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The Viability and Infectivity of *Toxoplasma gondii* Tachyzoites in Dairy Products undergoing Food Processing

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ABSTRACT

This study was undertaken to evaluate the viability and infectivity of the tachyzoites in different temperatures and times of milk by bioassay methods. Tachyzoites of RH strain were transferred into sterile milk and kept in different temperatures (4, 20 and 37°C) for different time intervals (10 and 30 min) and their viability was evaluated by vital stain (methylene blue) and the infectivity by intra-peritoneal and oral inoculation to different groups of inbred BALB/C mice (2-4 mice in each group obtained from Pasteur Institute, Tehran, Iran). Tachyzoites in saline were intra-peritoneally inoculated to inbred BALB/C mice as positive controls. All animals were kept in Laboratory Animal Center of Shiraz University of Medical Sciences in Shiraz, southern Iran. The touch smear of spleen and liver of dead mice were stained by Giemsa and were parasitologically evaluated. Eighty percent of tachyzoites were stained by methylene blue indicating identical parasite viability. All mice died after intra-preritoneal inoculation of tachyzoites present in sterile milk. Only one out of eight mice which was orally inoculated remained alive. All mice fed by tachyzoites in saline died. Duration of mice vitality was more in oral route than peritoneal inoculation ($n_1 = 11$, $Mean_1 = 9.55$ days and $n_2 = 17$, $Mean_2 = 6.65$ days, $p = 0.001$). Tachyzoites in drinks and foods undergoing heating up to 37°C for 30 min such as milk can be a possible route of transmission of toxoplasmosis to human. Therefore strict public health measures should be carried out to prevent transmission of disease in high risk areas.

Key words: Toxoplasmosis, food processing, viability, infectivity

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan that infects humans and a wide range of warm-blooded animals (Smith and Reduck, 2000). The parasite is known to cause congenital disease and abortion both in humans and livestock (Dubey, 2004; Sharif *et al*., 2007). Maternal toxoplasmosis during early pregnancy of human may leads to death of fetus or cause chorioretinitis, hydrocephaly, microcephaly and jaundice in neonates (Higa *et al*., 2010; Joynson and Wreghitt, 2001). However, acquired toxoplasmosis has mild flu like symptoms in immunocompetent humans, but the disease is severe in immunocompromised persons, for example 23% of HIV-positive patients will develop toxoplasmic encephalitis (Alvarado-Esquivel *et al*., 2010; Davarpanah *et al*., 2007; Oksenhendler *et al*., 1994).

The prevalence of toxoplasmosis in Fars Province, Southern Iran was reported 20.24% in bovine (Asgari *et al*., 2010) 26.5% in ovine, (Asgari *et al*., 2009) 14.02% in caprine, (Asgari *et al*., 2007) and 36.1% in chicken (Asgari *et al*., 2006). Human seropositivity in North and South of Iran using indirect fluorescent antibody method was shown to be 55 and 29%, respectively and a seroprevalence of 51.8% was reported in all regions of the Country (Ghorbani *et al*., 1978; Sedaghat *et al*., 1978; Assmar *et al*., 1997). Infections in man occurs because of ingestion of oocysts from the feces of contaminated cats and/or dogs (Clementino *et al*., 2007; Jittapalapong *et al*., 2007) and by ingesting raw or under-cooked products containing tissue cysts (Garcia *et al*., 2006; Gilot-Fromont *et al*., 2009). Milk may serve as a potential source for human toxoplasmosis (Ertug *et al*., 2005; Jones *et al*., 2009; Riemann *et al*., 1975; Skinner *et al*., 1990). Additionally, tachyzoites of *T. gondii* were found in the milk of several species, including sheep, goats, cows, mice and cats (Dubey, 1998; Inpankaew *et al*., 2010; Powell *et al*., 2001; Tenter *et al*., 2000).

Since bovine milk as a dietary food may be contaminated by *Toxoplasma gondii* tachyzoites, this study was undertaken to evaluate the viability and infectivity of tachyzoites in milk by bioassay methods.

MATERIALS AND METHODS

Inbred BALB/C mice were obtained from Pasteur Institute, Tehran, Iran at 10-14 weeks of age. Animals were kept at temperature of 22°C and 40-50% relative humidity in Laboratory Animal Center of Shiraz University of Medical Sciences in Shiraz, southern Iran. The protocol of all procedures and sacrifice were identical for all animals. During the experiments from June to December 2008, the animals were housed in cage and maintained under controlled environmental conditions (21±2°C, 65-70% RH and a balanced diet with free access to food and water). All experiments and the sacrifice procedure were adhered to the same guidelines under supervision of Animal Care Committee of Iran Veterinary Organization.

Toxoplasma gondii tachyzoites of the RH strain were obtained from Tehran University of Medical Sciences, Iran. Dye Test was based on the fact that live *Toxoplasma gondii* tachyzoites can actively absorb methylene blue dye from the culture medium and the dead ones remain colorless. Tachyzoites were obtained by mouse inoculation. *T. gondii* tachyzoites were injected intraperitoneally in Balb/C mice and the animals were killed 48 h after injection. Tachyzoites were collected after repeated flushing of the peritoneal cavity with phosphate-buffered saline (PBS; pH 7.4) and adjusted to a concentration of 25×10^6 mL^{-1}. Fifty micro litre of the sample (1.25×10^6 parasites) was transferred into eppendorf tubes, which already contained 150 μL of sterile milk or saline solutions. Three series of these tubes incubated in different temperatures (4, 20 and 37°C) for different times (10 and 30 min). After this incubation, 200 μL of methylene blue solution was added to a serial of the tubes and were incubated for 30 min. The viability of tachyzoites was evaluated by the percent of the stained tachyzoites under an invert microscope at x400 magnification.

The infectivity was compared by intra-peritoneal and oral inoculation of the tachyzoites transferred in milk to different groups of inbred Balb/C mice (2-4 mice in each group and 50 μL of milk containing 3×10^5 parasites for each mouse). All mice died and their touch smears of spleen and liver were stained by Giemsa and observed under light microscopy (X400) for detection of parasite. Tachyzoites in saline were also intra-peritoneally inoculated to mice as positive controls. Intra-peritoneal and oral inoculation of 2 mL of uncontaminated milk was considered as negative control. The data were analyzed using SPSS software (version 11.5, Chicago, IL, USA) by non-parametric test of Mann-Whitney. A $p<0.05$ was considered statistically significance.

RESULTS AND DISCUSSION

82.5 to 88% of tachyzoites in all media were stained by methylene blue whereas 93.5% of them in control group absorbed the dye and no significant difference was observed between the two groups indicating to the same rate of viable parasite.

All mice (n = 10) that were intra-preritoneally inoculated by tachyzoites with sterile milk died. Only one out of eight which was orally inoculated with milk was alive. Moreover, all of mice which were fed by tachyzoites in saline died (Table 1). Duration of the mice vitality in oral inoculation with Mean = 9.55 days was more than it in peritoneal inoculation with Mean = 6.64 days ($n_1 = 11$, $n_2 = 17$ and $p = 0.001$ by using Mann-Whitney test).

In this study, any significant differences between applied temperatures and times were not seen. The animals which were intraperitoneally and orally inoculated by bovine milk were not

Table 1: Infectivity of *Toxoplasma gondii* inoculations in different temperatures and times

Inoculation	Groups (No.)	Temperature (°C)	Incubated duration (min)	Alive (till one month)	Time of mice death (Day)
Only sterile milk	Intra-peritoneal (3)	37	10	+	-
				+	-
				+	-
Tachyzoite with sterile saline	Intra-peritoneal (3)	37	30	-	6
				-	6
				-	7
Tachyzoite with sterile saline	Intra-peritoneal (2)	20	30	-	6
				-	7
Tachyzoite with sterile saline	Intra-peritoneal (2)	4	30	-	6
				-	7
Tachyzoite with sterile milk	Intra-peritoneal (4)	4	10	-	6
				-	7
			30	-	8
				-	7
Tachyzoite with sterile milk	Intra-peritoneal (4)	20	10	-	7
				-	8
			30	-	6
				-	6
Tachyzoite with sterile milk	Intra-peritoneal (2)	37	30	-	6
				-	7
	Oral (3)	20	10	+	-
				+	-
				+	-
Tachyzoite with sterile milk	Oral (4)	4	10	-	11
				+	-
			30	-	9
				-	12
Tachyzoite with sterile milk	Oral (4)	20	10	-	7
				-	9
			30	-	7
				-	11
Tachyzoite woth saline	Oral (4)	20	10	-	8
				-	9
				-	10
				-	12

Values in parenthesis are no of groups, + = Alive, - = Died

shown clinical signs. The sources of infection for humans, worldwide, vary greatly with culture, ethnic, geographical location and food habits differences. Food animals such as pigs, sheep and goats have a highest frequency of tissue cysts in comparison with other animals. The many factors such as management and hygienic standards in breeding livestock, density of cats and environmental conditions are effective on the acquisition of *T. gondii* oocysts (Tenter *et al*., 2000). The association between infection and unpasteurised milk or milk products was unexpected (Cook *et al*., 2000). However, the milk of goat may serve as a potential source for human toxoplasmosis (Chiari and Neves, 1984; Ertug *et al*., 2005; Jones *et al*., 2009; Sacks *et al*., 1982; Skinner *et al*., 1990). *T. gondii* tachyzoites have been isolated from goats' milk and cows' colostrums (Dubey, 1988; Hiramoto *et al*., 2001; Jones *et al*., 2009).

Toxoplasma gondii was detected in the milk of five of six experimentally infected cats by either bioassay or PCR (Powell *et al*., 2001). Generally, Tachyzoites are not considered an important source of oral transmission of *T. gondii* because they are rapidly killed outside the host and because they are considered sensitive to proteolytic enzymes (Powell *et al*., 2001).

Conversely, tachyzoites were recently shown to survive up to 2 h in pepsin solutions and adult cats become infected when high numbers of tachyzoites are given orally (Dubey, 1998).

Pettersen, (1984) suggested that when the milk from lactating mother mice was exposed to HCl, that some of the parasites present were "acid-resistant" cystozoites. Furthermore, infectivity of bradyzoite in bovine milk was maintained even after storage for 20 days at refrigerator temperatures (Hiramoto *et al*., 2001). Moreover, Walsh *et al*. (1999) reported tachyzoites of RH strain survived for 3-7 days in goat milk at 4°C while this strain survived for up to 14 days in Hank´s Balanced Salt Solution (HBSS) at this temperature.

Hence there is an obvious risk of acquiring toxoplasmosis by consuming milk from infected donors. Alternatively, infection by tachyzoites might occur by penetration of the oropharyngeal mucosa (Sacks *et al*., 1982). Fusco *et al*. (2007) demonstrated that contamination of milk should not be underestimated since it can represent a critical point in food safety. Unhygienic water supplies were other sources of infection which was also reported Ertug *et al*. (2005).

This study showed that tachyzoites in all media were stained by methylene blue which indicates the parasite viability. Considering the our mentioned results, tachyzoites are not only resistant to milk media and conserve their infectivity for up to 30 min but were infective due to oral transmission. Moreover, the higher duration of the mice vitality in parasite oral inoculation showed this route has less virulent but is however infective.

Tachyzoites in drinks and foods undergoing heating up to 37°C for 30 min such as milk can be a possible route of transmission of toxoplasmosis to human. Therefore, strict public health measures should be carried out to prevent transmission of disease in high risk areas.

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