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Short communication

Survival of *Toxoplasma gondii* tachyzoites in simulated gastric fluid and cow's milk

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ABSTRACT

The protozoan parasite *Toxoplasma gondii* is one of the most important food-related pathogens worldwide. Besides contact to oocysts or ingestion of tissue cysts mainly by consumption of raw or undercooked meat from infected animals, raw milk is considered to be a risk factor and possible route of transmission for tachyzoites. This stage of the parasite is usually very sensitive to acidic pH and, therefore, considered unlikely to survive stomach passage. However, tachyzoites were shown to survive for several days in milk and there are also reports on transmission of toxoplasmosis via milk. Thus, the aim of the study was to examine retention of infectivity of tachyzoites in simulated gastric fluid (SGF) of different acidity and to elucidate whether addition of different shares of milk would affect survival of the parasites. Tachyzoites were exposed to SGF of pH 2.0 through 6.0 and their remaining infectivity was examined by cell culture. Furthermore, the impact on survival was investigated in different admixtures of milk to the SGF (25, 50, 75%) as well as in pure milk. Tachyzoites were shown to retain infectivity in SGF of pH 5.0 and 6.0 for at least 90 min while they were more sensitive to lower pH values. Admixture of milk resulted in extension of survival. The results support the hypothesis of tachyzoites to survive stomach passage and their retention of infectivity.

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1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects virtually all warm-blooded animals including humans (Dubey, 2010). Transmission of *T. gondii* to humans by tachyzoites is discussed to be an unlikely event in general (Tenter et al., 2000; Dubey, 2010) and is also discussed controversially in terms of milk. On the one hand, there are many indications that tachyzoites could be present in milk of infected animals (e.g. Sanger et al., 1953; Sanger and Cole, 1955; Beverley, 1959; Ishag et al., 2006; Dubey, 2010 (including additional references); Dehkordi et al., 2013; da Silva et al., 2015). On the other hand, tachyzoites were shown to be sensitive against low pH (Jacobs et al., 1960; Pettersen, 1979; Sharma and Dubey, 1981; Dubey, 1998) and may not survive in physiologic human gastric fluid which exhibits a pH of about 1–3 (Pocock et al., 2013). However, pH of gastric fluid will shift towards more or less neutral values due to intake of liquid and solid food depending on food composition (Savarino et al., 1988; Dressman et al., 1990). So, there may be a milieu where tachyzoites could still be infective since Dubey (1998) showed retention of infectivity of tachyzoites in neutralized artificial gastric fluid.

Several studies determined the consumption of milk as risk factor for toxoplasmosis (meta-analyzed by Boughattas, 2015) and some linked raw goat's milk consumption to actual human infections (Chiari and Neves, 1984; Dubey, 2010).

The aim of the present study was to gain experimental data on survival and infectivity of tachyzoites in simulated gastric fluid (SGF) of various pH values representing different constitutions. Furthermore, it should be examined whether addition of different shares of milk to SGF affects survival of tachyzoites to evaluate the probability of infection after oral uptake of milk contaminated with *T. gondii* tachyzoites and their stomach passage.

2. Materials and methods

2.1. Cell line and T. gondii strain

HEp-2 cells (CCVL RIE 141, Friedrich Loeffler Institute, Isle of Riems, Germany) were used for all experiments. The type II strain ME49 (Lunde and Jacobs, 1983) of *T. gondii* was kindly provided by the Institute of Parasitology of the University of Leipzig. The strain was maintained in HEp-2 cells in MEM with Earle's salts, 5 % fetal bovine serum (FBS), penicillin/streptomycin (media and

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supplements from PAA Laboratories, Austria). Free tachyzoites for experiments were prepared by centrifugation of cell culture supernatant (10 min at 185 × g), resuspension of the pellet in medium, and counting in a Neubauer chamber.

2.2. Simulated gastric fluid and milk

Simulated gastric fluid (SGF) was freshly prepared prior to respective experiments. It consisted of 2.6 g pepsin (806 U/mg; Sigma-Aldrich, Germany), 5.0 g NaCl (Carl Roth, Germany) and distilled water per 500 ml as described by Sharma and Dubey (1981). The pH-value was adjusted to 2.0, 3.0, 4.0, 5.0, and 6.0 by using $NaHCO_3$ (Merck, Darmstadt, Germany) or HCl (Carl Roth, Karlsruhe, Germany).

Commercially available UHT cow's milk with 3.5 % fat was mixed with simulated gastric fluid to gain 0, 25, 50, 75, and 100 % rates of milk.

2.3. Study design

In the first part of the study 500,000 tachyzoites were added to 50 ml prewarmed SGF of the desired pH in Erlenmeyer flasks and incubated for 15, 30, 60, or 90 min at 37 °C in a water bath. After incubation, SGF was centrifuged for 10 min at 185 × g to carefully pelletize tachyzoites. The pellet was resuspended in 3 ml medium containing 5 % FBS and then applied to the HEp-2 cells. Examinations for cytopathic effects (CPE) and DNA were done as described below.

In the second part of the study either 500,000 (high dose) or 10,000 (low dose) tachyzoites (Walsh et al., 1999) were added to 100 ml prewarmed SGF (pH 2.0), UHT cow's milk, or SGF premixed with UHT cow's milk at 25, 50, or 75 %. High dosage tachyzoites were incubated for 15, 30, 60, 90, or 120 min at 37 °C while low dosage tachyzoites were incubated up to 60 min. Subsequent procedure was analogous to the first study part.

One to seven cell cultures were examined per setting out of which one to four cultures were additionally examined by real-time PCR as described below.

2.4. Cell culture viability assay

Treated tachyzoite suspensions were administered to 80–90 % confluent HEp-2 cells in six-well plates. A positive (500,000 untreated tachyzoites), negative (no tachyzoites) and no-replication (500,000 tachyzoites, heat-treated for 30 min at 60 °C) control well were included on every six-well cell culture plate. After 24 h incubation, cells were first checked for CPE and medium was replaced. Afterwards CPE control was performed daily and medium was changed every two days. Final microscopic examination for CPE was done latest at five days post infection. Wells were considered positive (i.e. infected) when typical cytopathic effects were observed. These comprised an increase of detritus, presence of tachyzoite accumulations as well as free tachyzoites in the culture medium, and holes in the cell monolayer. If no such effects were visible, wells were scored negative.

2.5. Real-time PCR assay

To support cell culture findings, increase of *T. gondii* DNA was examined by real-time PCR. After microscopic examination, cells were washed three times with PBS (phosphate buffered saline), subsequently detached using accutase and pelleted by centrifugation (1450 × g, 5 min). The resulting pellet was resuspended in 200 µl PBS and DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), eluted in 100 µl PCR-grade water, and frozen at -20 °C until PCR examination.

Table 1
Survival of *T. gondii* in SGF at different pH values after different incubation times with 500,000 tachyzoites applied.

pH	15 min		30 min		60 min		90 min	
	culture[a]	PCR[b]	culture	PCR	culture	PCR	culture	PCR
6.0	n.d.	n.d.	5/5	2/2	4/4	2/2	4/4	2/2
5.0	1/1	n.d.	5/5	2/2	4/4	2/2	2/2	2/2
4.0	4/5	3/3	5/7	4/4	0/2	0/2	n.d.	n.d.
3.0	5/5	2/2	3/7	1/4	0/2	0/2	n.d.	n.d.
2.0	4/6	3/4	0/4	0/2	0/2	0/2	n.d.	n.d.

n.d. – not done.
[a] No. positive/no. assayed by cell culture.
[b] No. positive/no. assayed by real-time PCR.

The PCR targeted the 529 bp repetitive fragment (Homan et al., 2000) using primers 5′-CACAGAAGGGACAGAAGT-3′, 5′-TCGCCTTCATCTACAGTC-3′, and probe 5′-FAM-CTCTCCTCCAAGACGGCTGG-BHQ1-3′ (Edvinsson et al., 2006). A 27.5 µl PCR reaction consisted of PCR-grade water, 5.5 mM $MgCl_2$, 1x reaction buffer, 900 nM of each primer, 200 nM probe, 0.2 mM dATP, dCTP, dGTP, 0.4 mM dUTP, 0.01 U/µl UNG (uracil-N-glycosylase), and 0.025 U/µl Taq polymerase (AmpliTaq Gold, Life Technologies, Germany), and 2.5 µl sample DNA. Real-time PCR was performed on the StepOnePlus cycler (Applied Biosystems, Germany) and comprised initial UNG reaction for 10 min at 50 °C followed by 10 min denaturation at 95 °C and subsequent 45 cycles of denaturation (15 s, 95 °C) and annealing/elongation (60 s, 60 °C).

C_t values of cell cultures with heat-inactivated tachyzoites represent the maximum amount of detectable DNA present in samples without further parasite replication. Samples showing lower C_t values (< -2), indicating more initial DNA and thus *T. gondii* replication, were scored positive. Samples with C_t values which were between 0.5 and 2 lower than C_t values of the respective cell cultures with heat-inactivated tachyzoites were scored slightly positive. Samples with similar (±0.5) or higher C_t values were accordingly scored negative. All controls showed expected results.

3. Results and discussion

3.1. Survival of tachyzoites in SGF

Detailed cell culture and PCR results of tachyzoites in SGF of various pH values are listed in Table 1.

Tachyzoites died fast in SGF of low pH. We showed that they partially lost capability of cell culture infection in high acidic SGF (pH 2.0) within 15 min and were completely unable to infect cell cultures after 30 min. This is in accordance with findings of Dubey (1998) and Pettersen (1979), who reported inactivation of tachyzoites in HCl-pepsin (pH 1.0) within 15 min and in high acidic simulated gastric fluid after 25 min, respectively.

Retention of infectivity increased with higher pH, and was obtained partly for 30 min at a pH of 3.0 and 4.0 and for at least 90 min at a pH of 5.0 or 6.0. This general dependence of survival time on acidity of gastric fluid is in accordance with the findings of Wildführ (1956), however, such comparative analyses of survival and retention of infectivity at different pH have not been published, so far.

3.2. Impact of milk addition

The addition of 25, 50, or 75 % UHT cow's milk to SGF led to increased pH values of 4.8, 5.8, and 6.3, respectively. The pH of pure milk was 6.6. When 500,000 tachyzoites were added to pure SGF of pH 2.0, they were all inactivated within 15 min. Supplementation of as little as 25 % of milk to the acidic SGF and the accompanied increasing pH led to retention of infectivity for at least 120 min.

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