard system, blood was collected, and in the slaughterhouse, blood and tissue samples. There were sampled buffaloes in 10 villages, 1–2 animals/household, and in one farm. Households were selected based on the availability of buffaloes and to sample all three age categories. There was selected one slaughterhouse (AGRO-ARDEAL, Orheiu Bistriţei) as it is one of the few authorized slaughterhouses in the region for large ruminants, and most of the buffaloes to be slaughtered are directed here. In slaughterhouse, mainly adult animals are slaughtered during winter period in low number. In order to have a representative sample size for direct detection of

Fig. 1 Area of sample collection and number of samples/county

the *T. gondii* and because there a limited number of buffaloes slaughtered, we decided to sample all buffaloes that arrived in the slaughterhouse. The slaughterhouse was visited 12 times and between 4 and 8 animals were sampled each time. After, we checked the passports of the animals and we found that there were not double sampled farms.

Each animal was identified with a unique ID (number), and the information concerning the age (months), sex (male/female), origin (county), and rearing system (backyard/intensive farming) was collected. According to age, the buffaloes were divided into three age categories: buffalo calves (0–6 months), juveniles (6–30 months), and adults (> 30 months).

Table 1 Sampled animals with details for geographic origin, age, gender, rearing system, and sampling place

County	Age category			Gender		Rearing system		Sampling place		Total
	Buffalo calves	Juveniles	Adults	Females	Males	Backyard	Intensive farming	Field	Slaughterhouse	
Cluj	14	15	23	37	15	21	31	23	29	52
Sălaj	11	36	89	128	8	136	0	100	36	136
Bihor	0	0	3	2	1	3	0	0	3	3
Bistriţa-Năsăud	0	0	1	0	1	1	0	0	1	1
Maramureş	0	1	4	5	0	5	0	0	5	5
Total	25	52	120	172	25	166	31	123	74	197
Average age in months	5.0 ± 1.6	17.6 ± 8.3	129.9 ± 67.2	93.5 ± 78.4	21.9 ± 27.1	95.1 ± 77.9	27.5 ± 44.7	69.0 ± 71.9	110.1 ± 80.3	84.4 ± 77.6

Sample collection

Blood samples were collected either by puncture of jugular vein or at bleeding in the slaughterhouse. Sera (1 ml/sample) were obtained after clotting and were stored at − 20 °C until processing. Tissue samples (200 g of diaphragm, 50 g of heart and liver, 5 g of mesenteric and mediastinal lymph nodes) were collected in separate bags, at slaughterhouse, for each animal. Each sample was identified based on an alphanumeric system. Diaphragm samples were stored at + 4 °C and processed within 24–48 h after sampling, while heart, liver, and lymph nodes were stored at − 20 °C until processing.

Serological assays

Enzyme-linked immunosorbent assay

Detection of *Toxoplasma gondii* IgGs in buffaloes sera was performed by a commercial enzyme-linked immunosorbent assay (ELISA) test (Chekit Toxotest Antibody ELISA, Idexx-Bommeli, Switzerland). This test detects the antibodies against *T. gondii* in serum and plasma samples of ruminants. The test was performed according to the manufacturer's instructions. Briefly, serum samples were diluted 1:400 and the results were measured as optical density percentages (OD% = (OD sample − OD negative control)/(OD positive control − OD negative control) × 100). According to manufacturer's instructions, sera presenting an OD% higher than 30% were considered as positive, an OD% between 20 and 30% as ambiguous, and an OD% < 20% as negative.

Modified agglutination test

Buffaloes' sera were analyzed by the modified agglutination test (MAT) (Dubey and Desmonts 1987) for the detection of *T. gondii*-specific immunoglobulin (IgG) using an antigen prepared from formalin-fixed whole RH tachyzoites, provided by the National Reference Center for Toxoplasmosis in Reims (Villena et al. 2012). The starting dilution was 1:6, in accordance with a previous study in cattle (Opsteegh et al. 2016a). Eight further twofold dilutions were made, up to 1:768. To score animals positive or negative, we set up 2 cutoff values: ≥ 1:6 (Opsteegh et al. 2016a; Burrells et al. 2018) and ≥ 1:96 (Dubey et al. 1985).

Mouse bioassay

All diaphragms collected in the slaughter were bioassayed into 6–8-week-old female CD1 mice. Briefly, each diaphragm sample (200 g) was grounded, mixed, and incubated at 37 °C for 1.5 h with trypsin (final concentration 0.25%). The suspension was then filtered, pelleted by centrifugation, washed in saline, and resuspended in a saline solution containing penicillin G/, streptomycin/amoxicillin, and ciprofloxacin/cefotaxime/vancomycin. One millilter of this homogenate was stored at − 20 °C for further use and another 1 ml was inoculated intraperitoneally into two mice (1 ml/mice). Mice were bled 6 weeks post inoculation and their serum was tested for *T. gondii* antibodies with the MAT technique. Furthermore, mice were killed 42 days post inoculation and their brains (olfactory lobes and media regions) were examined (2 slides with 2 spots each) for tissue cysts by light microscopy. The remaining part of the brain was mixed up to 1 ml with physiological salt and stored at − 20 °C for further use. Detailed protocol of the mouse bioassay can be found in the Appendix A of the EFSA report "Experimental studies of *T. gondii* in the main livestock species" (Opsteegh et al. 2016a).

PCR assays

All tissue samples collected from buffaloes (heart, liver, mesenteric lymph nodes, mediastinal lymph nodes, diaphragm digest) and mouse brains from bioassay (homogenate with physiological salt) were submitted to a standard PCR (sPCR), nested PCR (nPCR), and real-time quantitative PCR (qPCR) amplification. An animal was considered positive if any of the collected samples were positive by any of the PCR techniques.

Genomic DNA extraction

Genomic DNA (gDNA) was extracted from 25 mg of tissue (heart, liver, mesenteric lymph nodes, and mediastinal lymph nodes), 200 μl of diaphragm digest, and 200 μl of mouse brain homogenate, using a commercial kit (Isolate II Genomic DNA Kit, Bioline, UK), according to the manufacturer's standard protocol for cultured cells and human or animal tissue. Genomic DNA from a known number of *T. gondii* (RH strain) tachyzoites (10^6 parasites) was extracted according to the same protocol and used as positive control for PCR amplification.

Standard PCR and nested PCR

All tissue samples were screened with standard PCR targeting the 529 bp DNA fragment (Homan et al. 2000), then by nested PCR. Three protocols of nested PCR were used: (a) targeting the ITS1 region (Vitale et al. 2013) (ITS1 nPCR); (b) targeting the B1 gene (Jones et al. 2000) (B1 nPCR); and (c) targeting the 529 bp DNA fragment (Fallahi et al. 2014) (529 bp nPCR). Standard PCR and nested PCRs were carried out in a 25 μl reaction mixture consisting of MyTaq Red HS Mix 2x master mix (Bioline, UK), 25 pM of primers (Table 2), and 4 μl of template DNA or 4 μl of first-round product in nested amplification. All amplifications were performed in Bio-Rad

Table 2 Sequence of primers used in standard PCR and nested PCR targeting 529 bp fragment, ITS1 region, and B1 gene respectively

Assay	Oligonucleotide primer	Oligonucleotide sequence	Size of nPCR product (bp)	Limit of detection	Reference
PCR	Tox-4	5′-CGCTGCAGGGAGGAAGACGA AAGTTG-3′	529	320 fg (4 tachyzoites)	Homan et al. 2000
	Tox-5	5′-CGCTGCAGACACAGTGCATC TGGATT-3′			
ITS1 nPCR	Outer primer (sense strand)	5′-TGCGGAAGGATCATTCACACG-3′	1000	200 fg (2 tachyzoites)	Vitale et al. 2013
	Outer primer (nonsense strand)	5′-CCGTTACTAAGGGAATCATAGTT-3′			
	Inner primer (sense strand)	5′-GATTTGCATTCAAG AAGC(G)TGATAGTAT 3′	313		
	Inner primer (nonsense strand)	5′-AGTT(T)AGGAAGCAATCTGA AAGCACATC-3′			
B1 nPCR	Outer primer (sense strand)	5′-GGAACTGCATCCGTTCATGAG-3′		50 fg (1 tachyzoite)	Jones et al. 2000
	Outer primer (nonsense strand)	5′-TCTTTAAAGCGTTCGTGGTC-3′			
	Inner primer (sense strand)	5′-TGCATAGGTTGCAGTCACTG-3′	193		
	Inner primer (nonsense strand)	5′-GGCGACCAATCTGCGAATACACC-3′			
529 bp nPCR	Outer primer (sense strand)	5′-TGACTCGGGCCCAGCTGCGT-3′		640 fg (8 tachyzoites)	Fallahi et al. 2014
	Outer primer (nonsense strand)	5′-CTCCTCCCTTCGTCCAAGCCTCC-3′			
	Inner primer (sense strand)	5′-AGGGACAGAAGTCGAAGGGG-3′	164		
	Inner primer (nonsense strand)	5′-GCAGCCAAGCCGGAAACATC-3′			

PCR standard PCR, *nPCR* nested PCR

C1000™ Thermal Cycler. Cycling conditions for each PCR assay are presented in Table 3.

Real-time quantitative PCR

All tissue samples were tested by the real-time qPCR targeting the RE gene (Opsteegh et al. 2010) by using the CFX96 Touch Real-time detection system (Bio-Rad). The 20 µl reaction mixture consisted of 0.7 µM of Tox-9F (AGGAGAGATA TCAGGACTGTAG) and Tox-11R (GCGTCGTCTCGTCT AGATCG) primers, respectively, 0.1 µM of Tox-TP1 (CCGGCTTGGCTGCTTTTCCT) probe, 10 µl of SsoAdvanced Universal Probes Supermix (Bio-Rad), and 4 µl of template DNA. The reaction was performed according to the protocol described by Opsteegh et al. (2010). The standard curve and the estimation of PCR efficiency (> 1.85) and error (< 0.05) were calculated with inclusion of *T. gondii* DNA standard dilutions in reaction (from 30 pg to 30 fg). Samples that show a smooth exponential-amplification curve and presented a positive Cp value (< 35) were scored positive.

Gel electrophoresis

PCR products from sPCR and nPCR (8 µl) were electrophoresed in 1.5% agarose gel in TAE buffer and stained with SYBR® Safe DNA gel stain (Invitrogen). Electrophoresis conditions were 100 V and 400 mA for 30 min in TAE buffer. DNA fragments were visualized under UV light on an image analyzer (Bio-Rad BioDoc-It™ Imaging System) and compared to a 100 bp molecular weight marker (GeneRuler 100 bp DNA Ladder, Fermentas).

Table 3 Reaction condition in standard and nested PCR, targeting 529 bp fragment, ITS1 region, and B1 gene respectively

		sPCR	ITS1 nPCR	B1 nPCR	RE nPCR
First-round amplification	Denaturation	95 °C, 15 s, 35 cycles	94 °C, 50 s, 40 cycles	93 °C, 10 s, 40 cycles	94 °C, 20 s, 30 cycles
	Annealing	60 °C, 15 s, 35 cycles	60 °C, 30 s, 40 cycles	57 °C, 10 s, 40 cycles	55 °C, 20 s, 30 cycles
	Elongation	72 °C, 10s, 35 cycles	72 °C, 60 s, 40 cycles	72 °C, 30 s, 40 cycles	72 °C, 20 s, 30 cycles
Nested amplification	Denaturation		94 °C, 50 s, 40 cycles	93 °C, 10 s, 40 cycles	94 °C, 20 s, 35 cycles
	Annealing		60 °C, 30 s, 40 cycles	62.5 °C, 10 s, 40 cycles	55 °C, 20 s, 35 cycles
	Elongation		72 °C, 60 s, 40 cycles	72 °C, 15 s, 40 cycles	72 °C, 20 s, 35 cycles

Statistical analysis

Frequency, apparent prevalence, and its 95% confidence interval (CI) were calculated for each method of detection. The chi-square test was used for comparison of the frequencies by age category, gender (male and female), and rearing system (backyard and intensive farming). Logistic regression analysis was performed to quantify the association between the presence of *T. gondii* antibodies and risk factors (age, gender, and rearing system). The risk factors remained in the logistic regression analysis if the goodness of fit of the model was significant ($p \leq 0.05$).

A p value of ≤ 0.05 was considered statistically significant. Statistical analysis was performed with EpiInfo 2000 software (Centers for Disease Control and Prevention: http://wwwn. cdc.gov/epiinfo/).

The degree of agreement between serological methods (ELISA and MAT) and between indirect (ELISA, MAT) and direct (bioassay, PCRs) methods was measured by Cohen's kappa (k) statistic in EpiTools (Watson and Petrie 2010; Sergeant 2017). Also, proportions of positive and negative agreement were calculated. The level of confidence was set at 0.95 and population status as unknown/mixed. The strength of agreement was defined based on the k value: < 0.00 poor agreement; 0.00–0.20 slight agreement; 0.21–0.40 fair agreement; 0.41–0.60 moderate agreement; 0.61–0.80 substantial agreement; and 0.81–1.00 almost perfect agreement (Landis and Koch 1977).

Results

Collected samples

In total, 197 animals were sampled in backyard system ($n = 123$) or in the slaughterhouse ($n = 74$). The sampled population represented 0.98% from total population of buffaloes in 2016 in Romania (National Institute of Statistics 2017).

Animals originated from 5 counties of Transylvania: Sălaj, Cluj, Bihor, Maramureş, and Bistriţa-Năsăud (Fig. 1, Table 1). Most of the samples ($n = 136$) were collected in Sălaj county. The number of sampled animals per county reflects the population size of buffaloes in these counties. The ages of the animals varied from 2 weeks up to 300 months (25 years), with an average of 84.4 ± 77.6 months (7 ± 6.5 years). According to the age, the samples were collected from 25 buffalo calves (0–6 months; average 5.0 ± 1.6 months), 52 juveniles (6–30 months; average 17.6 ± 8.3 months), and 120 adults (> 30 months; average 129.9 ± 67.2 months). Most of the sampled animals were raised in backyard conditions (166/197; 84.3%) and were females (172/197; 87.3%) (Table 1).

Serology

One hundred ninety-seven sera were tested for specific IgG detection by ELISA and MAT techniques. IgG antibodies against *T. gondii* were detected by MAT in 16 (8.1%) buffaloes when the cutoff was 1:6 and only in 3 buffaloes (1.5%) at a cutoff of 1:96 (Tables 4 and 5). The highest titer (1:768) was obtained in a juvenile female (30-month-old) buffalo (Table 5). By ELISA, 13 (6.6%) animals had *T. gondii* antibodies (Table 4).

The agreement between MAT and ELISA was fair (cutoff $\geq 1:6$ $k = 0.219$; cutoff $\geq 1:96$ $k = 0.36$), regardless of the MAT cutoff value. The proportion of positive agreement was fair (0.28 and 0.38 respectively), while the proportion of negative agreement was almost perfect (0.94 and 0.97 respectively).

The apparent seroprevalence was significantly ($p = 0.02$) higher in adults (12.5%) compared to calves (0.0%) and juveniles (1.9%) by MAT $\geq 1:6$. The apparent seroprevalence was not significantly different by gender and rearing system. It was found that adults are 2.73 (OR 95% CI 0.74–10.0) more likely to present antibodies against *T. gondii*, comparing with juveniles ($p = 0.05$).

Bioassay

Seventy-four bioassays were performed, corresponding to the animals collected in the slaughterhouse. No *T. gondii*-specific IgGs were detected by MAT technique in mice sera ($n = 148$). No *T. gondii* tissue cysts were identified by the light microscopy examination of the olfactory lobes and media regions of the mouse's brain ($n = 148$). The PCR amplifications (sPCR, nPCR, and qPCR) of the mice' brain homogenate displayed negative results. There was no agreement between indirect methods and bioassay detection of *T. gondii* in buffaloes.

PCRs

All tissue samples collected from buffaloes (heart, liver, mesenteric lymph nodes, mediastinal lymph nodes, and diaphragm digest) were submitted to a standard PCR (sPCR), nested PCR (nPCR), and real-time quantitative PCR (qPCR) amplification. *T. gondii* DNA was detected in 2 out of 74 animals (2.7%; 95% CI 0.33–9.42): a 252-month (21 years)-old female buffalo in mesenteric lymph node by RE nPCR and in a 30-month (2.5 years)-old male buffalo in diaphragm digest by real-time qPCR. All other PCR assays or samples gave negative results. There was no agreement between indirect methods and PCR detection since positive animals in nPCR and qPCR were seronegative in both methods (MAT regardless of the cutoff value and ELISA).

Table 4 Frequency and prevalence of *T. gondii* antibodies, in sera collected from buffaloes, as detected by ELISA, MAT, and overall serology, stratified by age category, gender, and rearing system

	MAT ≥ 1:6			MAT ≥ 1:96			ELISA		
	Frequency	Prevalence (95% CI)	*p*	Frequency	Prevalence (95% CI)	*p*	Frequency	Prevalence (95% CI)	*p*
Age category									
Calves (*n* = 25)	0	0 (0.0–13.3)	0.02	0	0 (0.0–13.3)	0.80	1	4.0 (0.7–2.0)	0.78
Juveniles (*n* = 52)	1	1.9 (0.3–10.1)		1	1.9 (0.3–10.1)		3	5.8 (2.0–15.6)	
Adults (*n* = 120)	15	12.5 (7.7–19.6)		2	1.7 (0.2–5.9)		9	7.5 (4.0–13.6)	
Gender									
Males (*n* = 25)	0	0 (0.0–13.3)	0.23	0	0 (0.0–13.3)	0.83	1	4.0 (0.1–20.4)	0.89
Females (*n* = 172)	16	9.3 (5.4–14.7)		3	1.7 (0.4–5.0)		12	7.0 (3.7–11.9)	
Rearing system									
Backyard (*n* = 166)	12	7.2 (3.8–12.3)	0.48	3	1.8 (0.4–5.2)	0.96	13	7.8 (4.2–13.0)	0.22
Intensive farming (*n* = 31)	4	12.9 (3.6–29.8)		0	0 (0.0–11.2)		0	0 (0.0–11.0)	
Total (*n* = 197)	16	8.1 (4.7–12.9)		3	1.5 (0.3–4.4)		13	6.6 (3.6–11.0)	

Discussion

Surveys of *T. gondii* in buffaloes were reported worldwide, in countries where buffaloes represent an important component of the national livestock (Supplementary files 1 and 2). Worldwide, *T. gondii* seroprevalence in buffaloes is generally low (< 25.0%), whatever method was used (DAT, IFAT, IHAT, LAT, MAT, ELISA). Higher seroprevalence was reported in local studies from Turkey, Egypt, Mexico, and Brazil (Supplementary file 1). In these studies, a faulty technique might have been employed or there is indeed an intense circulation of the parasite in those areas (due to specific environmental conditions like felid densities and climatic specificities). Similarly, the apparent overall seroprevalence of *T. gondii* in buffaloes from northwestern Romania is low regardless of the test used. However, the agreement between MAT and ELISA was fair (0.28), similarly to the poor agreement obtained in cattle for the same techniques (Opsteegh et al. 2016a). The apparent seroprevalence was significantly higher in adults than in calves and young buffaloes. The uptake of *T. gondii* oocysts from grasslands for a longer period of time is the most logical explanation. Age has already been significantly correlated with the seropositivity to *T. gondii*

Table 5 Sera dilution at MAT in positive buffaloes by age category and total

Titers	Calves	Juveniles	Adults	Total
1:24	0/25	0/52	8/120	8/197
1:48	0/25	0/52	5/120	5/197
1:96	0/25	0/52	2/120	2/197
1:768	0/25	1/52	0/120	1/197
Total	0/25	1/52	15/120	16/197

either in water buffaloes (Persad et al. 2011; Santos et al. 2013; Ahmad and Qayyum 2014) or in other species (Dubey 2010; Opsteegh et al. 2016b). However, the highest dilution in MAT (1:768), correlated with high titers of IgG antibodies, was obtained in a juvenile (30 months old), most of the adults (13/15) presenting low titers. Most of the authors report low titers of antibodies in buffaloes (Supplementary file 1). In an experimental study in buffaloes, early serological response was noticed but not marked, followed by an early decline, even below a significant level after 56 days post infection (Gautam et al. 1982). The seroprevalence was not significantly different by gender and rearing system in our study, while divergent results were obtained elsewhere: male prevalence higher than females (Hamidinejat et al. 2010), semi-intensive and extensive farming identified as risk factors for increased seroprevalence in Pakistan and Brazil (Ahmad and Qayyum 2014; Brasil et al. 2015). The most probable hypothesis to explain these differences among studies would be the management practices: (i) the females/males are more out on the pastures (possible sources of *T. gondii* infection) and (ii) there is no real management difference between the two rearing systems. In our case, the female buffaloes are more frequently on pasture and the intensive farming is mostly limited to 40–50 animals (Borghese 2013).

Most of the studies concerning *T. gondii* in buffaloes were focusing on specific IgG antibody detection, while few of them (*n* = 3) were interested in direct detection of parasite by PCR techniques and none, as far as we know, by (cat or mouse) bioassay (Supplementary files 1 and 2). In this study, along with ELISA and MAT, several PCR techniques (standard PCR, nPCR, and qPCR) and mouse bioassay on various tissues (diaphragm, heart, liver, mesenteric and mediastinal lymph nodes) were employed in order to identify *T. gondii* DNA or tissue cysts. We used a variety of direct detection

techniques in order to overcome the lack of sensitivity of these methods, which has been observed in cattle (Opsteegh et al. 2016a, b). For the same reason, we used a sensu *large* positive case definition: an animal was considered positive if any of the collected samples were positive by any of the PCR techniques. Nonetheless, this resulted in only 2 PCR-positive buffaloes.

T. gondii DNA was detected in two different animals (a 30-month-old male and a 252-month-old female), in only one distinct tissue (diaphragm digest and mesenteric lymph node), by different PCR techniques (qPCR and 529 bp nPCR). Gautam et al. (1982) found in experimental infection that mesenteric lymph nodes are the most consistently parasitized tissue (3/8) and they did not isolate the parasite from diaphragm. Anyway, the authors isolated the parasite from mesenteric lymph nodes after 11–32 days post infection and not later (Gautam et al. 1982). All other PCR assays or samples from these 2 buffaloes gave negative results. The results of these PCR assays do not overlap at all, except for the negative ones. The observed discordance might be explained by a non-homogenous distribution of *T. gondii* within an animal carcass in terms of tissue distribution or parasitic load. Similarly, in an experimental study performed in calves, *T. gondii* was detected by either MC-qPCR or mouse bioassay in pools of tissues, but failed to detect the parasite DNA in all individual tissues (Burrells et al. 2018). Also, earlier studies indicated that *T. gondii* persists for short term in the tissues of the experimentally infected buffaloes (Gautam et al. 1982).

Moreover, the two buffaloes were serologically negative in both indirect detection methods (ELISA and MAT). Similar results were found in bovines, where seronegative animals were giving a positive amplification by PCR, demonstrating that seroprevalence cannot be used as an indicator of the number of cattle and buffaloes carrying infectious parasites (Opsteegh et al. 2011; Opsteegh et al. 2016a). This may indicate that only recent *T. gondii* infections are detectable by PCR in cattle and buffaloes. Apparently, contradictory results were found by Hassanain et al. (2013) in buffaloes, pointing out that 2/11 (18.2%) of the ELISA-positive buffaloes were found positive by PCR on blood; however, no PCR was run on serological negative samples.

Up to now, direct detection of *T. gondii* in buffaloes was performed in three studies (Supplementary file 2) and it was limited to PCR from milk (3.7% positive results) or blood (18.2–22.0% positive results) (Chaudhary et al. 2006; Dehkordi et al. 2013; Hassanain et al. 2013). The prevalence of *T. gondii* DNA detection in present study (2.7%) is much lower than previous studies but close to what is generally reported in bovines (1.6%, Opsteegh et al. 2016a; 1.79%, Hosein et al. 2016). One explanation for this lower prevalence might be the geographical area of studies: in Europe, the *T. gondii* prevalence in bovines is much lower than in Asia or South America (Belluco et al. 2016), so the situation may be similar for buffaloes.

Also, negative results were obtained in mouse bioassay (MAT, PCR, and microscopic examination of brains), regardless of the corresponding buffaloes' serology or PCR result. Even the two mice that were inoculated with a *T. gondii* DNA-positive diaphragm digest did not seroconvert 6 weeks p.i. This might indicate the presence of *T. gondii* parasite in diaphragm tissue, in a non-infectious form. One should keep in mind however that due to economic reasons, the bioassay was performed only on diaphragm and on two mice. Since predilection sites in buffaloes are not known, a comparison of various tissues tested by bioassay would be more informative.

Detection of specific antibodies in sera may indicate the presence of infectious parasites within the host and therefore a potential risk of infection in humans by undercooked meat consumption. However, this is true only if there is a good correlation between antibody detection and the presence of *T. gondii* tissue cysts in various tissues. In our study, the seropositive samples were negative in direct detection tests, and the other way around, so overall, there was no agreement between detection methods of *T. gondii* in buffaloes. Therefore, both MAT and the Chekit Toxotest ELISA appear unsuitable to obtain an estimate of the prevalence of *T. gondii* in buffaloes. Moreover, in the 74 buffaloes tested by mouse bioassay, no viable *T. gondii* was detected; therefore, it is questionable whether there is a risk for consumers.

In conclusion, since no viable *T. gondii* was detected in buffaloes, the risk of human infection from buffalo meat is minimal. The same conclusion was pointed out by Gautam and co-workers in 1982 after an experimental infection with *T. gondii* in buffaloes. Buffaloes' biological response to a *T. gondii* infection appears to be very similar to another Bovinae subfamily member, the cattle, which raise questions about the evolution of parasite carriage over the course of the lifetime of animals.

Compliance with ethical standards

References

Ahmad N, Qayyum M (2014) Seroprevalence and risk factors for toxoplasmosis in large ruminants in northern Punjab, Pakistan. J Infect Dev Ctries 8:1022–1028

Belluco S, Mancin M, Conficoni D, Simonato G, Pietrobelli M, Ricci A (2016) Investigating the determinants of *Toxoplasma gondii* prevalence in meat: a systematic review and meta-regression. PLoS One 11(4):0153856

Belluco S, Simonato G, Mancin M, Pietrobelli M, Ricci A (2017) *Toxoplasma gondii* infection and food consumption: a systematic

review and meta-analysis of case-controlled studies. CRIT REV FOOD SCI NUTR 58:3085–3096. https://doi.org/10.1080/10408398.2017.1352563

Borghese A (2005) Buffalo production and research, Food and Agriculture Organization of the United Nations, Rome. Buffalo production and research. In: FAO (ed) REU Technical Series 67:1–315

Borghese A (2013) Buffalo livestock and products. CRA – Council for Research in Agriculture, pp 511 ISBN 978-88-97081-27

Bouwknegt M, Devleesschauwer B, Graham H, Robertson LJ, van der Giessen JW, The Euro-FBP workshop participants (2018) Prioritisation of food-borne parasites in Europe, 2016. Euro Surveill 23(9):17–00161

Brasil AW, Parentoni RN, Feitosa TF, Bezerra S, Vilela VL, Pena HF, de Azevedo SS (2015) Risk factors for Toxoplasma gondii and Neospora caninum seropositivity in buffaloes in Paraiba state, Brazil. Rev Bras Parasitol Vet 24:459–463

Burrells A, Taroda A, Opsteegh M, Schares G, Benavides J, Dam-Deisz C, Bartley PM, Chianini F, Villena I, van der Giessen J, Innes EA, Katzer F (2018) Detection and dissemination of Toxoplasma gondii in experimentally infected calves, a single test does not tell the whole story. Parasit Vectors 11(1):45

Chaudhary ZI, Ahmed RS, Hussain SMI, Shakoori AR (2006) Detection of Toxoplasma gondii infection in butchers and buffaloes by polymerase chain reaction and latex agglutination test. Pak J Zool 38: 333–336

Dehkordi FS, Borujeni MR, Rahimi E, Abdizadeh R (2013) Detection of Toxoplasma gondii in raw caprine, ovine, buffalo, bovine, and camel milk using cell cultivation, cat bioassay, capture ELISA, and PCR methods in Iran. Foodborne Pathog Dis 10(2):120–125

Dubey JP (2010) Toxoplasmosis of animals and humans, 2nd edn. CRC Press Taylor, Francis Group, Boca Raton

Dubey JP, Desmonts G (1987) Serological responses of equids fed Toxoplasma gondii oocysts. Equine Vet J 19(4):337–339

Dubey JP, Thulliez P (1993) Persistence of tissue cysts in edible tissues of cattle fed Toxoplasma gondii oocysts. Am J Vet Res 54:270–273

Dubey JP, Desmonts G, McDonald C, Walls KW (1985) Serologic evaluation of cattle inoculated with Toxoplasma gondii: comparison of Sabin-Feldman dye test and other agglutination tests. Am J Vet Res 46(5):1085–1088

Dubey JP, Verma SK, Ferreira LR, Oliveira S, Cassinelli AB, Ying Y, Kwok OCH, Tuo W, Chiesa OA, Jones JL (2014) Detection and survival of Toxoplasma gondii in milk and cheese from experimentally infected goats. J Food Prot 77:1747–1753

Fallahi S, Kazemi B, Seyyed Tabaei SJ, Bandehpour M, Lasjerdi Z, Taghipour N, Zebardast N, Nikmanesh B, Omrani VF, Ebrahimzadeh F (2014) Comparison of the RE and B1 gene for detection of Toxoplasma gondii infection in children with cancer. Parasitol Int 63:37–41

FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization] (2014) Multicriteria-based ranking for risk management of food-borne parasites. Microbiological risk assessment Series 23, Rome, pp 302

Gautam OP, Chhabra MB, Gupta SL, Mahajan SK (1982) Experimental toxoplasmosis in buffalo calves. Vet Parasitol 11(4):293–299

Giordano G, Guarini P, Ferrari P, Biondi-Zoccai G, Schiavone B, Giordano A (2010) Beneficial impact on cardiovascular risk profile of water buffalo meat consumption. Eur J Clin Nutr 64:1000–1006

Hamidinejat H, Ghorbanpour M, Nabavi L, Hajikolaie MRH, Jalali MHR (2010) Seroprevalence of Toxoplasma gondii in water buffaloes (Bubalus bubalis) in south-west of Iran. Trop Biomed 27:275–279

Hassanain MA, El-Fadal HA, Hassanain NA, Shaapan RM, Barakat AM, El-Razik KAA (2013) Serological and molecular diagnosis of toxoplasmosis in human and animals. World J Med Sci 9(4):243–247

Homan WL, Vercammen M, De Braekeleer J, Verschueren H (2000) Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR. Int J Parasitol 30:69–75

Hosein S, Limon G, Dadios N, Guitian J, Blake DP (2016) Toxoplasma gondii detection in cattle: a slaughterhouse survey. Vet Parasitol 228: 126–129

Jones CD, Okhravi N, Adamson P, Tasker S, Lightman S (2000) Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of T. gondii in aqueous humor. Invest Ophthalmol Vis Sci 41:634–644

Kondaiah N, Anjaneyulu ASR (2003) Potential of buffalo meat to processing different products. Proc. of Fourth Asian Buffalo Congress, New Delhi, India 28:200–204

Kos K (1975) Contribuţii la cercetarea etnografică a creşterii bivolilor, Anuarul Muzeului Etnografic al Transilvaniei, VII, seria 1974-1975, pp 121–136

Landis JR, Koch GG (1977) The measurement of observer agreement for categorical data. Biometrics 33:159–174

National Institute of Statistics (2017) Livestock and animal production in 2016. http://www.insse.ro/cms/sites/default/files/field/publicatii/efective_de_animale_si_productia_animala_in_anul_2016.pdf

Naveena BM, Kiran M (2014) Buffalo meat quality, composition, and processing characteristics: contribution to the global economy and nutritional security. Anim Front 4:18–24

Opsteegh M, Langelaar M, Sprong H, Den Hartog L, De Craeye S, Bokken G, Ajzenberg D, Kijlstra A, Van Der Giessen J (2010) Direct detection and genotyping of Toxoplasma gondii in meat samples using magnetic capture and PCR. Int J Food Microbiol 139: 193–201

Opsteegh M, Prickaerts S, Frankena K, Evers EG (2011) A quantitative microbial risk assessment for meatborne Toxoplasma gondii infection in the Netherlands. Int J Food Microbiol 150:103–114

Opsteegh M, Schares G, Blaga R, Van der Giessen J, on behalf of the consortium (2016a) Experimental studies on Toxoplasma gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01) Final report. EFSA supporting publication, EFSA

Opsteegh M, Maas M, Schares G, Van der Giessen J, on behalf of the consortium (2016b) Relationship between seroprevalence in the main livestock species and presence of Toxoplasma gondii in meat (GP/EFSA/BIOHAZ/2013/01) An extensive literature review. Final report. EFSA supporting publication, EFSA

Persad A, Charles R, Adesiyun AA (2011) Frequency of toxoplasmosis in water Buffalo (Bubalus bubalis) in Trinidad. Vet Med Int 705358

Santos LM, Damé MC, Cademartori BG, da Cunha Filho NA, Farias NA, Ruas JL (2013) Occurrence of antibodies to Toxoplasma gondii in water buffaloes and meat cattle in Rio Grande do Sul state, southern Brazil. Acta Parasitol 58:334–336

Sergeant ESG (2017) Epitools epidemiological calculators. Ausvet Pty Ltd. http://epitools.ausvet.com.au

Villena I, Durand B, Aubert D, Blaga R, Geers R, Thomas M, Perret C, Alliot A, Escotte-Binet S, Thebault A, Boireau P, Halos L (2012) New strategy for the survey of Toxoplasma gondii in meat for human consumption. Vet Parasitol 183:203–208

Vitale M, Galluzzo P, Currò V, Gozdzik K, Schillaci D, Di Marco Lo Presti V (2013) A high sensitive nested PCR for Toxoplasma gondii detection in animal and food samples. J Microb Biochem Technol 5: 39–41

Watson PF, Petrie A (2010) Method agreement analysis: a review of correct methodology. Theriogenology 73:1167–1179