Meat juice serology for *Toxoplasma gondii* infection in chickens

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Abstract

Toxoplasma gondii is an important food-borne zoonosis. Free-range chickens are at particularly high risk of infection and are also excellent indicators of soil contamination by oocysts. In the present study, hearts of 77 free-range chickens were collected at slaughter. *T. gondii* meat juice enzyme-linked immunosorbent assay was performed with a commercial kit, following validation with positive controls, from experimentally infected chickens, and negative ones. Out of 77 samples, only 66 gave sufficient meat juice for serology. Of these, 24 (36.4%) were positive for *T. gondii* considering the 5*standard deviation values (calculated on the optical density of negative controls), while all the samples were negative considering sample/positive% values. Parasite-specific polymerase chain reaction was carried out on all samples obtained from heart tissue and none were positive for the presence of *T. gondii* DNA. Results would suggest that further study on the use of meat juice with a validated serological test to detect *T. gondii* in chickens could lead to widespread epidemiological studies in this important intermediate host. However, sample collection and test specificity require further evaluation.

Introduction

Toxoplasma gondii is an Apicomplexan protozoa that is considered as one of the most important food-borne parasitic zoonoses globally (Tenter *et al*., 2000; McAllister, 2005; Hill and Dubey, 2013). Human infection is acquired through the ingestion of infective oocysts shed by cats, which contaminate the environment, or tissue cysts present in raw or undercooked meat of the many intermediate hosts of the parasite (Dubey, 2010). Birds, including chicken, play an important role in the epidemiology of *T. gondii*. While raw or undercooked chicken meat as a source of infection for humans is less likely compared to other meats, chickens are an important source of infection for cats that in turn shed oocysts into the environment. Furthermore, free-range chickens are an excellent indicator of soil contamination in that they feed from the ground (Dubey *et al*., 2015).

Several serological tests can be used to determine *T. gondii* infection in poultry (Dubey *et al*., 1993, 2002; Casartelli-Alves *et al*., 2014), including the modified agglutination test (MAT), indirect immunofluorence antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISA). Meat juice serology (MJS) has proven to be an excellent method for detection of *T. gondii* infection at slaughter in different species, including sheep and pigs, and has been confirmed to correlate well with serum serology (Basso *et al*., 2013; Meemken *et al*., 2014; Bacci *et al*., 2015). The application of meat juice serology would be ideal for large-scale investigations of *T. gondii* prevalence in poultry.

Thus, the aim of the present study was to determine seroprevalence for *T. gondii* with meat juice ELISA obtained from free-range chickens using a commercially available diagnostic kit, and to confirm the presence of the parasite with application of molecular biology techniques. Therefore a 200- to 300 fold repetitive fragment (529 bp) and a 35-fold repetitive target (B1) for amplification were used.

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Materials and Methods

Animals

From April to July 2015, a total of 77 chickens were sampled: forty chickens came from a backyard farm located in the Piacenza province (northern Italy), while 37 came from a large scale industrial farm with strict biosecurity measures located in Teramo province (central Italy). All animals were raised according to free-range standards, including outdoor access for a minimum of 8 hours a day and slaughter at a minimum of 54 days. The slaughterhouses were located near the poultry farms. Hearts (medium weight around 4-5 g) of 77 animals were collected, put individually in small bags, and immediately frozen at -20°C for 18-24 hours after slaughter. After thawing, meat juice was obtained for use in serology and each heart was conserved for molecular biology, according to Bacci *et al*. (2015).

Kit validation

A commercial ELISA test kit (ID SCREEN® Toxoplasmosis Indirect Multi-Species; IDvet, Grabels, France) was used. However, since this kit has been developed for detection of anti-*T. gondii* immunoglobulin G (IgG) antibodies in pigs, cats, ruminants and dogs, some adaptations were made for the diagnosis in chickens. Briefly, the conjugate of the kit was replaced with anti-chicken IgG (whole molecule) peroxidase conjugate (Sigma Aldrich®, St. Louis, MO, USA) and tested with positive control serum obtained from experimentally-infected chickens and negative uninfected controls (kindly provided by Dr. B. Bangoura, University of Leipzig, Germany). In order to define the best conditions for testing meat juice samples, positive controls were tested at different dilutions (1:40, 1:80, 1:160, 1:320). Negative controls were tested *in toto*. For each condition, the secondary antibody was used at three different concentrations (1:15,000, 1:20,000, 1:30,000). The highest optical density (OD) values for negative controls were considered as plate cut-off. We also modified the incubation temperature to 37°C, according to Sun *et al*. (2015). Plates were incubated for 1 h with serum samples and then for 45 min with the secondary antibody. All other conditions were met according to manufacturer's instructions. Optical densities were read at 450 nm with a spectrophotometer (Multiskan™ FC Microplate Photometer; Thermo Scientific, Swedesboro, NJ, USA) and results were expressed in two ways: i) as sample/positive (S/P)% values [$S/P\% = (OD_{sample} - OD_{negative\ controls})/(OD_{positive\ controls} - OD_{negative\ control}) \times 100$] greater than 50% (samples with S/P% ≤40% were considered negative, between 40 and 50% were considered doubtful); ii) as 5×the mean standard deviation (SD) of the mean of negative controls (5*SD). All the samples that presented an OD value higher than the value 5*SD were considered positive (Dubey *et al*., 2005).

Meat juice serology

All collected meat juice samples were tested *in toto*, according to conditions described above. Based on results of kit validation, secondary antibody was used at a dilution of 1:15,000. Positive serum controls were tested at a 1:40 dilution.

DNA extraction and *Toxoplasma gondii* identification with polymerase chain reaction

Hearts from all slaughtered chickens were sectioned and homogenised for DNA extraction. Briefly, hearts were surface-sterilised by submersion in 70% ethanol. All the cardiac muscle, cleaned from fat and connective tissue, was sampled and blended with the addition of 50 mL of phosphate buffered saline (PBS). Two-hundred μL of tissue were used for the DNA extraction carried out using a commercial kit (DNeasy Blood & Tissue Kit; Qiagen, Valencia, CA, USA).

Toxoplasma gondii presence was confirmed by a PCR targeting a 529 bp region, using the primers TOX4 and TOX5, as described by Homan *et al*. (2000). Amplification was performed by 2 min incubation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final 10 min incubation at 72°C. A second nested-PCR protocol, to confirm or not the presence of *T. gondii*, was performed essentially as described by Burg *et al*. (1989) using the internal and external primers amplifying the *B1* gene. The first round amplification was performed in a final volume of 25 μL, with 0.1 μM each primer, and 2× reaction mixture (MyTaqmix-ready-to-use; Bioline, Taunton, MA, USA). The amplification protocol foresaw a first denaturation step at 94°C for 2 min, followed by 40 cycles of 94°C for 10 sec, 57°C for 10 sec, 72°C for 30 sec. The nested amplification contained 1 μL of first-round product, 0.5 μM of each primer in a final volume of 25 μL. Nested reaction consisted of 40 cycles (after a first denaturation at 94°C for 2

Table 1. Results of enzyme-linked immunosorbent assay (meat juice) and polymerase chain reaction (heart) on the samples in the two farms.

Sample farm Piacenza	PCR	ELISA S/P%	ELISA 5*SD	OD value	Sample farm Teramo	PCR	ELISA S/P%	ELISA 5*SD	OD value
1	-	-	+	0.1387	2	-	-	-	0.0489
2	-	-	+	0.0710	4	-	-	-	0.0474
3	-	-	+	0.0720	5	-	-	-	0.0513
5	-	-	+	0.0772	6	-	-	-	0.0498
6	-	-	+	0.1107	8	-	-	-	0.0515
8	-	-	+	0.1886	9	-	-	+	0.0740
11	-	-	+	0.0838	10	-	-	-	0.0529
12	-	-	+	0.0854	11	-	-	-	0.0484
13	-	-	+	0.0668	12	-	-	-	0.0487
14	-	-	+	0.0625	13	-	-	-	0.0469
15	-	-	+	0.1113	14	-	-	-	0.0519
17	-	-	+	0.0643	15	-	-	-	0,0495
18	-	-	-	0.0577	17	-	-	-	0.0503
19	-	-	+	0.0769	19	-	-	-	0.0522
20	-	-	+	0.0881	20	-	-	-	0.0513
21	-	-	+	0.0636	21	-	-	-	0.0483
22	-	-	-	0.0580	23	-	-	-	0.0500
23	-	-	-	0.0586	24	-	-	-	0.0563
24	-	-	-	0.0576	25	-	-	-	0.0542
25	-	-	+	0.1024	26	-	-	-	0.0518
26	-	-	+	0.1203	27	-	-	-	0.0570
27	-	-	+	0.0671	28	-	-	-	0.0520
28	-	-	+	0.0632	29	-	-	-	0.0500
29	-	-	-	0.0585	30	-	-	-	0.0500
30	-	-	-	0.0588	31	-	-	+	0.0631
31	-	-	-	0.0556	32	-	-	-	0.0496
32	-	-	-	0.0562	33	-	-	-	0.0500
33	-	-	-	0.0573	34	-	-	-	0.0519
34	-	-	-	0.0554	35	-	-	-	0.0497
35	-	-	+	0.0742	36	-	-	-	0.0524
36	-	-	+	0.0850	37	-	-	-	0.0597
37	-	-	+	0.1370					
38	-	-	-	0.0556					
39	-	-	-	0.0517					
40	-	-	-	0.0574					

PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; S/P, sample/positive; SD, standard deviation; OD, optical density.

min) of 94°C for 10 sec, annealing at 62.5°C for 10 sec, 72°C for 15 sec. Negative controls from first round amplification and an additional second round negative control of sterile water were included in the nested reactions. Positive controls were included in each PCR reaction.

The PCR products were fractionated on a 1.5% agarose gel, stained with SybrSafe (Life technologies) and visualized by UV transillumination; the weight of the bands were identified using a marker of molecular weight (100 bp DNA ladder; Promega, Madison, WI, USA).

Results

Of the 77 hearts collected, only 66 gave sufficient meat juice for use in ELISA. Table 1 shows the results of meat juice serology. None of the meat juice samples (0%) were positive when considering S/P% values, while 24 (36.4%) were positive if OD values were expressed as 5*SD values (positive cut-off 0.062). None of the samples was positive for the presence of *T. gondii* DNA following PCR with two parasite-specific primers.

Discussion

A recent review has reported that prevalence of *T. gondii* in free-range chickens can be as high as 100% (Dubey, 2010), confirming their role in the epidemiology of infection and the related zoonotic risk through consumption of undercooked chicken meat.

Serum serology for *T. gondii* in chickens can be carried out with different methods, including MAT, IFAT and ELISA. Sensitivity and specificity vary and depend on different factors, including age, number of animals sampled, and test used. Currently, there are no available ELISA tests for *T. gondii* serology in chickens. However, Casartelli-Alves *et al*. (2014) recently reported sensitivity and specificity of 85 and 56% respectively, in naturally exposed chickens, using a commercial ELISA adapted to chicken sera. Meat juice serology (MJS) is increasingly used to determine the prevalence of various pathogens in different animal species, including *T. gondii* infection in pigs and sheep (Lundèn *et al*., 2002; Berger-Schoch *et al*., 2011; Glor *et al*., 2013; Bacci *et al*., 2015). There are currently no reports on the use of MJS for *T. gondii* infection in chickens at slaughter. Dubey *et al*. (2005) applied the ELISA technique to evaluate the presence of anti-*T. gondii* antibodies in tissue fluid obtained from retail breast meat, reporting a prevalence of 1.3%. In the present study, hearts were used for obtaining meat juice. A recent study has shown that comparison of *T. gondii*-specific antibody titers in meat juice and serum of pigs revealed a strong positive correlation for meat juice from heart tissue, making this the organ of choice for MJS (Wallander *et al*., 2015). The commercial kit used in the present survey has been validated for multiple species, but not for chickens, and the protocol was adapted by changing the species-specific conjugate and testing performance with serum from experimentally infected chickens. Serum from experimentally infected chickens was used both *in toto* for kit validation and at a 1:40 dilution when testing meat juice samples in order to decrease antibody concentration and to better mimic OD values in meat juice, which are consistently lower compared to serum (Wallander *et al*., 2015). Positive titers in meat juice collected from the hearts were established in two different ways, according to manufactures' instructions and according to Dubey *et al*. (2005), resulting in a prevalence of approximately 0 and 36.4% respectively. The results obtained from the two evaluation methods were very different. If the positive control had a very high concentration of immunoglobulin Y, thus giving a high cut off OD value, samples from chickens with low antibody titres may have resulted negative with the S/P% calculation. On the other hand, five times the mean standard deviation of the mean of all samples tested may have resulted in a higher number of false positive samples. It would be necessary to further study the validation of this kit with experimentally infected birds.

Considering 5*SD values, prevalence values were notably different between the two farms, probably due to different farm management. The large scale, industrial free-range chickens are kept under strict biosecurity measures, including barriers, which inhibit access by cats, thus making soil contamination by oocysts nearly impossible. The backyard farm in northern Italy, on the contrary, is family run and the grounds are open to the surrounding countryside.

Parasite-specific PCR was negative in all the samples, thus it was not possible to verify positive serology with the presence of *T. gondii* in myocardial tissue of naturally exposed, free-range chickens. Hill *et al*. (2006) reported similar results when comparing diagnostic methods in both *T. gondii*-experimentally and naturally infected pigs and retail pork products. The authors cited different reasons for DNA-negative samples, including limited tissue sample size and random distribution of tissue cysts. Geuthner *et al*. (2014) also reported a very low prevalence of *T. gondii* DNA in muscle tissue from experimentally infected chickens (2.1%), suggesting that *T. gondii* does not persist for long periods in this species. Dubey *et al*. (2005) found no positive samples of retail breast meat when tissue was bioassayed in mice. The results of the present study would confirm these previous reports and would suggest that while positivity in MJS may be an indicator of infection risk, it likely does not correlate with detection in the meat.

In Italy free range farms have increased in recent years and approximately 3,500,000 chickens were raised in 2013 (www.istat.it), making Italy one of the most important producers in Europe. For its features, free-range livestock could be a source of infection of *T. gondii* and an indicator of contamination, even if studies on infection prevalence in Italian poultry are still limited.

Conclusions

To conclude, we have shown that MJS with a commercially available ELISA kit, adapted for chicken serum, can be considered as a promising tool for wide-scale epidemiological studies for *T. gondii* in poultry. The development of a valid ELISA test for use in slaughtered chickens would be extremely useful since the important role that chickens play in *T. gondii* epidemiology, in particular for the risk of zoonotic infection and food safety.

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