INTRODUCTION

Toxoplasma gondii is a protozoan parasite and one of the most common parasitic infections of man and other warm-blooded animals (Dubey and Beattie, 1988). Nearly one-third of human population has been exposed to this parasite (Tenter et al., 2000). Hosts can acquire T. gondii infection by ingesting tissues of infected animals or ingesting food or drink contaminated with sporulated oocysts, or transplacentally (Tenter et al., 2000).

Horses are considered clinically resistant to toxoplasmosis and there are no confirmed cases of clinical toxoplasmosis in these animals (Dubey, 2010). However fever, ataxia, retinal degeneration and encephalomyelitis may occasionally occur, as well as abortion or stillbirth (Miao et al., 2013). Many serological tests have been used to detect antibodies against T. gondii in horses: Sabin-Feldman Dye test (DT) (Hejliček and Literák, 1994), indirect immunofluorescence test (IFAT) (Gupta et al., 2002; Locatelli-Ditrch et al., 2006),
enzyme linked immunosorbent assay (ELISA) (El-Ghaysh, 1998; Ghazy et al, 2007), indirect hemagglutination (IHA) (Riemann et al, 1975; Chhabra et al, 1985), modified direct agglutination test (MAT) (Dubey et al., 1999a,b), direct agglutination test (DAT) (Chhabra and Gautam, 1980; Jakubek et al., 2006), latex agglutination test (LAT) (Bártová et al., 2010) and complement fixation test (CFT) (Prosek and Hejlíček, 1980).

The purpose of this study was to evaluate three serological methods (two „in house” tests MAT and IFAT and one commercially ELISA) for detecting anti- T. gondii infection and determine T. gondii seroprevalence in horses.

**MATERIALS AND METHODS**

**Sample collection**

Serum samples were collected from 82 slaughtered horses reared in backyard system. The horses were originated from Centre and North-West of Romania, from four counties (Mureș, Harghita, Satu-Mare, Maramureș). Blood was drawn from the jugular vein just prior to slaughter, and sera were transferred in Eppendorf tubes and kept in a freezer at -20°C until use.

**Serological assays**

Enzyme-linked immunosorbent assay (ELISA) Toxoplasma gondii antibodies were detected by indirect ELISA, at a sera dilution of 1:10, using the commercial kit ID Screen Toxoplasmosis Indirect Multi-species (ID. vet, France). We followed the manufacturers’ instructions. The results were expressed as S/P percentages according to the formula: S/P% = ODsample / ODpositive control x 100. Sera with S/P ≤ 40% were deemed as negative, between 40% and 50% doubtful, between 50% and 200% positive, and ≥ 200% strong positive.

Immunofluorescence antibody test (IFAT)

The test used whole tachyzoites of T. gondii from peritoneal exudates of mice infected intraperitoneally with the T. gondii RH strain. The initial peritoneal exudate obtained from the mouse must be passaged every third or fourth day into fresh mice. The exudate for passage should be as a cellular as possible and must be free of bacterial contamination. It is diluted in antibiotic saline (to 500ml of sterile saline add 2ml of Heparin 5000units/ml and 5ml of penicillin/streptomycin solution) until the diluted exudate contains approximately 10^6 organisms/ml. Following, 0.5ml of this diluted exudate is inoculated intraperitoneally into fresh mice.

An anti-horses IgG fluorescein conjugated goat IgG fraction (Jackson Immunoresearch Laboratories Inc., catalog no. 108-095-003, lot 77231) served as conjugate. The cut-off of the immunofluorescence test was established at a dilution of 1:32. The test was carried out as described by Györke et al. (2011).

**Modified agglutination test (MAT)**

The modified agglutination test (MAT) for the detection of T. gondii-specific IgG antibodies was performed as previously described (Dubey and Desmonts, 1987), using an antigen prepared from formalin-fixed whole RH strain tachyzoites (Reims, France). Each serum samples was serially twofold diluted. The threshold dilution was 1:6.

**STATISTICAL ANALYSES**

**Evaluation of test characteristics and level of agreement**

This study determined sensitivity (Se), specificity (Sp), Jouden index (J), positive and negative predictive values (PPV and NPV) of the ELISA, IFAT, and MAT, for anti- T. gondii antibody detection, by analyzing serum samples from 82 naturally infected horses from backyard system, using WinEpiscope software. As golden standard (comparative test) we used a cumulated seropositivity (CP). We considered as positive those samples that were positive for at least two of the three applied methods.

Also, agreement between the all three serological tests and CP was assessed by the calculation of kappa statistic value.

Kappa has values from -0.25 to 1, and interpreted according to Petrie and Watson (1999):

- k ≤ 0.20 without consistency
- 0.21 ≤ k ≤ 0.40 poor agreement
- 0.41 ≤ k ≤ 0.60 moderate agreement
- 0.61 ≤ k ≤ 0.80 good agreement
- k> 0.80 very good agreement

Tests characteristics and level of agreement were performed by Win Episcope 2.0. program.

**Descriptive epidemiology**

Frequency, prevalence and its 95% confidence interval of anti- T. gondii antibodies, processed by ELISA, IFAT, and, MAT were established. All statistics were performed using the EpilInfo 2000 software.
RESULTS AND DISCUSSION

Serum samples collected from 82 naturally infected horses from backyard system, were tested by all three serological tests (ELISA, IFAT, MAT), for evaluation of test characteristics and level of agreement.

A good correlation of the diagnostic tests was observed. Fifteen positive serum samples and 37 negative were obtained by all three tests. ELISA (39%; 32/82; CI 95% 28.4-50.4) and MAT (37.8%; 31/82; CI 95% 27.3-49.2) had the most positive results, while IFAT had the fewest (34.1%; 28/82; 95% CI 24.0-45.4) (Tab. 1). ELISA was the test with the highest performance: sensitivity (96.8%), specificity (96.1%), NPV (98%), PPV (93.78%) and Youden index (0.93), followed by MAT (Tab. 2).

Similar to the results obtained by Petrie and Watson (1999), in our study, an excellent agreement was obtained between CP and ELISA (k = 0.923), CP and MAT (k = 0.844), a good agreement was observed between ELISA and MAT (k = 0.768), a moderate agreement was obtained between CP and IFAT (k = 0.445) and a poor agreement was between IFAT and ELISA (k = 0.371), IFAT and MAT (k = 0.286) (Tab. 2).

We aimed to evaluate three serological methods, two "in house" (IFAT and MAT) and one commercially ELISA (ID.vet) and, to establish which one is more sensitive and specific for anti- T. gondii antibodies detected in horse serum samples. IFAT and MAT may detect antibodies that appear at early stage of infection against components of the membrane of tachyzoites (Karim and Ludlam, 1975; Hirvela-Koski, 1990). Differently, indirect ELISA's are capable of detecting antibodies later in the time-course of infection (Hirvela-Koski, 1990). Moreover, ELISA and IFAT are able to distinguish IgG from IgM, while MAT does not.

In our study, the best agreement was obtained between the ELISA and IFAT method (k = 0.768). The high sensitivity and specificity was obtained by ELISA (Se = 96.77; Sp = 96.08). Our results suggest that ELISA and MAT would be useful for detecting anti-T. gondii antibodies in horse serum samples. These results are sustained by Ghazy et al. (2007) which, comparing the diagnostic efficiency of indirect ELISA in comparison to IFAT and MAT, reported highest diagnostic efficiency for ELISA followed by MAT and IFAT.

Tab.1. The seroprevalence of anti-T. gondii antibodies by ELISA, IFAT, MAT and CP

<table>
<thead>
<tr>
<th>Method</th>
<th>Frequency</th>
<th>Prevalence %</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>32</td>
<td>39</td>
<td>28.4-50.4</td>
</tr>
<tr>
<td>IFAT</td>
<td>28</td>
<td>34.1</td>
<td>24.0-45.4</td>
</tr>
<tr>
<td>MAT</td>
<td>31</td>
<td>37.8</td>
<td>27.3-49.2</td>
</tr>
<tr>
<td>CP</td>
<td>31</td>
<td>37.8</td>
<td>27.3-49.2</td>
</tr>
</tbody>
</table>

Legend: CP - cumulated seropositivity (were considered positive those samples that were positive at least two of the three applied methods).

Tab.2. The performance of the serological methods for anti-T. gondii antibodies detection and Kappa agreement between all three serological methods and between CP and serological methods

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>IFAT</th>
<th>MAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (%)</td>
<td>96.77</td>
<td>61.29</td>
<td>90.32</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>96.08</td>
<td>82.35</td>
<td>94.12</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td>93.78</td>
<td>77.78</td>
<td>90.32</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>98</td>
<td>67.86</td>
<td>94.12</td>
</tr>
<tr>
<td>Youden index</td>
<td>0.93</td>
<td>0.44</td>
<td>0.84</td>
</tr>
<tr>
<td>Kappa agreement</td>
<td>CP</td>
<td>0.923</td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>0.371</td>
<td>0.768</td>
</tr>
<tr>
<td></td>
<td>MAT</td>
<td>0.286</td>
<td></td>
</tr>
</tbody>
</table>

Legend: CP - cumulated seropositivity (were considered positive those samples that were positive at least two of the three applied methods).
CONCLUSIONS
This study recommended utilization for detection of \textit{T. gondii} antibodies in horses, of indirect ELISA and MAT, which proved better diagnostic potency compared with IFAT. Because the test evaluation indicated the commercial ELISA and “in house” MAT had the highest Jouden index, as well as because of financial reasons, MAT can be chosen for processing horse serum samples for epidemiological studies. MAT has the advantage that the results are obtained quickly and the interpretation of the results is facile, without special equipment required. The serial dilution of serum samples is helpful for establishing the titer of \textit{T. gondii} antibodies. Therefore, we recommend MAT testing for \textit{T. gondii} infections as both a qualitative and a quantitative test.

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REFERENCES