Infectivity of cysts of the ME-49 *Toxoplasma gondii* strain in bovine milk and homemade cheese*

Infecciosidade de cistos de *Toxoplasma gondii* ME-49 em leite bovino e queijo caseiro

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Keywords
Toxoplasmose, transmission.# Milk, microbiology.# Cheese, microbiology.# Food contamination.# Food hygiene, methods. Food preservation. Toxoplasmosis, animal, chemically induced. Mice, blood. Enzyme-linked immunosorbent assay. Antibodies, protozoan. – *Toxoplasma gondii*.

Abstract

Objective
Analyze the infectivity and storage resistance of cysts of the ME-49 strain of *Toxoplasma gondii* in artificially infected bovine milk and homemade fresh cheese.
Methods
Pasteurized bovine milk was infected with 10 cysts/ml of the ME-49 strain of *T.gondii* and inoculated in different groups of mice, immediately or after storage at 4ºC for 5, 10 and 20 days. Homemade fresh cheese was prepared with artificially infected milk, and also tested in groups of mice, using the same storage process. Infection was identified by the presence of cysts in the brain or serological testing in challenged mice after 5 weeks, confirmed by Western Blot and histology.
Results
The infectivity of cysts of the ME-49 strain of *T.gondii* was maintained in the milk even after storage for 20 days at refrigerator temperatures. Cysts were also able to survive the production process of homemade fresh cheese and storage for a period of 10 days in the same conditions.
Conclusions
These data demonstrated that milk and dairy products could be an important source of *T.gondii* in human contamination, reinforcing the importance of milk pasteurization before any processing or ingestion.

Descritores
Toxoplasmose, transmissão.# Leite, microbiologia.# Queijo, microbiologia. Contaminação de alimentos.# Higiene dos alimentos, métodos. Conservação de alimentos. Toxoplasma, patogenicidade. Toxoplasmose animal, induzido quimicamente. Camundongos, sangue. ELISA. Anticorpos antiprotozoários. – Toxoplasma gondii.

Resumo

Objetivo
Analisar a infecciosidade e a resistência de cistos de T. gondii *em leite e queijo fresco caseiro, pela infecção artificial de leite bovino.*
Métodos
O leite bovino pasteurizado foi infectado artificialmente com 10 cistos/ml de T.gondii *cepa ME49 e inoculado em grupos de camundongos, imediatamente ou após ser estocado por 5, 10 e 20 dias a 4ºC. Preparou-se queijo fresco caseiro com leite infectado, sendo testado em grupos de camundongos, utilizando a mesma conservação. A infecção foi detectada pela presença de cistos no cérebro dos*

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camundongos desafiados ou testes sorológicos após cinco semanas, também confirmada por Western Blotting e histologia.

Resultados

A infecciosidade dos cistos da cepa ME49 de T.gondii *foi mantida mesmo quando armazenado no leite até 20 dias de conservação em condições de refrigeração a 4ºC. Os cistos resistiram ao processo de fabricação do queijo e eram infectantes após um período de 10 dias nas mesmas condições.*

Conclusões

Os achados mostraram que o leite e seus derivados podem ser uma importante fonte de contaminação humana pelo T.gondii*, reforçando a importância da pasteurização do leite antes de qualquer processamento ou ingestão.*

INTRODUCTION

Toxoplasma gondii is an obligatory intracellular Apicomplexa parasite, with a complex life cycle, that causes toxoplasmosis in humans, and domesticated and wild animals. Infection is usually asymptomatic in people with a normal immune function and occasionally results in eye involvement. Toxoplasmosis can cause severe disease in fetuses of acutely infected pregnant woman, immunocompromised individuals (Aids) and therapeutically immunosuppressed patients, as cancer or transplant recipients.[2] In Brazil, *T.gondii* infects around 60% of the adult population.[13] Toxoplasmosis is acquired by ingesting food and water contaminated with oocysts from feces of infected cats or by the ingestion of raw or undercooked meat, containing tissue cysts, of infected intermediary host.

Milk was implicated as a source of infection of *T.gondii* in several reports. Toxoplasmosis was described in breast fed child whose mother had recently acquired toxoplasmosis[1] and ingestion of unpasteurized goat milk was considered a possible source of *T.gondii* infection in children of rural areas,[3,17,20] showing the importance of milk in the spread of *T.gondii* infection. Experimentally, there are reports of chronically infected lactating mice excreting cysts in their milk.[16]

This transmission was attributed both to tachyzoites in the milk and suckling trauma but also to tissue cyst excretion[16] due to specific exocytic lipid secretion in the mammary cell that allows the secretion of lipidic droplets with the luminal upper layer of the cell cytoplasm,[5] described in some works of cyst isolation in milk from acutely infected goats.[6] Despite of those few reports, studies were conducted to verify the infectivity of *T.gondii* cysts in milk, without any description of their infectivity in fresh homemade cheese, a common protein source in rural areas. The present study assesses the infectivity of cysts of the ME-49 strain of *T.gondii* in artificially infected bovine milk and derived fresh homemade cheese.

METHODS

Strains, material and animals

The ME-49 strain of *T.gondii* was cryopreserved in liquid nitrogen or by successive passages of cysts from infected brains, homogenized in phosphate buffered saline (PBS) and inoculated intraperitoneal (i.p.) in C57Bl/6j mice. RH strains have been maintained routinely in the Laboratório de Protozoologia in the past 15 years by cryopreservation or by i.p. passage in mice. All plastic ware, reagents and conjugates were purchased from one manufacturer and the solutions were prepared with high-quality water. Isogenic C57Bl/6j mice were obtained from São Paulo University's colony (Centro de Bioterismo/Faculdade de Medicina/USP) and kept in sterilized cages and absorbent media with food and water *"ad libitum"*. The management of these animals before or during the experiments followed the Principles of Laboratory Animal Care[14] and the Principles of Ethics in Animal Experimentation (COBEA).[4]

Experimental infection

Pasteurized bovine whole milk found in the market was infected with cysts of the ME-49 strain from brains of previously infected C57Bl/6j mice. The contaminated milk was administrated orally to groups of eight mice, fresh or stored at 4ºC for 5, 10 and 20 days. A dose of 12 cyst/mouse was used. Cysts kept and stored under the same conditions in sterile PBS were used as controls, with mice follow-up after 5 weeks.

Homemade fresh cheese was produced with manual technique, using products available in the market for Brazilian farmers. The pasteurized milk was contaminated with cysts of *T.gondii* of the ME-49 strain (12 cysts/1000 µl milk) in microcentrifuge tubes. After that, 1µl of commercial clot was added per tube and incubated for 30 min at 37ºC. After centrifuging at 800 *g*/5 min, the supernatant was discarded, the cheese washed out with saline solution by centrifugation, removed from tubes and stored in a refrigerator. The

cheese was offered individually to groups of four C57Bl/6j mice, fresh or stored at 4ºC for 5, 10 and 20 days. Ingestion was carefully observed for each mouse, usually occurring in the first half-hour. Mortality, morbidity and serological evidence of infection in C57Bl/6j mice were monitored by 40 days after the ingestion.

Mice blood samples

Blood samples (5 µl) were collected from the mice tails in standardized filter papers dried and stored at –20ºC until use. Before use, the soluble extract containing antibodies was obtained by extraction with 100 µl PBS during 18 hours at 4ºC, with gentle agitation. The soluble extract was recovered by centrifugation and considered a 1/20 dilution of blood.

***T.gondii* antigen preparation and ELISA**

Tachyzoites of the RH strain were harvested from the peritoneal cavity of previously infected mice in phosphate buffered saline (PBS), recovered by centrifugation, washed, counted and submitted to sonication for several periods of 4 cycles/30 seconds in an ice bath, until all parasites were destroyed, as detected by phase contrast microscopy. The solution was isotonized by 0.3 M NaCl addition (v/v) and cleared by centrifugation at 10,000 *g* for 3 min. The protein content of the supernatant was determined and aliquots were maintained at –70ºC until use as purified antigen. Ninety-six high binding plates (Multiwell Plate/polystyrene) were coated with 50 µl/well of a solution containing 10 µg/ml of the antigen extract in carbonate-buffered 0.1 M, pH 9.0, overnight at 4ºC. The plates were then washed 5 times with PBS containing 0.02% Tween 20 (PBS-T), and free protein binding sites blocked for 1 hour at 37ºC with PBS containing 2% fat-free lyophilized milk in humid chamber. Serum dilutions, 100 µl, were added to each well and the plates were incubated for 1 hour at 37ºC. After additional washing with PBS-T, bound IgG were detected by incubation for 1 hour with peroxidase-conjugated anti-mouse immunoglobulin G at 37ºC, followed by 5 washings with PBS-T. The bound conjugates were developed with 100 µl per well of orto–phenylenediamine dihydrochloride 1 mg/ml, H_2O_2 0.03% in phosphate-citrate buffer 0.2 M, pH 5.0, for 30 min, stopped with 25 µl per well of HCl 4N, and the optical density (OD) read at 492 nm in an ELISA reader.

Western blot

Specific antigen reactive antibodies were detected on Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) isolated antigens. Purified parasites, RH strain from peritoneal exudates of previously infected mice (10^8/ml) were sonicated to disruption in PBS and immediately centrifuged for 10,000 *g*/3 min. The supernatant, 400 µg protein/ml, was collected and submitted to SDS-PAGE in a 12,5% separating gel, with subsequent eletrophoretic transfer to nitrocellulose membranes. The membranes were blocked with a 5% fat-free lyophilized milk in PBS-T, cut in 3 mm strips and stored dried at –20ºC until use. Diluted serums were reacted with *T.gondii* antigen strips re-hydrated with PBS-T, for 18 hours at 4ºC. After careful washing with PBS-T, the antibodies were detected by sequential incubation with peroxidase-conjugated anti-mouse IgG, 60 min, with subsequent PBS washings, and developed with 4-cloro-1-naphtol 0.6 mg/ml and hydrogen peroxide 0.03% for 15 min, with constant agitation. After development, the strips were dried and scanned for documentation. Standard molecular weight protein markers were stained with Amido Black B.

Histology

All mice were killed under ethyl ether anesthesia 5 weeks after the challenge. Brain, heart, lung, liver and spleen were removed and fixed in 100 volumes of 10% formaldehyde in Sorensen buffer,with once solution replacement after 1 hour. The organs were included in paraffin, and a 7 µm thick section was obtained and stained with hematoxylin-eosin. When necessary, representatives fields were photographed in a Zeiss Axiophot microscope with planapochromatic optics.

Data analysis

A mouse was considered infected when there were *T.gondii* cysts identifiable in the squash preparation of central nervous system tissue, or when a positive serology was found in ELISA, confirmed by Western blot of specific bands. Comparison between frequencies was performed using Epi Info 6.01 statistical software, using 1-tail Fisher's exact test. Significance was considered when the probability of equality between frequencies was less than 0.05.

RESULTS

***T.gondii* cysts infectivity in milk**

Mice mortality induced by 12 cysts in milk, related to storage, can be seen in (Table 1). Deaths due to infection in most animals occurred by neurological symptoms after 20-30 days of infection, with a decaying pattern with longer storage periods. Interestingly, when compared with PBS stored cysts, the cysts maintained in milk induce a more severe disease,

specially when compared in 10 days storage time (p=0.038, 1-tailed Fisher exact test).

Table 1 - Mortality (%) of C57Bl/6j mice who were orally inoculated with milk infected with cysts of the ME-49 strain of *T.Gondii*, as compared to cysts inoculated in PBS.

Mortality (%)	Days of storage before inoculation 0	5	10	20
Milk	100	25	50	0
PBS	75	75	0	0

PBS: Phosphate buffered saline

Serology of animals, both from survivors or before death in all animals, tested tail blood samples using ELISA as described, could be seen in Table 2. All animals were infected up to five days of storage, regardless of medium, but cysts stored in milk had a prolonged survival, infecting all animals after 20 days of storage (p=0.038, 1-tailed Fisher exact test), when compared to PBS stored cysts in the same period. The positive serology was confirmed with Western Blot, with clearly identifiable bands in similar control infected mice (data not shown). The histological picture of one surviving mouse, sacrificed in the 40th day, show an intense encephalitic process with several *T.gondii* cysts dispersed in brain with foci of inflammatory cells.

Table 2 - Rate of infectivity (%) of cysts of the ME-49 strain in C57Bl/6j mice, after storage in milk or PBS at 4ºC, detected by the presence of specific antibodies using ELISA and Western Blot.

Infectivity (%)	Days of storage before inoculation 0	5	10	20
Milk	100	100	100	100
PBS	100	100	75	50

T.gondii infectivity in homemade cheese

Only one animal died in the group that ingested fresh cheese immediately after it was made. All other groups showed no deaths during the experiment. After 40 days, all mice groups were killed, followed by search of cysts of *T.gondii* in the brain, and serological testing. *T.gondii* cysts were found in the brain of all mice from the groups that had received homemade cheese fresh or stored during 5 or 10 days at 4ºC, a result also confirmed by specific serology, as seen in Table 3. Mice that received cheese stored for 20 days at 4ºC didn't show either brain cysts or seroconversion. The histological feature of a mouse infected with cheese stored for 10 days, confirmed by serology, presented fewer inflammatory foci and *T.gondii* cysts, despite evidence of encephalitis, suggesting a less intense disease. The infection was similar in both forms of contamination, as observed by the detection of tissue cysts, production of specific antibodies with recognition of the same protein bands, without any selective effect of the milk or cheese on the agent, varying only in parasite.

Table 3 - Rate of infectivity (%) of cysts of the ME-49 strain in C57Bl/6j mice, after storage in cheese at 4ºC detected by the presence of specific antibodies using ELISA, as compared to milk stored cysts.

Infectivity (%)	Days of storage before inoculation 0	5	10	20
Milk only	100	100	100	100
Cheese	100	100	100	0

DISCUSSION

These data show clearly that milk and homemade cheese could keep up the infectivity of *T.gondii* cysts for periods up to 10 days for homemade cheese and up to 20 days for milk, under regular refrigeration conditions. Transmission of human toxoplasmosis occurs mainly through the ingestion of food containing cysts of *T.gondii*, found in sheep, pigs, cow, hen and goats.[6-8] Toxoplasmosis outbreak has been mainly related to the ingestion of undercook meat,[1] but few outbreaks could be attributed to milk ingestion despite *T.gondii* was isolated in the milk of naturally infected cows.[19] The ingestion of raw milk has been considered as a possible source of the transmission of *T.gondii*,[18] and parasites were isolated, by mouse inoculation, in goat milk.[3] Goat milk is usually produced by small farms producers under poor conditions without pasteurization, only frozen, with low safety for contaminations,[15] but most milk sold in urban areas is pasteurized, a process that eliminates the *T.gondii* infectivity.[10]

These data also show that milk promoted higher infectivity and mortality, revealing probably better preservation or efficiency of *T.gondii* cysts when compared to PBS as diluent solution, even in the first periods of storage. This fact could be ascribed to nutrients in the milk that contributed for maintaining the cysts viable. Another explanation is related to the first steps of host invasion. The bradyzoites, the form found in the cysts, are resistant to the gastric digestion, a method for differentiating the tachyzoites.[12] Milk proteins can generate a competitive effect in the gastric juice, resulting in better infectivity of bradyzoites, both by reducing the acid content and allowing a more restrict pepsin digestion, as demonstrated in infected milk artificially exposed to gastric conditions.[16]

Homemade cheese was also a good vehicle for *T.gondii* cysts. The home processing of cheese involves bacterial acidification and coagulation of the casein, but this should be a mild process to maintain the palatability of the cheese. The clot could retain

the agent during washing but the entire process reduces the viability of the cysts, as demonstrated by the cessation of transmission after 10 days of storage in the refrigerator. Despite its infectivity, the disease was quite less intense in mice that ingested experimentally infected cheese, a fact that could explain the absence of related deaths on this form of transmission that could induce a less symptomatic disease in humans.

The cheese contamination could occur in two ways, first the exogenous one, associated with low hygiene conditions, allowing the contact of milk with oocysts from cats. Endogenous contamination of the milk in the mammary gland is facilitated by cellular exocytosis of milk secretion. During pre-lactation, relatively stable mammary cells could harbor *T.gondii* cysts. Those silent cysts could be secreted from the mammary gland cells by exocytosis, coated by host cell membranes, similar to milk fat globules secretion,[5] allowing milk contamination inside the gland. This contamination is worsened due to smaller concentration of proteolytic enzymes in the intestine of children and suckling animals, increasing the survival of *T.gondii* forms.[9]

Toxoplasmosis transmission by milk or fresh cheese, unpasteurized or inadequately processed, can be one of the important ways of contamination for this agent, as they areimportant food source in rural areas. Milk and cheese are one of the main sources of proteins for children, and cheese is a cheap and easy way of getting food proteins. Any reference about the viability of the cysts of *T.gondii* in fresh cheese was not found, just in milk, mainly of ovine.[6] The agent is destroyed after pasteurization when appropriately exposed to larger temperatures than 70ºC for 10 min, but pasteurization is sometimes not appropriate or the milk is processed in large volumes with some areas without adequate temperature, a frequent problem.[11] Cheese production after boiling is a time consuming and expensive process, especially in small farms, despite its efficiency in the elimination of this contaminant.

The present study showed that *T.gondii* cysts could survive in the milk and fresh derived cheese, which could be considered a potential source of *T.gondii* transmission, especially in rural areas where there's no pasteurization.

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