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**Serosurvey of *Toxoplasma gondii* infection in Hungarian sheep and goat
flocks**

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1. Introduction

The obligate intracellular protozoan parasite *Toxoplasma gondii* can be found worldwide and is capable of infecting all warm-blooded vertebrates including humans. *Toxoplasma gondii* replicates asexually in mammalian intermediate hosts and sexually within the intestinal mucosa of the definitive feline hosts (Garcia, 2001). The feline hosts which can also be intermediate hosts of the parasite can excrete oocysts in their faeces for maximum two-three weeks. The oocysts develop into the infective stages, sporulated oocysts in the environment where they can survive under favouring conditions up to several months.

Cats, pigs, sheep and goats are the domestic animal species most seriously affected by the protozoan parasite.

The infection may result in significant reproductive and therefore economic losses in the sheep and goat industry worldwide. It is considered to be one of the main causes of infectious abortions among small ruminants (Buxton et al., 2007). Infection of small ruminants can follow consumption of forage and feedstuff contaminated with sporulated oocysts and it can also be transferred transplacentally (Abu-Dalbouh et al, 2012).

Toxoplasma gondii is one of the most important zoonotic agents in the world. Humans can be infected with tachyzoites of the parasite occurring in unpasteurized milk of infected goats and sheep or with bradyzoites found in infected meat. The other way of infection is with sporulated oocysts through a faecal-oral route. Especially pregnant women, young children and immunocompromised humans are in danger of getting infected with the protozoan parasite.

At present knowledge, no information is available about the incidence of *T. gondii* infection sheep and goats in Hungary. The primary objective of this study, therefore, was to obtain data about the occurrence of *T.gondii* infection in the local sheep and goat flocks, by detection of antibodies developed against the protozoan parasite species in small ruminants.

2. Literature review

2.1 History of *Toxoplasma gondii*

The intracellular parasite *T. gondii* belongs to the Apicomplexa phylum, Sporozoa class, Eucoccidian order and Sarcocystidae family (Abu-Dalbouh et al., 2010).

This protozoan parasite was first discovered 1908 by Charles Nicolle and Louis Manceaux in the tissues of *Ctenodactylus gundi*, a North African rodent. In the same year Alfonso Splendore announced the identification of this organism in the tissues of a rabbit in Brazil.

The genus was named one year later by Nicolle and Manceaux as *Toxoplasma* for its bow-like appearance, in Greek: `toxos` means bow and `plasma` means creature (Dubey, 2010).

The medical importance of *T. gondii* for humans was discovered in 1939 when it was found in the brain of a congenitally infected child who died three days after birth. Wolf, Cowen and Page identified subsequently toxoplasmosis as a human disease (Dubey, 2009). In 1942 Sabin described the typical clinical signs of congenital toxoplasmosis as hydro- or microcephalus, intracerebral calcification and chorioretinitis (Dubey, 2008). The serological method, the Sabin–Feldman dye test was developed in 1948 by Albert Sabin and Harry Feldman (Dubey, 2008).

In 1954 Bill Hartley and his colleagues detected *T.gondii* within the placenta of aborting ewes and they could also isolate the parasite from an aborted foetus in New Zealand (Dubey, 2008). Hartley and Marshall recognised that this parasite species is a major cause of abortion storms in sheep worldwide, especially in the UK, Scandinavia, Europe and Australia (Innes, 2010).

The life cycle of *T. gondii* was completely discovered in 1970. There is a sexual and asexual cycle of the parasite, but only infected felids are the definitive host and excrete huge amount of environmentally resistant oocysts in their faeces (Innes, 2010). Therefore it could be explained how herbivorous animals or people with a vegetarian diet could get infected with the protozoan (Dubey, 2008).

2.2 The life cycle of *Toxoplasma gondii*

The life cycle showing the infective stages, can be seen in Figure 1.

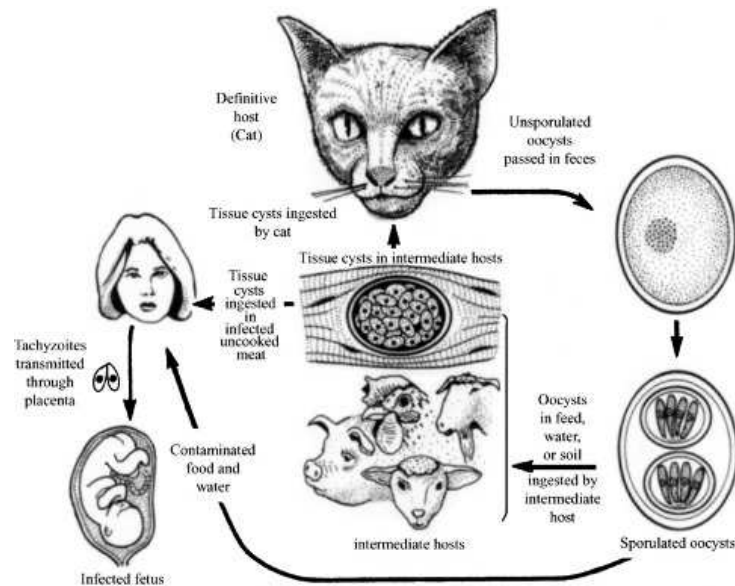


Figure 1: Life cycle of *Toxoplasma gondii* (Dubey, 1996)

The infective stages are the sporulated oocysts, the tachyzoites (active form) and the bradyzoites inside the tissue cysts (resting stage).

The three important infectious stages are:

- 1) Approximately 10-12 μm sized **sporulated oocysts**, consists of two sporocysts and each contains four sporozoites.
- 2) **Tachyzoites** are rapidly multiplying and infect any tissue throughout the body. They measure 2-6 μm in length with a centrally placed prominent nucleus, a pointed anterior end and a rounded posterior end. "Tachy" means fast in Greek, they are named like this because they occur in the acute phase of the disease and replicate fast.
- 3) **Bradyzoites** occurring within thin-walled tissue cysts are about 20-100 μm long. 'Brady' means slow in Greek, followed by the fact that bradyzoites replicate slowly inside the tissue cysts. They appear in the chronic phase of the disease. At the beginning there are a few but later thousands of bradyzoites will be in the tissue cysts which are round and frequently found in groups mostly in the muscular and neural tissues, but also may develop in visceral organs (Garcia, 2001).

Toxoplasma gondii is transmitted by consumption of sporulated oocysts or meat containing tissue cysts. The other way of infection is transplacental transfer of tachyzoites from mother to foetus (Dubey, 2010).

Unexposed cats/wild feline get infected most efficiently with the parasite due to carnivorism after ingestion of tissue cysts from an intermediate host, like a rodent (Dubey, 2010). It has been discovered that *T. gondii* infection of mice leads to a changed behaviour in their nature and they get easier caught (Sykes, 2013).

Bradyzoites are released from the tissue cysts after the wall is dissolved by proteolytic enzymes of the stomach and small intestine. The bradyzoites invade the epithelial cells of the small intestines of the cat and become merozoites. They undergo several asexual replications, called schizogony.

The next stage of the life cycle is the gametogony when the replicated merozoites turn into microgametes and macrogametes. During the sexual cycle when a fertilized zygote is formed, a makrogamete gets fertilized by a mikrogamete. Around the zygote two walls are formed after a while to build the oocyst. The matured oocysts are discharged into the intestinal lumen as unsporulated, about 10-12 µm diameter sized oocysts which are shed in the faeces of the definitive hosts. Each infected feline may shed millions of oocysts to the environment for as long as one to three weeks.

Sporulation of the oocysts occurs in the environment; the oocysts become infectious after one to five days following excretion (Dubey, 2010). Sporulated oocysts are remarkably resistant to adverse environmental conditions. They are the invasive form of *T. gondii* and remain infectious for several years, depending on humidity and temperature (Bostedt and Dedie, 1996). The definitive hosts generally develop a very good immunity after the first exposure with the parasite and therefore they shed oocysts only once in their lifetime. Soil, plants and even water contaminated with sporulated oocysts are the source of infection for intermediate and definitive hosts (Dubey, 2010).

Toxoplasma gondii can use a broad host range as intermediate hosts, including all warm blooded animals, birds and humans. In an intermediate host, the parasite only undergoes an asexual extraintestinal replication (Bowman, 2013).

The intermediate hosts can get infected with sporulated oocyst (Weissmann, 2003). The ingested sporulated oocysts excyst in the small intestines and the sporozoites are released which invade the intestinal mucosa and start an asexual replication cycle. At first 2 daughter cells are formed within the parent cell due to endodyogenie. Followed by endopolyogenie until the host cell ruptures and multiple now called tachyzoites are released and spread

haematogenously or by lymph (Tenter et al., 2000). This parasitaemia lasts from three to eight days. Tachyzoites invade various tissues throughout the body and multiply fast intracellular until the cells burst, resulting in tissue necrosis. The haematogenous spread of tachyzoites establishes a generalized infection and is responsible for the infection of the foetus via the placenta. Tachyzoites will be shed in the milk of sheep or goats ten days after the infection for three to six weeks and furthermore will be shed shortly in sperm and other se- or excretions (Bostedt and Dedie, 1996).

The infection gets under control according to the host immune response. Young and immunocompromised animals may capitulate to this generalized toxoplasmosis (Dubey, 2013). According to the developing immunity following parasitaemia, the tachyzoites are vanished from the blood and bradyzoites will remain in the tissues as cysts. These thin walled tissue cysts will remain lifelong and can harbour up to hundreds of bradyzoites and they can be formed in many organs and tissues, e.g., brain, heart and muscles.

The tissue cysts maintain the infection and a lifelong immunity. The immunity of the host may not protect from a reinfection but at least will prevent further shedding of tachyzoites in the milk and protect the foetus from an infection. These infectious cysts are also the source of infection for carnivorous intermediate and definitive hosts.

2.3 Clinical importance of *Toxoplasma gondii* in small ruminants

In the acute phase of the disease, caused by the rapidly replicating tachyzoites, tissue damage can be found in various organs. The severity of the clinical signs depends on the number of tachyzoites released when the hosts cell rupture.

In adult immunocompetent animals, toxoplasmosis usually occurs as a subclinical illness. A non-pregnant ewe exposed to *T. gondii* for the first time will gain immunity to the parasitic organism and this animal becomes resistant to *Toxoplasma*-induced abortion during the following pregnancies but not to reinfections. The outcome of the disease associated with *T. gondii* is influenced by the stage of gestation at the time of infection (Dubey, 2013).

The pregnant ewe can suffer of *Toxoplasma*-induced reproductive losses when parasitaemia occurs and tachyzoites invade the placenta and the uterus, leading to infectious placentitis (Weissmann, 2003). Approximately five days after infection, pregnant ewes develop pyrexia that lasts for 4 days, due to the parasitaemia. Fever, tremor, dyspnoea and abortion in the last

4 weeks of pregnancy may develop in up to 50% of sheep. Only 200 sporulated oocysts are needed to cause a congenital disease in unexposed sheep (Abu-Dalbouh et al., 2010).

If the tachyzoites reach the placenta and uterus between day 45 and 60 of gestation, foetal resorption will result; in between day 60 and 100 of gestation, stillbirth and foetal mummification will be seen; and abortion occurs after the third and fourth month of gestation. Abortion usually occurs only one time in first lambing ewes (Figure 2).

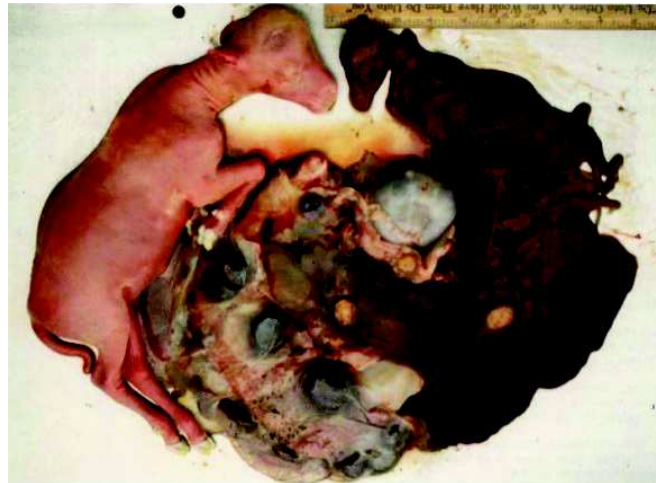


Figure 2: *Toxoplasma* induced abortion of sheep

The darker aborted foetus and its associated foetal membranes are severely autolyzed. The lighter aborted foetus and its associated foetal membranes are not autolyzed. The tachyzoite multiplication results in focal necrosis in the placenta (Gardiner et al., 1988).

About five days after the infection of a pregnant ewe with the protozoan parasite, necrosis and mineralization can be seen in the placental cotyledons of a pregnant ewe (Figure 3) (Weissmann, 2003).

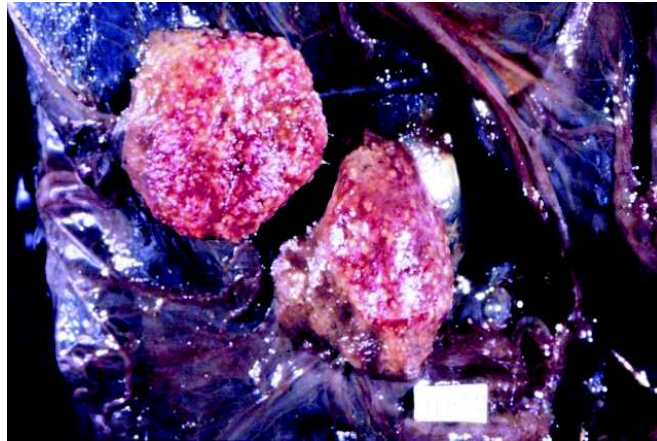


Figure 3: *Toxoplasma* infected placenta of sheep

All cotyledons are usually affected in *T.gondii* infection with the classical 1 or 2 mm white necrotic foci (Agerholm and Dubey, 2014).

Birth of weak lambs will be seen if the infection is happening between day 100 and 120 of gestation. They may show movement disorders and will not suck (Bostedt and Dedie, 1996). If the pregnant ewe gets infected in a later stage of pregnancy, the foetus may have developed a well established immune system without clinical effects. The lambs are born normal but will be latent infected and are immune for life (Buxton, 2007). Following such an infection ewes develop a protective cell-mediated immunity but will remain persistently infected with bradyzoites in tissue cysts found in brain and muscles and are seropositive for life (Buxton, 2007).

In adult goats the clinical toxoplasmosis often occurs and it is more severe than in sheep. The pyrexia, following parasitaemia lasts longer, for about 10 days. At the same time decreased appetite, cough and nasal secretion will be seen. Goats are capable of aborting multiple times without getting reinfected by *T. gondii* (Bostedt and Dedie, 1996).

2.4 Public health issues of *Toxoplasma gondii* infection

Toxoplasmosis is a widespread parasitic zoonosis, affecting approximately 25% of the human population (Weiss and Dubey, 2009).

Humans can get infected with the coccidian parasite by several routes;

- (1) eating undercooked or raw meat containing the infectious tissue cysts,
- (2) drinking non-pasteurized sheep or goat milk containing *T. gondii* tachyzoites,
- (3) ingesting food or water contaminated with sporulated oocyst (Bahia-Oliveira et al., 2003),
- (4) transplacentally or even by blood transfusions or organ transplantations (Gilot-Fromont et al., 2012).

The clinical toxoplasmosis in humans is linked epidemiologically to ingestion of *T. gondii* in food. The food-borne transmission is one of the major sources of *T. gondii* infection among humans. Several epidemiological studies have proved that undercooked meat is of high risk for *T.gondii* infection. Raw lamb meat has been linked to symptomatic toxoplasmosis in a family in New York. Furthermore it was observed that unpasteurized goat's milk to be a *T. gondii* infection risk in family clusters in the United States and the United Kingdom (Griffin et al., 2012). A recent study carried out in the United States, the seroprevalence of *T. gondii* antibodies found in goat meat marketed for human consumption was 53.4% (Griffin et al., 2012).

Infection with the pathogenic *T.gondii* can cause disease in the developing foetus, like mental retardation and loss of vision and is dangerous for people whose immune systems are compromised through disease or chemotherapy and can even result in death (Hill and Dubey, 2002).

Congenital infections only occur after the exposure with *T. gondii* during pregnancy. Most severe congenital infections acquired during the first trimester of pregnancy result in abortion meanwhile the pregnant woman having a temporary parasitaemia usually does not show any clinical symptoms. Infection during the second or third trimester of pregnancy will result in stillbirth or has a foetopathogenic effect. The congenitally infected children do suffer from retino-chorioditis, hydrocephalus, convulsions and intracerebral calcification. Subsequently can a transplacental infection result in abortions, malformations, blindness and mental retardation of the foetus. Focal lesions can be found in the placenta (Hill and Dubey, 2002).

Toxoplasmosis in immunocompromised individuals causing a fatal toxoplasmic encephalitis (Black and Boothroyd, 2000) resulting in mortality as a result of uncontrolled *T. gondii* infection, which is considered to be a consequence of reactivation of a latent infection (Bobić et al., 2012).

2.5 Laboratory diagnosis of toxoplasmosis in small ruminants

Since the introduction of the classic dye test of Sabin-Feldman in 1948, a method for the detection of anti-*Toxoplasma* antibody in serum using a methylene blue dye to detect tachyzoites (Dubey, 2008), several tests have been suggested to diagnose the infection with the pathogenic parasite.

There are several direct methods available to detect *T.gondii* infection in small ruminants. Such as to identify tachyzoites or tissue cysts by histopathology or by immunohistochemistry (Rossi et al., 2011), the polymerase chain reaction (PCR) to detect gene sequence specific for *T.gondii* from tissues and fluids of aborted foetuses and bioassays in mice (Nunes et al., 2015).

Serological tests such as the enzyme-linked immune sorbent assay (ELISA), which uses an enzyme linked to an antibody or antigen as a marker; the indirect fluorescent antibody test (IFAT) using a fluorescent dye which is linked to an antibody; the modified agglutination test (MAT), using formalin fixed *Toxoplasma* tachyzoites to detect IgG antibodies; the Western blot (WB) or immunoblot detects specific proteins by transfer (blotting) them to a carrier membrane; the indirect haemagglutination test (IHA) works with red blood cells which are coated with an antigen; and the latex agglutination test (LAT) is seen as latex beads which are coated to a specific antibody or antigen. These indirect serological tests are generally highly sensitive and have been largely used worldwide (Glor et al., 2013).

The IFAT, MAT and ELISA tests have been modified to detect immunoglobulin M (IgM) antibodies in the infected host, which is detectable about one week after the infection and remain for several months or years. The IgM/IgG ratio can be used to differentiate between the acute and chronic infections (Abu-Dalbouh et al., 2010).

“The IgM antibodies appear sooner after the infection than the IgG antibodies and disappear faster than IgG antibodies after recovery” (Hill and Dubey, 2002).

2.6 Serological studies of toxoplasmosis in small ruminants

In Germany, the serological investigation of 1732 sheep samples for detecting IgG antibodies against *T. gondii* revealed 804 (46%) samples negative, 105 (6%) samples inconclusive and 823 (48%) samples positive. Unfortunately, the method for serological investigation was not specified in the study. Moreover positive samples were found in all flocks and the prevalence of positive samples was 100% in 10 flocks (Ganter, 2008).

In Scotland, the seroprevalence of 3333 sera from 125 sheep flocks were examined to determine the *T. gondii* positivity in relation to age of animals. Using ELISA the overall seroprevalence was 56.6%. Each flock had at least a single positive animal and all ewes were seropositive in four flocks. The seroprevalence was lower (37.7%) in one year old stock of ewes comparing with older ones, over 6 year-old (73.8%) (Katzner et al., 2011).

In 6 regions of Lithuania, blood samples were taken from 354 sheep and tested for *T. gondii* using a commercial ELISA method. The total seroprevalence was 42.1% (Stimbirys et al., 2007).

In Italy serum samples from 630 milk sheep, in 33 dairy flocks were tested for the presence of antibodies to *T. gondii* using an indirect immunofluorescence antibody test. The overall seroprevalence in the district, adjusted for sampling weights and for test sensitivity and specificity, was 33.3%. At least one seropositive animal was found in 32 of the 33 flocks tested (Cenci-Goga et al., 2013).

In Sicily the sera of 1961 sheep were examined for *T. gondii*. The total seroprevalence was 49.9% (937/1876) by ELISA and 87% (54/62) of the farms had at least one seropositive animal (Vesco et al., 2007).

In Greece serum samples of 1501 sheep and 541 goats were examined with ELISA. A higher seroprevalence (48.6% [729/1501]) for *T. gondii* was determined in sheep in comparison to goats (30.7% [166/541]) (Tzanidakis et al., 2012).

In Poland a survey was carried out on a herd-level detecting the seroprevalence of *T. gondii* infections in goats. 1060 sera samples were collected from 49 breeding goat herds using two commercial indirect ELISA kits. The true herd-level seroprevalence was 100% and the true individual-level seroprevalence in these herds ranged from 30.2% to 100% (Czopowicz et al., 2011).

In Romania 735 serum samples were collected from dairy goats during 2007-2010. Using an ELISA kit 388 (52.8%) goats presented *T. gondii* antibodies (Iovu et al., 2012).

In Jordania, 169 biological samples were collected from aborted sheep and 86 from goats during the lambing season from September 2009 to April 2010. A total of 76 (29.8%) samples (45 blood and 31 tissue samples) were positive for *T. gondii* using PCR technique. The positive samples were derived from 43 sheep and 23 goats (Abu-Dalbouh et al., 2012).

In Central Ethiopia, a cross sectional study was carried out from September 2011 to November 2012 to detect IgG antibodies specific to *T. gondii* with Direct Agglutination Test in 628 randomly selected samples of small ruminants. The overall seroprevalence was 17.68% (111/628), 20% of sheep (61/305) and 15.48% of goats (50/323) were seropositive. There was a significantly higher risk of infection in adult than in young sheep (Gebremedhin et al., 2014).

In Sudan, 305 blood samples from sheep were collected at 3 slaughterhouses of which 125 samples were seropositive for *T. gondii* (40.9%) using a commercial ELISA kit (Medani and Kamil, 2014).

In West Indies antibodies to *T. gondii* were detected in sera from 26% of 116 sheep and 34% of 66 goats tested with an in-house ELISA (Hamilton et al., 2015).

In Marocco, 261 sera from sheep intended for human consumption were analysed with ELISA for the detection of anti-*T. gondii* specific IgG to confirm a past infection. Of the overall tested, 72 (27.6%) sera were positive (Sawadogo et al., 2005).

In Pakistan, a total of 200 serum samples from sheep and goats were collected and tested with a commercial latex agglutination kit. The overall seroprevalence of *T. gondii* infection was 19%. Goats had a higher prevalence (25.4%) compared to the sheep (11.2%). The infection rate was higher in females (24%) than in males (19%) for both animal species. The prevalence was significantly higher in adult sheep and goats than in younger animals (Ramzan et al., 2008).

In China, a total of 1732 Tibetan sheep were tested for *T. gondii* antibodies by a modified agglutination test (MAT) and 352 (20.3%) animals were positive (Yin et al., 2015).

In Korea, 610 sera samples of goats were collected from 60 herds between 2009 and 2011. A commercial ELISA kit for detection of anti-*T. gondii* IgG antibodies was used, 5.1% (31/610) of animals and 38.3% (23/60) of the herds were seropositive (Jung et al., 2014).

3. Materials and Methods

3.1 Study area and sampling

Blood samples were collected from 21 sheep and 8 goat flocks kept in different parts of Hungary (Figure 4) between May and November 2015.



Figure 4, Location of sheep (green pins) and goat (blue pins) flocks sampled

The sheep and goat flocks consisted of 100-600 and 20-200 animals, respectively. The sheep breeds were Hungarian merino, Lacaune and Suffolk-Charolaise crossbred. The goat breeds were Alpine, Hungarian milking, Saanen and Boer. A total of 990 female animals (740 ewes and 250 does) were randomly sampled. Only adult ewes and milking does were sampled, their ages ranged between 3 and 10 years. Ten to sixty-five sera samples were taken from each sheep farm and 10 – 45 sera samples were analysed from each goat farm.

Approximately 3 ml of blood were taken from the jugular vein of each animal using a vacuum tube. The samples were transported in a cool box with thermal packs to the Department of Parasitology and Zoology, Faculty of Veterinary Science, in Budapest. The samples were centrifugated for 10 minutes at 1,000-2,000 x g for collecting sera which were stored in Eppendorf tubes with an individual mark at minus 20° C until the examination.

3.2 Serological method

The new commercial ELISA kit (PrioCHECK® Toxoplasma Ab SR ELISA) marketed by Prionics (Lelystand B. V., the Netherlands) was used for *in vitro* detection of antibodies against *Toxoplasma gondii* in serum, plasma and meat juice samples from small ruminants. The ELISA plate is coated with cell culture derived tachyzoite antigen. The test has a high sensitivity of 98% and specificity of 99.6 % and clearly differentiates between positive and negative blood samples. Besides positive and negative control samples 90 sera can be analyzed within 150 minutes in each plate. The diagnostic test is easy to use, fast and efficient.

The ELISA-kit uses a four-step protocol, consisting of Sample Preparation, Sample Incubation, Conjugate Incubation and Detection, and uses coloured buffers to reduce handling errors.

For the detection of antibodies bound to the *Toxoplasma* antigen a peroxidase (POD) labeled antibody is used. Colour development occurs, after using a TMB (tetramethyl benzidine) substrate. This was measured optically with a photometer at a wavelength of 450nm which shows the presence of antibodies directed against *T. gondii*.

The ELISA-kit should be stored at $5\pm 3^{\circ}\text{C}$ until expiry date. The following components are included in the package:

- Component 1: 5 Test Plates are delivered in vacuum bags containing a desiccant bag
- Component 2: Sample Diluent (ready-to-use), 2 bottles containing 60 ml of Sample Diluent.
- Component 3: Washing Fluid (20x) (20x concentrate, dilute before use), 2 bottles containing 60 ml Washing Fluid (20x).
- Component 4: Conjugate (30x) (30x concentrate, dilute before use), 1 vial containing 2 ml Conjugate (30x). Dilute the amount of Conjugate necessary to run the test just prior to use.
- Component 5: Conjugate Diluent (ready-to-use), 1 bottle contains 60 ml Conjugate Diluent.
- Component 6: Positive Control, 1 vial containing 0.5 ml of Positive Control.
- Component 7: Weak Positive Control, 1 vial containing 0.5 ml of Weak Positive Control.
- Component 8: Negative Control, 1 vial containing 0.5ml of Negative Control.

- Component 9: Chromogen (TMB) Substrate (ready-to-use), 1 bottle containing 60 ml Chromogen (TMB) Substrate.
- Component 10: Stop Solution (ready-to-use), 1 bottle containing 60 ml Stop Solution.
- Additional Kit Contents: Package Insert

Additional Material which is required:

- Demineralised water or water of equal quality must be used
- Dummy plate, used for sample dilution or equivalent; non binding
- Single channel pipettes
- Multi channel pipettes suitable to pipette the required volumes *f*
- Pipette tips (as recommended by pipette manufacturer) *f*
- Solution reservoirs *f*
- Vortex
- Plate Reader, e.g. Tecan Sunrise or equivalent. The reader has to have an appropriate filter set to read the plates at 450 nm.

Solution preparation of:

- Washing Fluid Working Solution: to prepare 1 liter of the solution, mix 50ml of the washing fluid (20x, Component 3) with 950 ml demineralised water and mix until a clear solution is obtained.
- Conjugate Working Solution: to prepare Conjugate working solution for 1 plate, mix 0,4ml of Conjugate (30x, Component 4) with 11,6ml of Conjugate Diluent (Component 5). For the preparation of conjugate for 2 plates use the doubled amount of reagent.

Sample dilution for serum and plasma samples:

The final dilution of serum and plasma samples is 1:100.

The final dilution for the control samples is 1:10.

1.1 Use a Dummy Plate for first sample dilution.

1.2 Add 20 µl of Positive Control to wells A1 and B1 of the Dummy Plate.

1.3 Add 20 µl of Weak Positive Control to wells C1 and D1 of the Dummy Plate

1.4 Add 20 µl of Negative Control to wells E1 and F1 of the Dummy Plate.

- 1.5 Add 10 µl of serum or plasma samples to the remaining wells of the Dummy Plate.
- 1.6 Add 90 µl of Sample Diluent to the wells of the Dummy Plate except the wells containing controls (A1 to F1) and mix by pipetting up and down 5 times.
- 1.7 Add 90 µl of Sample Diluent to each well of the Test Plate (see figure 4).
- 1.8 Transfer 10 µl of the diluted samples and controls from the Dummy Plate to the Test Plate and mix by pipetting up and down 5 times, or shake gently for 1 minute.

Sample incubation:

- 2.1 incubate the samples on the Test Plate for 60±1 minutes at room temperature (22±3°C).
- 2.2 Wash the Test Plate four times with 300 µl Washing Fluid working solution per well.

Conjugate Incubation

- 3.1 Add 100 µl of the diluted Conjugate to each well of the Test Plate.
- 3.2 Incubate the Test Plate for 60±1 minutes at room temperature (22±3°C).
- 3.3 Wash the Test Plate four times with 300 µl Wash Fluid working solution.
- 3.4 Remove remaining liquid by clapping out the plate on a paper towel.

Detection, Substrate reaction:

- 4.1 Add 100 µl of the Chromogen (TMB) Substrate to each well on the Test Plate.
- 4.2 Incubate the Test Plate for 15±1 minutes at room temperature (22±3°C).
- 4.3 Add 100 µl of the Stop Solution to each well of the Test Plate. Remark: Add the Stop Solution in the same order and place as the Chromogen (TMB) Substrate solution was dispensed.

Reading of the test and calculating the results:

- 5.1 Shake the Test Plate shortly (5 -10 s) either on an orbital shaker (~300 rpm) or manually on the working bench.
- 5.2 Measure the optical density (OD) of the wells at 450 nm within 15 minutes after colour development has been stopped.
- 5.3 Calculate the mean OD_{450nm} value of wells A1 and B1 (Positive Control = OD_{450nm} PC).

5.4 Calculate the mean OD450 value of wells E1 and F1 (Negative Control= OD450nm NC).

5.5 The percentage positivity (PP) of the Controls and the test sera are calculated according to the formula below.

The corrected OD450 values of all samples are expressed as Percentage Positivity (PP) relative to the corrected OD450 max.

$$PP = \left[\frac{OD\ 450nm(Sample) - OD450nm(Neg.Control)}{OD450nm(Pos.Control) - OD450nm(Neg.Control)} \right] * 100$$

Result interpretation, validation criteria:

6.1 The mean OD450 of the Positive Controls (PC) must be ≥ 1.2 .

6.2 The PP of the Weak Positive Controls (WPC) must be $\geq 35\%$.

6.3 The mean OD450 of the Negative Controls (NC) must be >0.15 .

If these criteria are not met, the results are invalid and the plate has to be retested.

Results obtained above or equal to the cut-off of 20 Percent Positivity (PP) are considered positive. Results obtained below the cut-off of 20 PP are negative.

3.3 Statistical analysis of data

The statistical data were calculated, taking into account the specificity (98%) and sensitivity (99.6%) of the ELISA kit, by using the true seroprevalence with 95% confidence interval according to the method published by Reiczigel et al. (2010). This on-line calculator estimated the prevalence of a disease, corrected for sensitivity and specificity of the diagnostic test with exact confidence intervals.

4. Results and Discussion

Sheep flocks

The result of sampling sheep flocks can be found in Table 1.

Location of flocks	Number of examined animals	Number of infected animals	True prevalence (%)	95% confidence interval
Iharosberény	10	3	30.3	0.085 - 0.631
Csurgó-Avaspuszta	20	9	45.7	0.241 - 0.697
Somogybükkösd	30	14	47.4	0.241 - 0.697
Lábod, Kőröspuszta	20	10	50.8	0.296 - 0.721
Dóc	10	7	71.3	0.386 - 0.931
Nagyegyháza	20	10	50.8	0.296 - 0.721
Sárrét	10	2	20.1	0.034 - 0.565
Sárbogárd	20	4	20.1	0.069 - 0.428
Hortobágy, Görbehodály	65	48	75.2	0.628 - 0.852
Hortobágy, Halastó	45	7	15.6	0.068 - 0.289
Solt (Járáspuszta)	45	40	90.7	0.776 - 0.975
Angyalháza	45	37	83.8	0.694 - 0.936
Dunapataj	45	40	90.7	0.776 - 0.975
Karcag	60	48	81.6	0.690 - 0.907
Bucsa	30	15	50.8	0.328 - 0.688
Üllő	45	16	36.1	0.230 - 0.520
Biharkeresztes (1)	50	2	3.7	0.003 - 0.133
Biharkeresztes (2)	50	50	100	0.955 - 1.000
Biharkeresztes (3)	50	35	71.3	0.571 - 0.831
Biharkeresztes (4)	55	41	75.9	0.622 - 0.868
Fadd	15	11	74.7	0.472 - 0.922
Total	740	449	61.8	0.581 - 0.654

Table 1: The result of sheep sera samples checked for anti-*T. gondii* IgG.

Overall sera samples of 740 animals were collected from 21 sheep farms in Hungary (Table 1), of which more than half of them, 449 (60.68%) were seropositive for *T. gondii*. Seropositive ewes were found in each of the 21 sheep flocks tested. The true seroprevalence of *T. gondii* infection among the tested 740 animals was 61.8%. These values ranged between 3.7 % (Biharkeresztes 1) and 100% (Biharkeresztes 2). The overall true seroprevalence of *T. gondii* infection of sheep obtained in this study is higher comparing to the data published in other European countries like in Germany (Ganter, 2008), Scotland (Katzer et al., 2011) and

Lithuania (Stimbirys et al., 2007) where they found with ELISA 48.0, 56.6 and 42.1%, respectively.

In Italy where the infection of sheep caused by *T. gondii* was studied with IFAT (Cenci-Goga et al., 2013) and ELISA (Vesco et al., 2007) methods the seroprevalence was also smaller (33.3% and 49.9%) than in this study.

In non-European countries like in Marocco (27.6%) (Sawadogo et al., 2005) and Sudan (40.9%) (Medani and Kamil, 2014) the overall seroprevalence for *T. gondii* was as well lower as in Hungary. In these countries the sera samples of sheep were tested for the presence of anti-*T. gondii* IgG with ELISA.

In China, where 1732 sheep samples were examined using a modified agglutination test 352 (20.3%) animals were positive (Yin et al., 2015).

Goat flocks

The result of sampling goat flocks can be found in Table 2.

In goats, 250 sera samples were acquired from 8 goat farms in Hungary, 108 (43.2%) does were seropositive and 142 (56.8%) tested animals were seronegative (Table 2), using the new ELISA kit. Seropositive milking does were found in each of the 8 goat flocks tested. The overall true seroprevalence of *T. gondii* infection of goats was 43.9% with values ranging from 1.8% (Izsák) to 100% (Csetény).

Location of flocks	Number of examined animals	Number of infected animals	True prevalence (%)	95% confidence interval
Nagyréde	10	7	71.3	0.386 - 0.931
Valkó	45	30	67.9	0.520 - 0.810
Csetény	24	24	100	0.884 - 1.00
Izsák	45	1	1.8	0 - 0.113
Tét	33	1	2.7	0 - 0.156
Tés	35	11	31.8	0.179 - 0.493
Sümeg	38	26	69.7	0.522 - 0.840
Kisvásárhely	20	8	40.6	0.210 - 0.642
Total	250	108	43.9	0.377 - 0.502

Table 2: The result of goat sera samples checked for anti- *T. gondii* IgG

The overall true seroprevalence of *T.gondii* infection in goats received in the current study is lower comparing with the data published in Poland where 1060 sera samples were examined and found a true herd-level seroprevalence of 100% and the true individual-level seroprevalence ranged from 30.2% to 100% (Czopowicz et al., 2011).

In Romania 735 goat samples were analysed using an ELISA kit and 388 (52.8%) samples were found seropositive (Iovu, 2012). In Korea, this data was very small, only 31 (5.1%) of 610 goats were seropositive with ELISA technique (Jung et al., 2012).

Comparing the seroprevalence of sheep and goats in this study, a slightly higher portion (60.68%) of ewes had anti-*T. gondii* IgG antibodies than goats (56.8%) (Figure 5). Similar results were reported from some other countries. In Greece sheep had a higher seroprevalence (48.6%) for *T. gondii* than goats (30.7%) (Tzanidakis et al., 2012). In Jordania 71.01% of sheep and only 26.74% of goats were seropositive (Dalbouh et al., 2012). In Central Ethiopia the difference between the seropositivity of the two ruminant species was big, but more sheep (20%) were infected than goats (15.48%) (Gebremedhin et al., 2014).

In contrary to other studies carried out in West Indies (Hamilton et al., 2015) and Pakistan (Ramzan, 2008) higher percentages of goats (34% and 25.4%) were seropositive than sheep (26% and 11.2%).

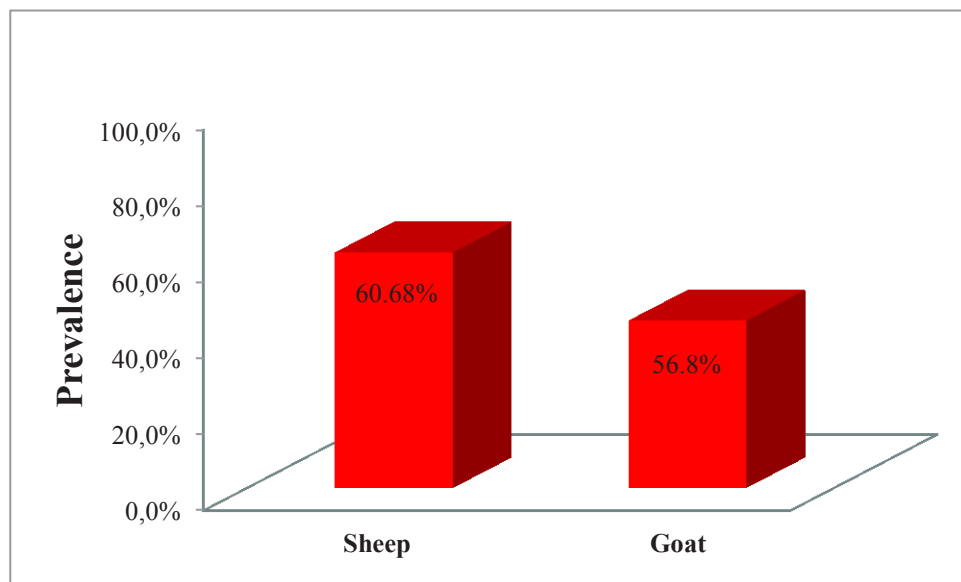


Figure 5: Seroprevalence of sheep and goats infected with *T.gondii*

There are some important factors which can influence the results of such kind of serological studies. The sensitivity and specificity of the different ELISA and other methods are not the same therefore this should be taken into account when the reported data are compared with each other and the results obtained in this study.

Another factor is the age of the animal. Seemingly there is a higher chance of getting infected with *T.gondii* among older animals in comparison to younger ones, like in Scotland where the seroprevalence was lower (37.7%) in one year old stock of ewes than older ones, over 6 year-old (73.8%) (Katzner et al., 2011), Central Ethiopia (Gebremedhin et al., 2014) or Pakistan (Ramzan, 2008) showed similar results.

The keeping system (in stables and/ on the pasture) of sheep and goats is also an important factor which can influence the results.

Furthermore is the number of tested animals per flock of importance as more animals tested will increase the chance of finding seropositive animals in a flock.

To the best of our knowledge this is the first serological survey of *T. gondii* infection of Hungarian sheep and goat flocks. The IgG antibodies detected in both small ruminant species in all the farms indicates the animals became infected earlier. The question is how these small ruminants acquired the infection.

The environment around the animals may be contaminated with sporulated oocyst originated from the infected definitive hosts, probably stray as well as domestic cats which occur in stables and pastures. The main infection route of small ruminants is by the faecal-oral route after ingesting contaminated water or feed.

Once the oocysts sporulate in the environment, which are shed in huge amounts by infected felines, they will remain infectious even for years under good conditions (Bostedt and Dedie, 1996).

Although the owners of sheep and goat flocks did not know whether any abortion due to *T. gondii* had been diagnosed and this risk should be considered in all these farms because following *T. gondii* exposure of pregnant animals for the first time foetal resorption, stillbirth, foetal mummification, abortion or birth of weak lambs will be seen, according to the stage of gestation at the day of infection (Bostedt and Dedie, 1996). Goats are overall more sensitive than sheep to the infection and can abort multiple times, without getting reinfected by *T. gondii* (Bostedt and Dedie, 1996).

Besides the animal health and economical aspects of sheep and goat toxoplasmosis the infected animals can be the source of human infection. Taking the food hygiene and safety into account it is very important to inform people about the possible routes of infection to receive toxoplasmosis. The main source of infection is the ingestion of undercooked meat of seropositive animals, which harbours the infectious tissue cysts. Meat from these animals is an important source of human *T. gondii* infection especially in countries and regions where sheep and goat meat is regularly eaten (Tzanidakis et al., 2012).

Raw or undercooked lamb meat is regarded a delicacy in certain countries, like in France and is therefore considered an important source of infection in that country. The other important route of human infection is the consumption of raw goat's milk and milk products which has been linked to cases of toxoplasmosis in humans and pigs. That's why raw milk should only be consumed after pasteurisation, especially pregnant and immunocompromised individuals should take special care. A transplacental infection will result in abortions, malformations, blindness and mental retardation of the foetus (Hill and Dubey, 2002). In immunocompromised humans fatal toxoplasmic encephalitis will be seen and can result in death (Black and Boothroyd, 2000).

Goats are continuative a fundamental source of meat and milk in many undeveloped countries (Kijlstra and Jongert, 2008). The main aim is to prevent the infection with *Toxoplasma* by making sure to cook meat properly, pasteurize milk and always wash hands appropriately after working in the garden, cleaning litter boxes of cats, or wear gloves during working with the mentioned tasks.

The education of the farmers about the disease and the route of infection is also very important and should be practised on a regular basis.

To prevent oocyst contamination of food or stables a theoretical solution would be to strictly ban cats out of these areas and to control the stray cat populations. The ban of cats from the farms to prevent the infection of small ruminants seems to be a more theoretical and not a very realistic solution.

In the case of environmental contamination with oocysts, farm animals intended for meat production should be kept inside to prevent the infection. This would be a very extreme way of management and is not desirable, as they are grazing animals.

Infection rates could be moreover reduced by keeping the feed of small ruminants covered and to heat all feed administered to a temperature of at least 70 °C and provide sterile drinking water (Dubey, 1996).

A higher probability of solving the contamination and reinfection problem would be to vaccinate the cats of the specific farm or area. Currently the development of live vaccines to prevent feline oocyst shedding is ongoing and in use is now the S48 strain 'Toxovax', which is a live vaccine originally developed for use in sheep, but when used in cats inhibits the sexual development of *T. gondii* (Verma and Khanna, 2012). This vaccine is commercially not available yet for felines.

Continuing to prevent *Toxoplasma* induced abortion in small ruminants and therefore the congenital toxoplasmosis is the usage of the vaccine 'Toxovax' which is based on the principle that exposure to the live parasite before pregnancy will result in immunity, that's why only one injection is needed to ensure a lifetime protection. The developed vaccine consists of live organisms of an attenuated strain of *T.gondii* (strain 48) which induces immunity in small ruminants (Verma, 2012).

Another way of immunisation is the intranasal vaccination which is using a crude extract of *T.gondii* tachyzoites and induces humoral and cell-mediated immunity in sheep (Stanley et al., 2004).

Up-to-now no reliable methods have been used during the meat inspection of slaughtered animals to detect *Toxoplasma* cysts in the meat. Therefore effective methods can be used for killing the tissue cysts such as freezing at -12°C, cooking with reaching internal temperature of 67°C, or gamma irradiation (Dubey, 1996).

In summary the control of *T. gondii* infection relies on the prevention of contamination of pasture, fodder and water with faeces of cats, which is a very difficult task as cats are highly present among the sheep and goat barns and show as well a seroprevalence for *Toxoplasma* in Hungary (Hornok et al., 2007). An overall improved management of farm cats should be done to prevent the infection of sheep and goats and therefore reduce the risk of human infection.

This kind of serological survey should be extended to other farms of the country including younger animals too, in order to get more data about the epidemiology of toxoplasmosis. It would also be important to check the infection rate of milking animals and to investigate the abortion of pregnant sheep and goats. Further studies would be needed on how to minimize the infection of *T.gondii* in cats and finally to the intermediate hosts and humans.

5. Abstract

Toxoplasma gondii is one of the most important zoonotic agents in the world. Humans can be infected with tachyzoites of the parasite occurring in unpasteurized milk of infected goats and sheep or with bradyzoites found in infected meat. This protozoan infection may result in significant reproductive and therefore economic losses in the sheep and goat industry worldwide. It is considered to be one of the main causes of infectious abortions among small ruminants

Before this study no information was available about the incidence of *T. gondii* infection sheep and goats in Hungary. Therefore the aim of the current study was to detect *T. gondii* antibodies in the sera of 990 small ruminants kept in different parts of Hungary. Seven-hundred forty sheep and two-hundred fifty goat samples were collected in 21 sheep and 8 goat flocks, respectively. A new commercial ELISA kit (PrioCHECK® Toxoplasma Ab SR ELISA) marketed by Prionics (Lelystand B. V., the Netherlands) has been used for *in vitro* detection of antibodies against *T. gondii* in serum samples. Taken into account the sensibility (98.0%) and the specificity (99.6%) of the ELISA kit the true prevalence was calculated with 95% confidence interval.

Of the total tested, 449 (60.68%) ewes and 108 (43.2%) does were seropositive for *T. gondii*. The true seroprevalence was 53.93% in sheep and 48.22% in goats. In sheep and goat flocks tested these values ranged between 3.7-100% and 1.8-100%, respectively. All the small ruminant flocks had at least one seropositive animal.

This kind of serological survey should be extended to other farms of the country including younger animals too, in order to get more data about the epidemiology of toxoplasmosis. It would also be important to check the infection rate of milking animals and to investigate the abortion of pregnant sheep and goats. Further studies would be needed on how to minimize the infection of *T. gondii* in cats and finally to the intermediate hosts and humans.

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