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Preliminary assessment of usefulness of cELISA test for screening pig and cattle populations for presence of antibodies against *Toxoplasma gondii*

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Abstract

Serology testing is an appropriate method for the detection of slaughter animals infected with *Toxoplasma*, which remain one of the main reservoirs of this parasite in the environment. Competitive ELISA (cELISA) in indirect modification was worked out and optimized for detecting antibodies to *Toxoplasma gondii* in pigs and cattle. Preliminary validation process showed that the sensitivity and specificity of cELISA obtained in pigs was better than in cattle (88.1% and 94.5% vs. 76.9% and 93.4%, respectively). Sera of 861 pigs and 865 cattle were examined with newly worked out cELISA test and modified agglutination test (MAT) (Toxo-Screen DA, bioMérieux, France). In the total of 1,726 examined animal sera, seropositive results were obtained in 15.0% by cELISA (15.4% in pigs and 14.6% in catttle), and in 13.6% by MAT (14.3% in pigs and 12.8% in cattle). Significant differences in percentages of positive results among populations of the studied animals from various areas of Poland were noted. Obtained results showed the usefulness of cELISA for examining sera of slaughter animals (especially pigs). The considerable rates of infection of slaughter animals with *T. gondii* in the area of research indicate a potential threat to human health.

Key words

Toxoplasma gondii, pigs, cattle, serology, cELISA, MAT

INTRODUCTION

Toxoplasmosis is a zoonosis widespread throughout the world, caused by a parasitic protozoan *Toxoplasma gondii*. Infection with *T. gondii* in humans proceeds mostly in a chronic, asymptomatic form, manifested only by the presence of specific antibodies. However, the primary infection of women during pregnancy may cause a congenital toxoplasmosis associated with the damage to the neural system and/or sight organ of the foetus. Similarly, in immunocompromised persons, infection with the parasite could be a cause of severe pathological changes in various organs and tissues.

Invasion with *T. gondii* in animals may constitute a serious veterinary problem associated with the occurrence of stillbirths or pathological symptoms in the newborns, especially in sheep [1-5]. Studies undertaken to date in Poland have shown a significant relationship between the breeding conditions of pigs and cattle, and frequency of *T. gondii* infection in these animals. The breeding of slaughter animals on small, often neglected farms, where proper zoohygienic standards are not assured, increased the rate of *T. gondii* infection [5,6].

Serological tests are basic methods enabling determination of the infection rate with *T. gondii*, also in slaughter animals. The usefulness of serological examinations of these animals for estimation of infection risk in meat consumers has been proved by various authors who demonstrated a significant relationship between seropositivity of animals and the presence of live parasites in their tissues [7-10]. However, the serological techniques used to date are limited either by the subjectivity of test reading (as in agglutination tests), or by confinement to only one animal species.

The competitive ELISA (cELISA) is a simple technique, suitable for diagnostics of a multiplicity of pathogens and hosts [11,12]. The advantage of this test is species independency, which makes it possible to use it in seroepidemiological surveys of animals and humans. Thus, the newly described cELISA test could be a promising alternative to other techniques used in the screening examinations of animals for the presence of anti-*Toxoplasma* antibodies.

The objectives of this study were: firstly, to make a preliminary assessment of the usefulness of the newly elaborated cELISA test in the diagnostics of toxoplasmosis in slaughter animals; and secondly, to estimate the infection rate with *T. gondii* in the selected populations of pigs and cattle in Poland.

MATERIALS AND METHODS

Serological tests. The studies were conducted with the use of the newly elaborated competitive ELISA test (cELISA) in indirect modification. This technique consists of the competition between antibodies in diagnosed sera and monoclonal antibody (hyperimmune serum) added to reaction, which is directed against antigen of *T. gondii*

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coated on the surface of the plate. For performing the cELISA test, the following components were used: flat-bottomed microplates (96 wells, PolySorp, NUNC); sonicated antigen of *T. gondii* (Fitzgerald Ind. Intern., Cat. No. 30-AT56); rabbit hyperimmune sera anti-*T. gondii* (Fitzgerald Ind. Intern., Cat. No. 20-TR19); goat Anti-Rabbit IgG conjugate with horse radish peroxidase (HRP) (Sigma, Cat. No. A6154); OPD (o-Phenylenediamine) substrate (Sigma, Cat. No. P5412); 30% H_2O_2; PBS (phosphate buffered saline, pH 7.2); solution of Tween 80 in PBS (0.01%); 10% solution of milk in 0.05 M carbonate-bicarbonate buffer, pH 9.6; and citrate buffer (pH 5.0).

The parameters of the cELISA test, established in the early stages of the study, were as follows: the *T. gondii* antigen was diluted with PBS 1:3,000 (to concentration ≈1.7 μg/ml), and the microplates coated with the diluted antigen in the proportion of 100 μl/well; tested sera were diluted 1:20; hyperimmune rabbit sera anti-*T. gondii* were diluted 1:1,000; and Anti-Rabbit HRP Conjugate was diluted 1:1,500.

Plates with coated antigen were incubated at 4°C for 16 h. After removing excess antigen from the plate, blocked buffer (milk in carbonate-bicarbonate buffer) was dropped and the plate incubated at 37°C for 30 min. After this, the tested and control sera (100 μl/well) were dropped into wells and the plate incubated for 1 h at 37°C. Then, hyperimmune rabbit sera anti-*T. gondii* were added (100 μl/well) and incubated for the next 40 min at 37°C. After washing (3 cycles with 0.01% Tween 80 in PBS), anti-Rabbit HRP conjugate was added (100 μl/well) and incubated for 1 h at 37°C. After washing as above, substrate solution (1 tablet of OPD, 20 μl H_2O_2 in 50 ml citrate buffer) was added and the plate was incubated in the dark for 10 min at room temperature. For stopping the reaction, 0.5 M sulfuric acid was added and the optical density (OD) at 490 nm was read (MRX II, DYNEX).

The maximal differences between optic density (OD) values of positive and negative control sera obtained in these conditions of reaction were: 4-fold and 2.5-fold for pig and cattle sera, respectively. The final result of the test was presented as a percent of inhibition (PI) of the control positive serum, calculated from the formula: PI = (OD of negative control – OD of tested serum/ OD of negative control – OD of positive control serum) × 100%.

As control sera were used: serum of pig immunized with the RH strain of *T. gondii* (positive control), and selected sera of pigs and cattle, for which positive or negative results with the use of the below-mentioned tests were confirmed:

- latex agglutination test (LAT, commercial kit Pastorex-Toxo, BIO-RAD, France);
- indirect immunofluorescence test (IFAT), with the use of slides coated with *T. gondii* antigen (BioMèrieux), and conjugates (Sigma);
- modified agglutination test (MAT) detecting anti-*T. gondii* IgG antibodies (commercial kit Toxo-Screen DA, BioMèrieux, France).

Commercial tests were performed according to producers' manuals, and IFAT as previously described [26].

Stages of the study

Stage 1: a total of 326 blood serum samples from pigs and 299 blood serum samples from cattle were examined for the presence of anti-*T. gondii* antibodies. Blood sera were collected at an abattoir during slaughter and routine veterinary examinations. For selection of positive and negative sera, the MAT test – commonly used for screening – was performed [14-16]. To confirm the results obtained with other sera (to exclude the presence of IgM antibodies in the sera negative in MAT, and to confirm the positive result in the sera weakly positive in MAT), LAT and IFAT tests were also used, respectively.

As a result of examination, 59 positive pig sera and 26 positive cattle sera, as well as 267 negative pig sera and 273 negative cattle sera, were selected. In the next step, the sensitivity and specificity of the cELISA test, and the quality and semi-quantitative correlation between the results of cELISA and MAT (with use of the tests LAT and IFAT in doubtful cases), were determined. The specificity and sensitivity of cELISA (p=95%) were calculated according to the patterns:

$$\text{Specificity (SP): NA / N- } \times 100\%$$
$$\text{Sensitivity (SE): PA / N+ } \times 100\%$$

where:
NA: number of negative samples detected in examination as 'negatives'
PA: number of positive samples detected in examination as 'positives'
ND: number of false negative samples
PD: number of false positive samples
N-: total number of negative results (SUM of NA + PD)
N+: total number of positive results (SUM of PA + ND).

Stage 2: in order to estimate the usefulness of cELISA in toxoplasmosis diagnostics for slaughter animals, selected populations of pigs and cattle from various regions of Poland were examined with the use of the newly elaborated cELISA test, and (in order to compare the results) with MAT. The prevalence of *T. gondii* infection was estimated among 861 pigs (Polish Landrace and Polish Large White, aged up to 1 year) from 8 provinces of Poland, and 865 cattle (Black-and-White Lowland races, aged up to 18 years) from 11 provinces of Poland.

Blood serum samples were obtained from the diagnostic veterinary laboratories. Until examination, the sera were preserved at -20°C. The prevalence of seropositive results in individual provinces and the relationships between conditions of keeping and age of animals (cattle), on the one hand, and the rate of infection with *T. gondii* on the other, were determined.

Statistical analysis was performed by Spearman's range order correlation test, χ^2 test, and Wilcoxon matched pairs test, using Statistica for Windows *vs.* 8.0 package (Tulsa, OK, USA).

RESULTS

Specificity and sensitivity of cELISA, correlation of the cELISA with MAT (test sera of pigs and cattle). Comparison of the results obtained with the newly elaborated cELISA test with standard tests (MAT, LAT, and IFAT) is shown in Tables 1-2, while the specificity and sensitivity of the cELISA test is presented in Table 3. As seen in the Tables, the cELISA test showed a highly significant correlation with the standard tests (Tab. 1-2) and revealed a high specificity and sensitivity (Tab. 3).

Table 1. Comparison of cELISA test for detection of anti-*T. gondii* antibodies in pigs with standard tests MAT, LAT, and IFAT

		cELISA		Total
		+	-	
Standard tests*	+	52	7	59
	-	11	256	267
Total		63	263	326

* MAT and/or LAT and/or IFAT
χ^2 (with Yates correction) = 213.42
p<0.0001

Table 2. Comparison of cELISA test for detection of anti-*T. gondii* antibodies in cattle with standard tests MAT, LAT and IFAT

		cELISA		Total
		+	-	
Standard tests*	+	20	6	26
	-	18	255	273
Total		38	261	299

* MAT and/or LAT and/or IFAT
χ^2 (with Yates correction) = 99.60
p<0.0001

Table 3. Characteristics of cELISA in examination of blood sera from pigs and cattle

Species	Specificity	Sensitivity
Pigs	94.5%*	88.1%*
Cattle	93.4%*	76.9%*

*p≤0.05

The cELISA test showed a higher specificity and sensitivity for sera from pigs compared to those from cattle (Tab. 3). In testing pig sera, a highly significant correlation was found between the titers of the test MAT and the values of inhibition index in the cELISA test (R=0.83, p<0.001). In testing cattle sera, the correlation was also significant but weaker (R=0.61, p< 0.001).

Seroprevalence of *T. gondii* infection in selected populations of pigs and cattle from various regions of Poland. Among the 1,726 animals examined (861 pigs and 865 cows), the seropositive results in MAT and cELISA tests were found in 13.6% and 15.0% of the total populations, respectively (Tab. 4). The rates recorded in MAT and cELISA tests of seropositive results in pigs (14.3% and 15.4%, respectively) were slightly greater compared to those recorded in cattle (12.8% and 14.6%, respectively). The majority of seropositive results constituted these of low titers (40-60) in MAT, and of low inhibition index (50-65%) in cELISA, which accounted for 51.2% and 63.2%, respectively, of seropositive samples in pigs and 83.8% and 85.8% of seropositive samples in cattle.

The distributions of seropositive results in pigs and cattle found in individual Polish provinces showed a significant variation depending on the province (χ^2>30.0, p<0.001) (Tab. 4). The percentages of positive results obtained in particular provinces by cELISA test were slightly but steadily greater, compared to those obtained by MAT. As shown by the Wilcoxon matched pairs test, these differences were significantly greater in pigs and total slaughter animals (p<0.05), but not in cattle (0.2>p>0.1).

Table 4. Results of serological examinations of slaughter animals for presence of anti-*Toxoplasma* antibodies in individual provinces of Poland

Species	Province	No. of animals examined	Seropositive results			
			MAT		cELISA	
			No.	%	No.	%
Pigs	Lubelskie	86	12	14.0	13	15.1
	Wielkopolskie	99	1	1.0	2	2.0
	Kujawsko-Pomorskie	156	33	21.2	33	21.2
	Dolnośląskie	80	15	18.7	16	20.0
	Lubuskie	41	8	19.5	11	26.8
	Opolskie	69	5	7.2	5	7.2
	Mazowieckie	180	35	19.4	37	20.6
	Zachodniopomorskie	150	14	9.3	16	10.7
Total No. of pigs		861	123	14.3	133	15.4
Cattle	Małopolskie	73	6	8.2	8	11.0
	Podkarpackie	62	9	14.5	8	12.9
	Lubelskie	156	39	25.0	41	26.3
	Świętokrzyskie	40	8	20.0	9	22.5
	Wielkopolskie	79	4	5.1	4	5.1
	Podlaskie	69	4	5.8	8	11.6
	Kujawsko-Pomorskie	78	10	12.8	14	17.9
	Pomorskie	73	8	11.0	12	16.4
	Dolnośląskie	50	4	8.0	2	4.0
	Mazowieckie	110	14	12.7	14	12.7
	Zachodniopomorskie	75	5	6.7	6	8.0
Total No. of cattle		865	111	12.8	126	14.6
Total No. of slaughter animals		1,726	234	13.6	259	15.0

As can be seen in Table 4, in the examined pigs the greatest frequencies of positive results in the MAT and cELISA tests were found in the provinces: Lubuskie (19.5% and 26.8%, respectively), Kujawsko-Pomorskie (21.2% and 21.2%), Mazowieckie (19.4% and 20.6%) and Dolnośląskie (18.7% and 20.0%). The smallest seroprevalences were found in the Wielkopolskie province (1.0% and 2.0%) and in the Opolskie province (7.2% and 7.2%) (Tab. 4).

In the examined cattle, the greatest frequencies of positive results in the MAT and cELISA tests were found in the provinces: Lubelskie (25.0% and 26.3%, respectively), Świętokrzyskie (20.0% and 22.5%), and Kujawsko-Pomorskie (12.8% and 17.9%). Smaller figures were found in the provinces: Pomorskie (11.0% and 16.4%), Podkarpackie (14.5% and 12.9%), and Mazowieckie (12.7% and 12.7%). The smallest were found in the provinces: Wielkopolskie (5.1% and 5.1%), and Dolnośląskie (8.0% and 4.0%) (Tab. 4). The relatively large differences between the frequencies of seropositive results obtained in the MAT and cELISA tests were noted in cattle from the provinces: Podlaskie (5.8% *vs.* 11.6%), Kujawsko-Pomorskie (12.8% *vs.* 17.9%), Pomorskie (11.0% vs. 16.4%), and Dolnośląskie (8.0% *vs.* 4.0%).

In the 2 subgroups of examined animals (205 pigs and 331 cows), their origin from small (≤ 20 animals) or large farms (with hundreds or thousands of animals) was documented. Statistical analysis of the results (in MAT) demonstrated that the prevalence of anti-*Toxoplasma* antibodies was significantly greater in animals that originated from small farms, compared to those from large farms (pigs: 17.6% *vs.*

2.2%; cattle 23.8% *vs.* 10.8%) ($p<0.001$). Comparison of the results obtained from cattle in selected categories of age (group I: up to 4 years; group II: between 4-10 years; group III: above 10 years), showed significantly lower percentages of seropositive results in group I (11.5%), compared to results obtained in group II (19.0%) and in the rest of the animals (groups II + III) (19.1%) in MAT. Similar results were obtained from cELISA - in group I (12.2%) compared to group III (24.4%) ($p<0.05$). Because of low age of pigs (up to 1 year), the correlation between their age and frequency of infection was not determined.

DISCUSSION

Of the various research methods, serological techniques are most often used for the assessment of the infection of animals with the parasite *Toxoplasma gondii*. The newly elaborated, not species specific test cELISA, could find universal application for the examinations of both humans and animals. The demonstration in the present study of slightly greater but significant frequencies of seropositive results in farm animals by cELISA test, compared to MAT and other standard tests (LAT, IFAT) used to confirm the results of MAT, may indicate a greater sensitivity of cELISA, resulting from the possibility to detect a wide range of the antibody classes (IgM, IgG, IgA, IgE). On the other hand, it is possible that some part of seropositive results obtained in cattle with cELISA could be due to cross reaction with anti-*Neospora* or anti-*Sarcocystis* antibodies. Therefore, the aim of the next stage of the present study will be the estimation of probability of such reactions. The preliminary results obtained indicate the particular usefulness of the cELISA test for examination of pig sera, and somewhat less for examination of cattle sera. The lower sensitivity and specificity of cELISA in the examination of cattle sera may be partly due to the presence in the serum of this species of so-called 'natural antibodies' which may bind not specifically with the *T. gondii* antigen used in diagnostic tests [3].

With respect to the common consumption of meat, the testing for toxoplasmosis of slaughter animals, mostly pigs, is very important. Pork containing tissue cysts of the parasite is regarded as a main source of infection with *T. gondii* in European populations [1, 17, 18]. On the other hand, some serological surveys based on abattoir samples, may not provide a completely true assessment of risk to humans; this is because some post- slaughter treatments of meat (storage, treatments with salts) can affect the viability of tissue cysts.

The role of cattle as an important source of the parasite for humans is not unequivocally determined, despite commonly stated high frequencies of seropositive findings in these animals [19]. The results of the present study indicate a considerable degree of infection with *T. gondii* of slaughter animals in some regions of Poland. The significant differences between frequencies of seropositive results in individual provinces may be due to differences in the contamination of agricultural environment with invasive forms of *T. gondii*, in breeding conditions, and size of farms. The significantly smaller incidence of seropositive results on large farms, compared to small ones, confirms the opinion of many authors that the rearing of farm animals in confined buildings with proper zoohygienic conditions and limited access of cats, effectively decreases the possibility of infection with the protozoan *T. gondii* [6,18,20-22].

The percentage of seropositive pigs found in the present study (14.3%-15.4%) is similar to those noted in Poland during last 20 years in the provinces Lubelskie (10.5-15.0%) [6,22] and Wielkopolskie (13.2%) [23], but lower compared to the results obtained earlier by the Polish authors Ramisz and Zemburowa (26.3-46.0%) [24], Umiński *et al.* (21.2-53.0%) [25], and Krupa and Bartoszcze (35.2%) [26]. A similar tendency of decreasing frequency of *T. gondii* infection in recent years could be observed in cattle reared in Poland. The percentage of seropositive cows noted in the present study (12.8-14.6%) is lower compared to those obtained in earlier own studies in Lubelskie (54.0-55.5%) [6,21,25], and to those reported by Ramisz and Zemburowa from Małopolskie (22.5-54.6%) [24]. The recently observed drop of serological reactions to *Toxoplasma* in pigs and cattle in Poland may be due to progressive improvement of zoohygienic conditions after the economic transformation.

In spite of improvement, the recently noted frequency of seropositive results in pigs reared in Poland is greater compared to those recorded in Western European countries, such as Germany (4.1%) [27], the Netherlands (0.4-10.9%) [28,29], Italy (10.4%) [18], and Finland (3.0%), Norway (3.0%), Portugal (5.0%), Austria (1.0-3.0%) [5], which apply more rigorous zoohygienic regulations in the rearing of slaughter animals,.

On the other hand, this frequency is smaller compared to the countries in which pigs are often kept in a free-range backyard, such as Serbia (28.9%) [30], or the Czech Republic (35.0%) [5].

The seroprevalence of anti-*Toxoplasma* reactions in cattle stated in the present study is greater compared to those reported from Spain (7.3%) [31] and Norway (5.0%) [5], and similar to that reported from Switzerland (14.0%) [5]. However, it is, much smaller compared to the results obtained in countries where the pasture type of rearing cattle is often used, such as Serbia (76.3%) [30], the Czech Republic (22.0%), Greece (40.0%), Portugal (43.0%), and Turkey (66.0%) [5].

Direct comparison of the epidemiological situation of toxoplasmosis in individual countries could be difficult because of the variety of research techniques applied, and the differences in examined animal populations (age, mode of rearing). The recent decrease in the frequency of *T. gondii* infection among slaughter animals in Poland could be due to changes in rearing animals, such as increase in number of large confinement buildings, and to the steady improvement of zoohygienic conditions. Total elimination of the parasite does not seem feasible because it is so widespread in the agricultural environment, and reveals variable routes of infection. Nevertheless, the experiences of other European countries -such as Austria, the Netherlands and Germany – indicate that the introduction of rigorous hygienic regulations in animal breeding may effectively limit the risk of *T. gondii* invasion and ensure a better protection of human health.

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