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New strategy for the survey of *Toxoplasma gondii* in meat for human consumption

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ABSTRACT

Monitoring of *Toxoplasma* infection in animals destined for human consumption is a great challenge for human toxoplasmosis prevention. This study aimed to compare results obtained from a naturally infected population of sheep using different tests and targeting an original matrix: meat samples and muscle fluids collected at the slaughterhouse. A commercial ELISA test was performed on diaphragm fluids from 419 ovine carcasses collected at the slaughterhouse. A MAT (modified agglutination test) was performed on heart fluids obtained from the same animals. In addition, all hearts were bioassayed in mice. Serological test agreement, the relative sensitivity of ELISA MAT and mouse bioassay as well as a correlation between titres and parasite isolation probability were statistically evaluated. The overall agreement (kappa coefficient = 0.64) of ELISA on diaphragm fluids and MAT on heart fluids is substantial and subsequently both tests can be used for epidemiological studies. Relative sensitivity was higher for MAT performed on cardiac fluids (90%) than ELISA on diaphragm fluid (61%). For both serological tests, relative sensitivity is lower in lambs younger than 12 months. Relative sensitivity of mouse inoculation was 42%. A significant correlation was obtained between increasing MAT titres and probability to isolate live parasite from the heart. When the fluid titre was higher than 1:16, parasites were isolated in 65% of cases. When it was lower, isolation failed in 95% of the cases. According to our results, cardiac fluids appear to be a relevant matrix for toxoplasmosis survey in meat.

1. Introduction

Toxoplasma gondii is a common and cosmopolitan parasite of all warm-blooded animals including humans (Dubey and Beattie, 1988). The major route of toxoplasmosis transmission to human is the consumption of contaminated food, especially undercooked meat containing bradyzoites cysts. In Europe up to 63% of human infections are attributable to the consumption of undercooked or cured meat products (Cook et al., 2000). Moreover, ovine meat is a significant risk factor for acquiring *Toxoplasma* infection in certain areas of France (Berger et al., 2008).

Prevention of toxoplasmosis transmission in humans depends on meat safety strategies. Monitoring infection in

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animals destined for human consumption is a great challenge for toxoplasmosis control (Kijlstra and Jongert, 2009). Work at the slaughterhouse level offers the opportunity to investigate the very last "gate" of transmission from animal to human. Screening carcasses at the slaughterhouse for several food borne pathogens, such as *Salmonella* sp. or *Campylobacter* sp., has been introduced in many Western countries (Kijlstra and Jongert, 2008). Carcass surveys present several advantages. Work on early necroptic pieces is possible. In addition, control plans for a pathogen can easily be conducted by veterinary teams, as carcasses are grouped. Nationwide standardised, systematic samplings can be proposed easier than at the herd level. However, the kind of samples available is different: blood samples are not feasible and sera are therefore not available.

For serological analysis, muscle fluids offer an interesting alternative matrix because it is simpler to collect than drawing and processing blood for serum. Monitoring programs based on muscle fluid serological tests have been successfully implemented for *Salmonella* (Mousing et al., 1997; Nielsen et al., 1998), *Trichinella* (Nöckler et al., 2005) or Aujeszky virus (Le Potier et al., 1998) diagnosis in slaughter pigs. The production of muscle fluid is very suitable for large-scale analyses, mainly because the procedure is simple and may be automated. Moreover, collected pieces of meat also offer the opportunity for parasitological evidence. For *Trichinella*, digestion methods are used for routine meat inspection in pigs (Nöckler et al., 2005). For *T. gondii*, the presence of tissue cysts is less constant in animals. Direct visualisation from tissues is hazardous, and the gold standard remains parasite isolation through bioassays in mice or cats (Dubey and Beattie, 1988). But it is time consuming and, although 100% specific, lacks sensitivity.

Difficulty of toxoplasmosis monitoring in farm animals is due to the asymptomatic and chronic expression of the disease. Moreover, despite the broad range of serological tests available routinely used to determine *T. gondii* infection status in populations of different animal species (Dubey and Beattie, 1988), no standardized validated serological tests are currently available that correlate seropositivity to the presence of infectious parasites in animal meat (Kijlstra and Jongert, 2008). Due to the difficulty of obtaining a suitable gold standard test, the diagnostic accuracy of the different methods is usually assessed by comparing their results to those from either bioassays in cats or mice, considered the "gold standard" for infection (Dubey et al., 1995; Wingstrand et al., 1997), or other serological tests with expected high sensitivity and specificity, such as the Sabin–Feldman dye test or the modified agglutination test (MAT) (Werre et al., 2002).

This study aimed to compare results obtained from a naturally infected population of sheep using different tests and targeting an original matrix: meat samples and muscle fluids collected at the slaughterhouse. Different approaches were used and compared in order to evaluate the feasibility of such a strategy for toxoplasmosis control. A commercial ELISA test performed on muscle fluids obtained from diaphragms was used. This technique is a very simple one to develop because the diaphragm is a piece of meat easy to collect, available from all fresh carcasses, including the ones from abroad and has no exchange value. In addition, ELISA tests can be performed by neophytes (Nöckler et al., 2005) and would be easy to standardise. We thus compared this ELISA test with a MAT (modified agglutination test) performed on muscle fluids obtained from the heart of the same animal. In addition, all hearts were bioassayed in mice in order to compare the two serological tests on meat juice, as well as to evaluate the relative sensitivity and correlation between indirect serological approaches and direct detection of the parasite by mouse bioassay.

2. Materials and methods

2.1. Meat samples

Diaphragms and hearts of 419 ovine carcasses were collected in slaughterhouses (337 lambs younger than 12 months, 75 adults older than 12 months and 7 animals of unidentified age) (Halos et al., 2010). Hearts were collected and placed in individual plastic boxes and kept refrigerated (approximately 4 °C) for 1–2 days and transported to the Parasitology Laboratory of CNR in Reims. Diaphragms were collected in plastic bags and kept frozen after transportation to LNR in Maisons-Alfort.

2.2. Fluid collection

Fluids from each heart were directly collected in each individual plastic box. Fluids from each diaphragm were obtained from 25 g of meat which were cut into small pieces and frozen overnight at −20 °C in a plastic bag. After thawing at room temperature, the muscle fluid was collected with a pipette into a microtube as previously described (Nöckler et al., 2005).

2.3. Study design

For each animal, two samples were available: diaphragm, for which an ELISA test was performed on extracted fluids ($ELISA_{DF}$) and the heart for which a MAT was performed on the extracted fluid (MAT_{CF}). When the two results were discordant, a MAT was performed on diaphragm fluids (MAT_{DF}) as well as an ELISA test on cardiac fluids ($ELISA_{CF}$) (48 animals). All hearts were bioassayed in two mice.

2.4. Modified agglutination test

The MAT (modified agglutination test) for the detection of *T. gondii*-specific immunoglobulin (IgG) antibodies was performed as previously described on all cardiac fluids using an antigen prepared from formalin-fixed whole RH tachyzoites (Dubey and Desmonts, 1987). Cardiac fluids were serially two-fold diluted. The threshold dilution was 1:4.

2.5. ELISA test

The ELISA test, Elisa Pourquier® (Institut Pourquier, France) was performed on diaphragm fluids according to the manufacturer's instructions except that fluid dilution was 1:4 and not 1:20 due to the weaker concentrations in

body fluids compared to sera (Nielsen et al., 1998; Nöckler et al., 2005). Dilution 1:4 was chosen as compromise between the weak antibody concentration and the high protein concentration which may lead to a non-specific reaction at a lower dilution (Le Potier et al., 1998). After incubating antigen-coated microplates with the tested fluids at 1:4 dilution, *T. gondii*-specific antibodies were detected by binding the antigen/antibody complex with a peroxidase-labelled anti-ruminant IgG monoclonal antibody conjugate. Both the positive and negative controls provided in the kit were sheep sera. The optical density (OD) of the reaction was read on a Multiscan EX (Thermo Electron, France). Results were measured as optical density percentages ($OD\% = 100 \times (ODsample - ODnegative\ control)/(ODpositive\ control - ODnegative\ control)$). According to the manufacturers, samples presenting an OD% greater than 50% should be deemed as positive, an OD% between 40% and 50% as ambiguous, and an OD% < 30% as negative.

2.6. Bioassay for T. gondii in mice

Hearts were bioassayed in outbred female Swiss Webster mice (Charles River Laboratory, France). Briefly, each whole heart (180 g for an ovine adult and 120 g for a lamb) was mixed and incubated at 37 °C for 2.5 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 and streptomycin, leading to a final volume of 6 ml. This homogenate was inoculated intraperitoneally into two mice (1 ml per mouse) (Villena et al., 2004; Afonso et al., 2007; Aubert et al., 2010). Mice were bled four weeks post inoculation and their serum was tested at 1:25 dilution for *T. gondii* antibodies with the MAT. Mice were killed 60 days post inoculation and their brains were examined for tissue cysts.

2.7. Statistical analysis

2.7.1. Relative sensitivity

A global status was computed for each animal: positive if one (or more) of the three performed tests (MAT_{CF}, $ELISA_{DF}$, mouse bio-assay) had given a positive result, and negative otherwise. Relative sensitivities were calculated using this global status as a gold standard. Values obtained in lambs and adults were compared.

2.7.2. Serological test agreement

MAT_{CF} and $ELISA_{DF}$ were compared using the kappa coefficient. Its value was interpreted according to the usual scale (<0.2: slight agreement, 02–0.4: fair agreement, 0.4–0.6: moderate agreement, 0.6–0.8: substantial agreement, >0.8: almost perfect agreement) (Dohoo et al., 2003). The Kappa coefficient was separately computed for two different ovine populations: lambs and adults. Both values were compared using a bootstrap approach (McKenzie et al., 1996; Vanbelle and Albert, 2008). Proportions of positive results were compared using McNemar's χ^2 test.

As the two primarily used serological techniques (MAT_{CF} and $ELISA_{DF}$) have been performed on fluids extracted from two different organs (cardiac and diaphragm fluids), discordant results could be attributed to a test effect (sensitivity of one of the two tests is higher than that of the other test, whatever the organ), to an organ effect (sensitivity of a test performed on a given organ is higher than that of the same test performed on the other organ, whatever the test), or to both effects. These hypotheses could be investigated in discordant animals (for MAT_{CF} and $ELISA_{DF}$ results) as these were also tested with the two complementary techniques (MAT_{DF} and $ELISA_{CF}$). Two logistic mixed models were used: a test-oriented model and an organ-oriented model. In the test-oriented model, the independent variable was the test result obtained on cardiac fluids and the fixed effect was the result obtained on diaphragm fluids using the same test (MAT or ELISA). In the organ-oriented model, the independent variable was the MAT result obtained on a given organ and the fixed effect was the ELISA result obtained on the same organ. In both cases, the animal was included as a random effect.

2.7.3. Correlation between serological titres and bio-assay

The relationship between bio-assay and quantitative serological results was finally studied in seropositive animals. For each of the two primarily used techniques (MAT_{CF} and $ELISA_{DF}$), a generalised linear model was defined to investigate the relationship between the quantitative serological result (log(titre) for MAT and OD% for ELISA) and two binary dependent variables: the result of the mouse bio-assay and the age of the animal. The association between quantitative serological results and mouse bio-assay results, as revealed by the model, was further studied constructing and analysing a receiver operating curve (ROC) to evaluate the predictive value of the corresponding test for the result of the mouse bio-assay.

3. Results

3.1. Relative sensitivities

Of the 419 tested animals, 115 (27%) gave a positive result one (or more) of the three primarily used tests (MAT_{CF}, $ELISA_{DF}$, mouse bio-assay) (Table 1).

Of the two serological techniques studied, the relative sensitivity of MAT_{CF} was the highest with a value of 90% (95% confidence interval [CI]: 83–95%). A slight difference between lambs and adults was observed, with a lower value in lambs (85%, 95% CI: 73–93%) than in adults (96%, 95% CI: 97–99%) (Fisher's test: $p = 0.05$).

For $ELISA_{DF}$, the relative sensitivity was lower: 61% (95% CI: 52–70%). It was significantly lower in lambs (41%, 95% CI: 28–54%) than in adults (84%, 95% CI: 71–92%) (Fisher's test: $p < 0.0001$).

The relative sensitivity of mouse bio-assay was clearly lower than that of both serological techniques: 42% (95% CI: 33–52%). It was significantly lower in lambs (24%, 95% CI: 13–38%) than in adults (60%, 95% CI: 46–74%) (Fisher's test, $p = 0.0002$). Of the 48 carcasses positive for bio-assay (10 with only one of the two mice used positive), 2 were negative for both serological techniques ($ELISA_{DF}$ and MAT_{CF}).

Table 1
Results of modified agglutination test (MAT) on cardiac fluids, ELISA on diaphragm fluids and mouse bio-assay on hearts samples for the detection of *T. gondii* in sheep, France, 2008.

MAT (cardiac fluids)	ELISA (diaphraph fluids)	Mouse bio-assay	Number of animals		
			Lambs	Adults	Total
Positive	Positive	Positive	8	27	36[a]
		Negative	8	16	24
		NA[d]	1	1	2
	Negative	Positive	3	4	7
		Negative	26	4	30
		NA[d]	4	1	5
Negative	Positive	Positive	0	1	1
		Negative	7	1	8
		NA[d]	0	0	0
	Negative	Positive	2	0	2
		Negative	235	17	256[b]
		NA[d]	43	3	48[c]

[a] Age missing for one animal.
[b] Age missing for four animal.
[c] Age missing for two animal.
[d] Non available result because of death of inoculated mice due to bacterial contamination.

Both animals were lambs, with low $ELISA_{DF}$ OD% (−3.4 and 3.4) and with a MAT_{CF} titre of 1:2, just below the threshold.

3.2. Comparison of serological tests

The overall kappa coefficient was 0.64 (95% CI: 0.54–0.73%), indicating a substantial agreement between the results of MAT_{CF} and those of $ELISA_{DF}$. The cross-classification of the results of the MAT and ELISA for both lambs and adults is shown in Table 1.

Discordant results showed an imbalance in favour of MAT_{CF}-positive and $ELISA_{DF}$-negative animals (MacNemar's χ^2 test: $p < 0.0001$), thus confirming the higher sensitivity of MAT on cardiac fluids. In lambs, agreement between MAT_{CF} and $ELISA_{DF}$ was 0.40 (95% CI: 0.23–0.58%), indicating a moderate agreement. It was conversely higher in adults: 0.67 (95% CI: 0.50–0.85%). The difference between lambs and adults was significant ($p < 0.05$).

In animals for which discordant results were obtained (Table 2), the test-oriented logistic mixed model did not show any significant link between the result of a given test (MAT or ELISA) performed on cardiac fluids and that of the same test performed on diaphragm fluids ($p = 0.36$). Conversely, the organ-oriented logistic model revealed a significant link between the results of tests performed on the same organ ($p = 0.003$). This suggests that the main determinant of discordant results between MAT on cardiac fluids and ELISA on diaphragm fluids could be the organ rather than the test.

3.3. Correlation between serological titres and bio-assay

In the 104 MAT_{CF}-positive animals, the general linear model showed a significant association between the log(titre) and the mouse bio-assay result ($p < 0.0001$) but no significant effect of age on log(titre). Conversely, in the 71 $ELISA_{DF}$-positive animals, neither the mouse bio-assay result nor the animal age was significantly linked with ELISA OD%.

ROC analysis was thus conducted to analyse, in MAT_{CF}-positive animals, the value of the titre for predicting the mouse bio-assay result (Fig. 1). The area under the curve was 0.83, indicating a moderate predictive ability. The optimal cut-off was 1:16, and the corresponding sensitivity and specificity values were 0.95 and 0.59, respectively: 95% of bio-assay-positive animals showed a ≥16 MAT_{CF} titre, and 59% of bioassay-negative animals gave a <16 MAT_{CF} titre. Positive and negative predictive values were 65% and 94%, respectively; a positive animal with a MAT_{CF} titre ≥16 thus had a 65% probability to give a mouse bio-assay positive result, while a positive animal with a MAT_{CF} titre <16 had a 94%probability to give a bio-assay negative result.

Table 2
Results of modified agglutination test (MAT) on diaphragm fluids and ELISA on cardiac fluids for the detection of *T. gondii* in sheep having given discordant results for MAT on cardiac fluids and ELISA on diaphragm fluids, France, 2008.

MAT (cardiac fluids)	ELISA (diaphragm fluids)	MAT (diaphragm fluids)	ELISA (cardiac fluids)		
			Negative	Positive	Total
Positive	Negative	Negative	8	15	23
		Positive	6	11	17
		Total	14	26	40
Negative	Positive	Negative	0	2	2
		Positive	4	2	6
		Total	4	4	8

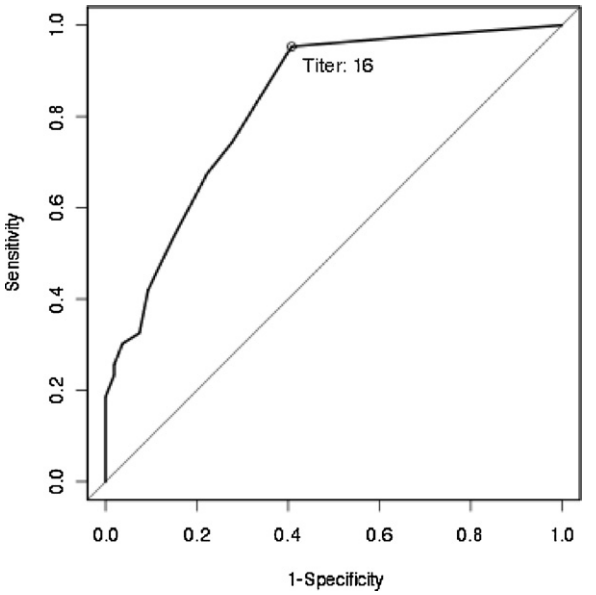

Fig. 1. Receiver operating curve for the prediction of mouse bio-assay result from the titre obtained using modified agglutination test (MAT) on cardiac fluids for the detection of *T. gondii*, France, 2008 (the proposed threshold maximizing sensitivity + specificity is indicated on the graph).

4. Discussion

Muscle fluids thus appear to be a relevant matrix for survey of toxoplasmosis seroprevalence in meat, as previously described for other food borne pathogens (Mousing et al., 1997; Le Potier et al., 1998; Nielsen et al., 1998; Nöckler et al., 2005). Moreover, the overall agreement (kappa coefficient = 0.64) of ELISA on diaphragm fluids and MAT on heart fluids is substantial and the two tests can be used for epidemiological studies.

Nevertheless, some points need to be considered. Relative sensitivity estimates show better accuracy for MAT performed on cardiac fluids (90%). This value is close to those previously described for MAT among naturally infected animals (Mainar-Jaime and Barberán, 2007; Dubey et al., 1995; Gamble et al., 2005; Shaapan et al., 2008). Values obtained for ELISA on diaphragm fluid is lower (61%) than MAT, as previously described, but is also lower than expected from previous studies (Mainar-Jaime and Barberán, 2007; Dubey et al., 1995; Gamble et al., 2005). However this difference appears to be induced by the organ and the way of obtaining the muscle fluids than by the test; tests performed on fresh cardiac fluids appear to be more sensitive than tests performed on diaphragm fluids, which were obtained after freezing and thawing the muscle. The use of 1:4 dilution may have led to an under-estimation of positive samples. Nevertheless, results obtained with a 1:2 dilution were similar (data not shown). Relative sensitivity of mouse bioassay is the lowest (42%) as expected (Dubey and Beattie, 1988) but is the only test that detects live parasites and infected meat.

An increase of relative sensitivity with age was observed for both serological tests. They both harbour a lower sensitivity for lambs (i.e. animals younger than 12 months). This difference appears larger for ELISA tests performed on diaphragm fluids. Since the *Toxoplasma* primo-infection leads to a one month period of IgM antibody production (Dubey and Beattie, 1988; Esteban-Redondo and Innes, 1997), early infection with no IgG production can occur during a short time interval, mostly observed in young animals that have never encountered the parasite before. This could explain the two samples for which live parasites were isolated but no serological detection was available either with MAT or ELISA. Both samples were 3 month-old lambs. The reason why this bias is much more significant with ELISA test remains unclear.

Interestingly, in animals with a MAT result on cardiac fluids above the threshold, a significant correlation was obtained between increasing MAT titres and the probability of isolating live parasites from the heart. When the fluid titre was higher than 16, parasites were isolated in 65% of the cases. When it was lower, isolation failed in 95% of the cases. This suggests that MAT titres obtained on cardiac fluids could be used to select animals submitted to bioassay. Further studies are needed to correlate with more accuracy the presence of the parasite in consumed meat with the serological titre.

Given the overall results the ELISA test on muscle samples can be used for epidemiological sero-survey. It offers an acceptable sensitivity linked to simplicity of use. Results obtained with this kind of technique should nevertheless be interpreted with consideration for its limits. The moderate sensitivity of ELISA tests for lamb as well as the absence of correlation between the ELISA OD% and the isolation of the parasite would justify, when feasible, the selection of the MAT rather than ELISA for its use in toxoplasmosis control schemes.

Strategies to work on muscle fluids offers a great potential for toxoplasmosis studies on farm animals, especially for large scale control studies. We cannot yet offer any serological test asserting the absence of *Toxoplasma* in meat and offering the guarantee that undercooked lamb can be consumed without posing an unacceptable risk for people at risk and freezing the meat remains necessary.

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