



Milk as a Non-Invasive and Alternative Sample for Serum in the Diagnosis of Animal Toxoplasmosis: A Systematic Review

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(Received 04 Aug 2021; accepted 15 Nov 2021)

Abstract

Background: Toxoplasmosis is one of the most important zoonotic parasitic diseases worldwide with a high impact on human and animal health. Body fluids such as milk are used to diagnose many parasitic diseases, including toxoplasmosis. Therefore, this study aimed to investigate the role of milk as a non-invasive and alternative sample for serum in the diagnosis of animal toxoplasmosis.

Methods: Five English-language databases (ScienceDirect, PubMed, ProQuest, Scopus, and Web of Science) were explored for published articles before Dec 2020.

Results: In total, 42 out of 2256 published articles were included in this systematic review. In 21 articles, serum and milk samples were evaluated simultaneously with serological or molecular tests, and the results were compared. The results of descriptive studies and a review of nine experimental studies showed that milk could be used as a non-invasive and alternative sample for the serum in the diagnosis of toxoplasmosis.

Conclusion: Due to the relatively high prevalence of *Toxoplasma gondii* (*T. gondii*) infection in milk, consumption of raw milk from infected animals can be a potential source of human infection and a significant threat to public health. On the other hand, due to the ease and cheapness of collecting milk samples, the use of milk is recommended for the diagnosis of toxoplasmosis.

Keywords: *Toxoplasma gondii*; Non-invasive samples; Milk; Serological; Molecular

Introduction

Toxoplasma gondii is a zoonotic apicomplexan that can infect all warm-blooded animals (1, 2). This parasite has a cosmopolitan distribution (3) and about one-third of the world's population have antibodies to this intracellular protozoan (4). The definitive hosts, including domestic cats and other felids, contaminate the environment by shedding the unsporulated oocysts through feces (1,

5). Humans and many animals, as the intermediate hosts, could be infected by eating food contaminated with oocysts shed by cats or by consumption of tissue cysts of the parasite after eating raw or undercooked meat (2). *Toxoplasma* infection in immunocompetent humans is generally asymptomatic (5), but in immunocompromised individuals may cause more intense consequences



such as chorioretinitis, encephalitis, and congenital birth defects (6). In a pregnant woman who was first infected by the parasite, *T. gondii* reaches the fetus via the placenta (2). Treatment of toxoplasmosis requires appropriate diagnostic tools and specimens. The diagnosis of *T. gondii* infection may be established by serological, biological, histological, and molecular methods, or by a combination of some of them (7).

Today, most invasive specimens such as serum or cerebrospinal fluid are used for the diagnosis of toxoplasmosis. Many studies have discussed the use of non-invasive specimens such as milk (8), urine (9), and tear (10) to diagnose *T. gondii* infection. Milk samples have certain advantages over serum samples. The collection of serum samples is more invasive and difficult than the collection of milk samples. In addition, serum sampling requires qualified veterinary expertise and specific equipment as well as may cause stress in animals. Therefore, we aimed to evaluate whether milk is a suitable sample to replace serum according to the available literature.

Methods

Study Design and Protocol Registration

This study was accomplished according to the preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines (11). The study protocol has been registered in PROSPERO with the registration number CRD42021236783.

This article is an approved plan from Student Research Committee of Mazandaran University of Medical Sciences, Sari, Iran (number: 9032). The code of ethics of this plan is IR.MAZUMS.REC.1400.028.

Search Strategy

To evaluate data on the role of milk in the diagnosis of toxoplasmosis, five English databases (ScienceDirect, PubMed, ProQuest, Scopus, and Web of Science) were searched from 1990 to 2020. Search terms used alone or in combination were *Toxoplasma gondii*, *T. gondii*, toxoplasmosis,

non-invasive samples, milk, diagnosis, detection, serological, and molecular.

Inclusion and Exclusion Criteria

The articles were included according to the following criteria: 1) articles performed on milk as a non-invasive sample by serological and molecular methods, 2) the articles performed on animals and humans, and 3) original papers and short communications with available full texts in only English language. Duplicate articles, articles in languages other than English, non-original publications as well as thesis and conference papers were excluded.

Study Selection and Data Extraction

At first, all the recovered articles were imported to EndNote. Then the two authors independently assessed the selected articles. The third author resolved any disagreements in the included studies by arbitration and discussion with other authors. The relevant studies data were imported into a Microsoft Excel datasheet. The data extracted from every study included first author, host, type of sample, sample size, antibody type, as well as the molecular and serological results of milk and serum.

Results

Study Characteristics and Search Results

The preliminary search in the five databases yielded 2256 relevant studies. After discarding the duplicate articles, 2136 publications remained. In the next stage, two researchers reviewed the titles and abstracts of full texts independently. Eventually, 54 articles were chosen for the precise evaluation of full texts, of which 43 papers were eligible. Out of 43 studies, one study was excluded due to the lack of reporting of positive samples with *T. gondii* (12). Finally, 42 studies were included in this systematic review. Nine articles had an experimental design and 35 studies had a descriptive design. Two articles contained data used in both designs (experimental and descriptive design) (13, 14) (Fig. 1).

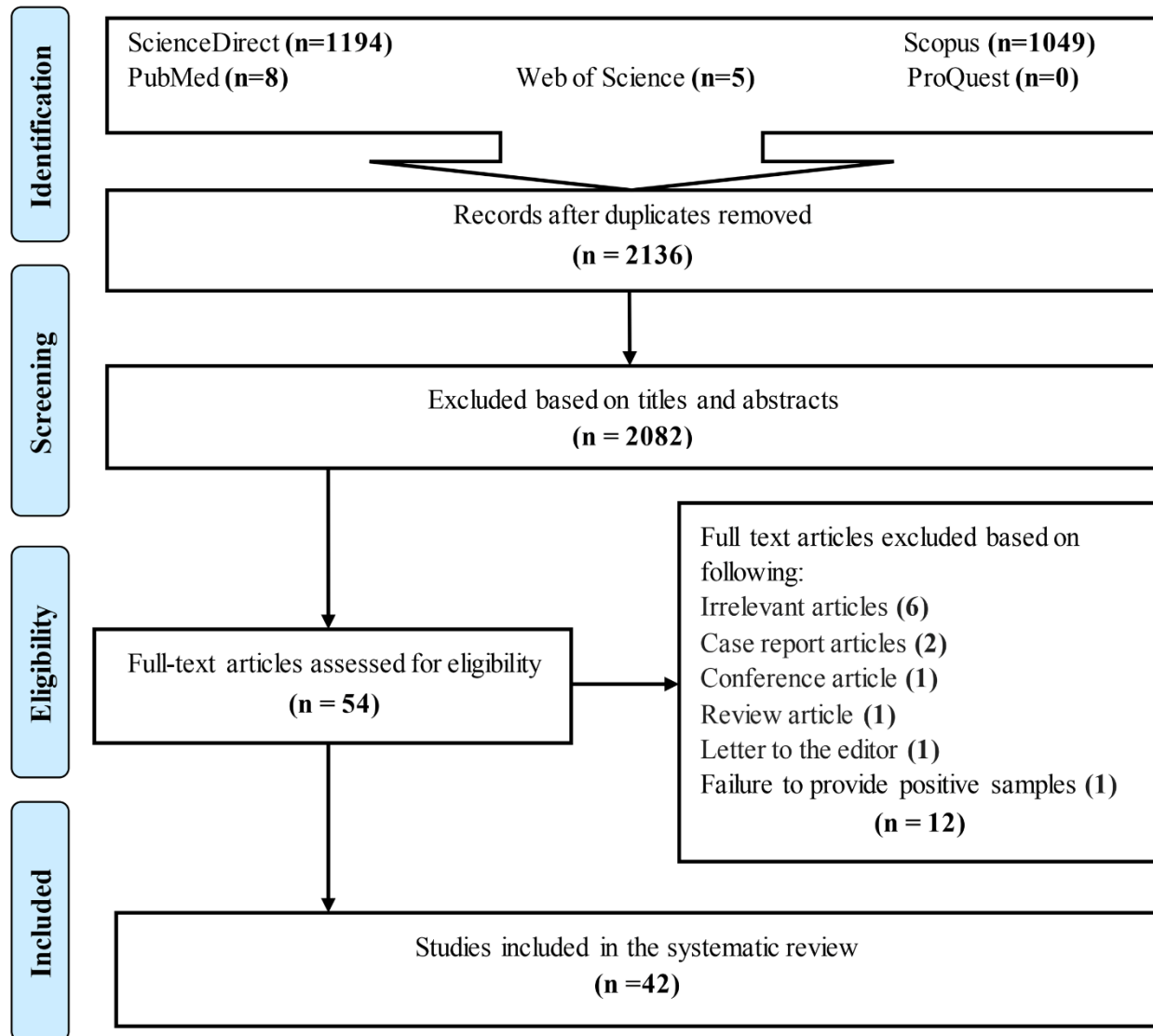


Fig. 1: Flow diagram of the study design process

Results of Descriptive Studies on Milk and Serum Samples

The selected articles were performed in the UK (15), Italy (16-23), Slovak Republic (24, 25), Tunisia (26), Poland (27, 28), Malaysia (6), Mongolia (29), China (8), Iran (14, 30-33), Brazil (34-39), Egypt (13, 40-44), and Iraq (45).

In 28 descriptive studies, 6576 milk samples were evaluated using different molecular techniques such as polymerase chain reaction (PCR), real-time PCR, reverse-transcriptase PCR (RT-PCR), nested-PCR, and loop-mediated isothermal am-

plification (LAMP) for identification of the *T. gondii* DNA that 332 out of these samples (5.05%) were positive. In addition, the number of 2453 milk samples in 9 articles was assessed using various serological techniques such as modified agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA), latex agglutination test (LAT), indirect fluorescent antibody test (IFAT), indirect hemagglutination test (IHAT), indirect immunofluorescence reaction (IIR), direct agglutination test (DAT), dye test, and rapid test cassette for detection of the *T. gondii* and 557

cases (22.7%) were positive. Moreover, 21 studies were conducted on serum samples by serological techniques including 6106 samples, out of which 1517 were positive for toxoplasmosis (24.84%),

and five studies containing 302 serum samples, out of which 36 were positive for toxoplasmosis (11.92%) by molecular techniques (Table 1).

Table 1: Baseline of descriptive studies on milk and serum samples included in this systematic review

<i>First author</i>	<i>Host (s)</i>	<i>Sample (s)</i>	<i>Methods</i>	<i>Detected antibodies</i>	<i>Sample size (n)</i>	<i>Serological results of milk n (%)</i>	<i>Serological results of serum n (%)</i>	<i>Molecular results of milk n (%)</i>	<i>Molecular results of serum n (%)</i>
Skinner	Goat	Milk	IHAT, Dye test, and bio-assay	IgM and IgG	6	--	4 (66.66)	--	--
Fusco	Sheep	Milk and serum	IFAT and PCR	IgG	1170	--	333 (28.5)	4/117 (3.4)	--
Ludmila	Goat	Milk and serum	ELISA, PCR, and nested-PCR	IgG	87	--	43 (49.43)	14/43 (32.56)	--
Camossi	Ewe	Milk and serum	PCR and MAT	IgG	139	--	70 (50.36)	7 (5.03)	--
Abdel-Rahman	Goat	Milk and serum	IHAT	IgM and IgG	73 milk and 182 serum	43 (58.90)	77 (42.30) [IgG: 55 (30.22) and IgM: 22 (12.09)]	--	--
Bezerra	Goat	Milk and serum	IIR and PCR	IgG	248 milk and 248 serum	--	56 (22.58)	15 (6.05)	--
Dehkor di	Bovine, ovine, caprine, buffalo, and camel	Milk	Cell line culture, cat bio-assay, ELISA, and PCR	IgG	889	41 (4.61)	--	46 (5.17)	--
Manci-anti	Goat	Milk and serum	MAT and nested-PCR	IgG	77 milk and 127 serum	--	77 (60.62)	10 (13)	10 (13)
Tavassoli	Sheep and goats	Milk	PCR	--	625	--	--	19 (3.04)	--
Ahmed	Goat, sheep, and cow	Milk	PCR	IgM and IgG	150	--	--	5 (3.33)	--
Da Silva	Ovine	Milk and serum	IFAT	IgG	40 milk and 40 serum	10 (25)	18 (45)	--	--
Da Silva	Sheep and goats	Milk and serum	IFAT and PCR	IgG	243	--	16 (6.58)	5 (2.02)	--

Luptakova	Sheep	Milk and serum	ELISA and real-time PCR	IgM	80 milk and 80 serum	--	25 (31.25)	9 (11.25)	5 (6.25)
Mancianti	Donkey	Milk and serum	IFAT and nested-PCR	IgG	44 milk and 44 serum	--	11 (25)	3 (6.81)	6 (13.63)
Martini	Donkey	Milk and serum	IFAT and PCR	IgG	18 milk and 18 serum	--	4 (22.22)	4 (22.22)	4 (22.22)
Sadek	Sheep and goats	Milk	Microscopic examination, LAT, and PCR	--	105	41 (39)	--	8 (16)	--
De Santana Rocha Amairia	Sheep	Milk and serum	PCR and IFAT	IgG	275	--	114 (41.5)	18 (6.5)	--
	Goat	Milk and serum	ELISA and nested-PCR	IgG	77 milk and 77 serum	--	24 (31.2)	6 (7.8)	--
Attia	Goat	Milk and serum	ELISA and MAT	IgG and IgM	600 milk and 600 serum	ELISA:120 (20) and MAT:110 (18)	ELISA:132 (22) and MAT:120 (20)	--	--
Cisak	Sheep, goat, and cow	Milk	PCR	--	119	--	--	12 (10.1)	--
Ossani	Ewes	Milk	IFAT, PCR, and bioassay	IgG	42 ewes or 108 milk samples	--	42 (100)	13/42 (30.95) or 13/108 (12.04)	--
Razmi	Cattle	Milk	ELISA	--	123	14 (11.38)	--	--	--
Sroka	Goat	Milk and serum	DAT, real time PCR, and nested-PCR	IgG	60 milk and 73 serum	--	51 (70)	Real time PCR: 39 (65) and nested-PCR: 26 (43)	--
Vismarra	Sheep	Milk	PCR and real time PCR	--	21	--	--	PCR: 1/10 (10) and real time PCR: 9/10 (90)	--
Abdullah Alipour	Cow	Milk	PCR	--	14	--	--	0 (0)	--
	Bovine, ovine, caprine, buffalo, camel, and donkey	Milk and traditional dairy product	Nested-PCR	--	880	--	--	70 (7.95)	--
Gazzonis	Goat	Milk and serum	ELISA	IgG	383 milk and 383 serum	242 (63.18)	245 (63.96)	--	--
Saad	Sheep, goat, and	Milk	ELISA and qPCR	IgG	90	46 (51.11)	--	2 (4.34)	--

Gazzo-nis	camel Goat	Milk and serum	ELISA and PCR	IgG	30	--	19 (63.3)	8/30 (26.66) or 13/63 (20.63)	--
Iacobucci	Sheep, goats, and Bactrian camels	Milk	Nested-PCR	--	126	--	--	9 (7.14)	--
Mo-hamed	Ewes, does, and cows	Milk and serum	ELISA	IgM and IgG	150 milk	79 (52.7) [IgM: 56 (37.3) IgG: 0 (0), and IgM & IgG: 23 (15.3)]	--	--	--
A.A. Abadi	Buffalo, cow, sheep, goat, donkey, and camel	Milk and dairies	Nested-PCR	--	230 raw milk	--	--	18 (4.86) [milk: 14 (6.08) and dairies: 4 (2.85)]	--
Alkanaq	Goats and pregnant women	Milk and serum	PCR and rapid test cassette	IgM and IgG	150 milk and 150 serum	--	33 (22)	6/150 (4)	14/33(42.42)
Ranucci	Ewe	Milk and cheese	LAMP and RT-PCR	--	16 milk and 32 cheese	--	--	LAMP [milk:16 (100) and cheese: 32 (100)] and RT-PCR [milk: 16 (100) and cheese: 0 (0)]	--
Wang	Cattle	Milk and serum	ELISA and semi-nested PCR	IgG	2092 milk and 2092 serum	--	123 (5.88)	22 (1.05)	--

Results of Experimental Studies on Milk Samples

These studies were conducted in France (46), the USA (47, 48), Brazil (49, 50), Sudan (51), Egypt (13), Iran (14), and Greece (52). The animals used in these studies were mice (46, 49), cat (47), she-camel (51), rat (50), goat (13, 14, 48, 52), ovine (14, 52), bovine, buffalo, and camel (14). *Toxoplasma* antibodies were determined using ELISA, LAT, and MAT. Furthermore, molecular methods used in this study were PCR and nested PCR. The strains of the *T. gondii* that were used to infect the animals in the experimental studies were

included 76K (46), Mozart, Maggie (47), ME-49 (47, 49), and BTU4 (50) as well as TgGoatUS26 and GT1 (48). The amplification of B1 (47, 50) and Ncl8s-ITS1 (48) genes of the *T. gondii* was performed in three studies. *T. gondii* infection was evaluated in milk by flotation technique for detection of *Toxoplasma* oocysts in fecal samples of cats, serological and molecular methods as well as bioassay following oral infection of the animal model (mice, cat, she-camel, and goat) with different strains of the parasite (46-48, 50-52) or infection of animal models (cats and mice) through fed with the milk from infected animals

with *T. gondii* (13, 14, 49). Moreover, the transmission of *T. gondii* infection through contami-

nated milk with *Toxoplasma* to offspring was examined (51) (Table 2).

Table 2: Baseline of experimental studies on milk samples included in this systematic review

First author	Host (s)	Sample size (n)	Type sample (s)	Methods	Detected antibodies	Results
Chardes	Mice	6 to 10 for each experiment	Milk, blood, and intestinal secretions	ELISA and western blotting	IgA, IgG, and IgM	The IgA antibody response began earlier in serum and milk than in intestinal secretions. Nevertheless, at the intestinal level, the IgA antibody response was the first humoral response, whereas, in milk and serum, IgA, IgG, and IgM production all commenced at the same time after infection. Initially, the IgA and IgM antibody titers in serum and intestinal secretions rose in parallel but the IgM antibody titers peaked earlier than the IgA antibody titers.
Powell	Cat	6	Milk	PCR and bioassay	--	The milk of one cat was bioassay positive only, one was PCR positive only, and three were bioassay and PCR positive.
Hiramoto	Mice	Groups of eight mice	Milk and cheese	ELISA, western blot, and histology	IgG	The infectivity of cysts of the ME-49 strain was maintained in the milk even after storage for 20 days at refrigerator temperatures.
Ishag	She-camel	3	Milk	LAT, histopathology, and bioassay	IgG	Tachyzoites and cysts were detected in the brains of all inoculated mice (6/9) and suckling calves (2/3). <i>Toxoplasma</i> antibodies were detected in the sera of mice and calves.
Costa	Rat	18	Milk	PCR, IFAT, MAT, bioassay, and PCR	--	Rat milk samples were PCR-positive, pups were serum reactive to <i>T. gondii</i> and tissue samples presented positive DNA results through PCR.
Abdel-Rahman	Goat	2 (milk from IgG seropositive goats and milk from IgM seropositive goats)	Milk	Bioassay in cats	--	Experimental infection in cats showed that only one cat out of 4 given milk from IgG seropositive goats (chronically infected) shed oocysts 5 days post-infection, whereas all 4 cats given milk from IgM seropositive goats (acutely infected) shed oocysts in their feces 5-7 days post-infection.
Dehkordi	Bovine, ovine, caprine, buffalo, and camel	51 (bovine: 8, ovine: 13, caprine: 8, buffalo: 7, and camel: 5)	Milk	Bioassay in cats	--	The oocyst of <i>T. gondii</i> was detected the feces of all 51 cats.
Dubey	Goat	8	Milk and cheese	MAT, bioassay in mice and cat, and nested-PCR	--	By mouse bioassay, <i>T. gondii</i> was detected in milk from all eight goats. With respect to the infectivity of cheese, <i>T. gondii</i> was detected in mice inoculated subcutaneously with cheese stored for 3 days at 4 °C but not in any mouse inoculated orally with the cheese suspension. One cat fed cheese shed oocysts 7 to 11 days after consuming

Lafi	Sheep and goat	36 [sheep: 18 (12 infected and 6 control) and goats: 18 (12 infected and 6 control)]	Milk	ELISA and PCR	--	cheese. Also, of the 20 samples of uncentrifuged milk from all four goats, 18 were positive for <i>T. gondii</i> DNA. All infected animals started to show an increase in the antibody titers on day 14 after infection and continued to rise steadily until day 60 of infection and then started to decline. <i>T. gondii</i> DNA was detected in tissue samples (95%) collected from aborted fetuses. <i>T. gondii</i> DNA was detected in 94% of blood samples that were collected from infected animals and live newborn lambs and kids. <i>T. gondii</i> DNA was detected in blood on the 3th day after infection in all infected animals and continued for 21 day after infection. PCR detected <i>T. gondii</i> DNA in maternal blood of infected animals 3-5 days before abortion occurred. Eighty-eight percent pre-colostral udder secretion and 12.5% of colostrum and milk samples collected during the 21 days period following the infection were PCR positive. No <i>T. gondii</i> DNA was detected in tissues, milk samples of the control groups or in milk samples obtained from infected animals after 28 days of infection.
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Discussion

The current systematic review shows the value of raw milk instead of serum for diagnosis in humans and animals around the world. According to Table 1, the lowest and highest seroprevalence rates of anti- *T. gondii* antibodies in milk samples were related to the studies as 4.61% (41/889) (14) and 63.18% (242/383) (22). Additionally, the lowest and highest prevalence rates of *T. gondii* in milk samples using molecular methods were observed in studies as 0% (0/14) (6) and as 100% (16/16) (23). Differences in prevalence may be attributed to different climatic characteristics, the examined populations, sensitivity and specificity of detection techniques, cultural, hygienic, and nutritional habits, breeding conditions and management of animals, immune status, the timing of infection, the genetic composition of the host and the organism, or distribution and behavior of cats (38, 40, 53-56). The lack of evaluation of these factors in most eligible studies can be considered a basic gap.

T. gondii can be a source of infection for humans and animals due to the consumption of meat and milk from infected animals. This pathogen causes

significant human and animal health problems (57). Some researchers have demonstrated the milk of sheep, goats, cattle, buffalo, camel, donkeys, and mice have tachyzoites, which favor vertical transmission (31, 35, 39, 43). Tachyzoites can survive in goat's milk at 4 °C for about three to seven days (58). In addition, the infectivity of cysts persisted in cow's milk even after 20 days of refrigerated storage (49). These studies confirm the possibility of human infection due to the consumption of raw milk as well as the possibility of tachyzoite shedding in milk. In addition, an experimental study confirmed the transmission of *T. gondii* to milk and offspring in rats infected with the parasite (50). Reactivation of tissue cysts during an acute infection or low immunity during pregnancy results in the release of tachyzoites in milk (33, 38). However, tachyzoite is not an important source of oral transmission of *T. gondii* because they are sensitive to the proteolytic enzyme in milk and are immediately destroyed by gastric proteolytic enzymes (54). Gastric enzymes easily destroyed tachyzoite; however, due to the low concentration of gastric enzymes, the consumption of tachyzoite in milk, especially in infants, leads to infection (59). Moreover, the sur-

vival of tachyzoite in acid pepsin solution for up to 2 hours can support the achievement of infection via the consumption of milk and other foods (60). Another explanation is that infection may occur by penetration of tachyzoite into the mucosal tissues before reaching the stomach (59, 61, 62). Likewise, the transmission of *T. gondii* tachyzoite in milk is attributed to suckling trauma and tissue cyst excretion (63). Endogenous contamination of the milk and excretion of *T. gondii* tachyzoite in the mammary gland is facilitated by cellular exocytosis of milk secretion (64). During pre-lactation, cysts of *T. gondii* could harbor relatively stable mammary cells. Silent cysts are secreted from mammary gland cells by exocytosis, similar to the secretion of milk fat globules, which are covered by the host cell membrane, and then contaminate the milk inside the gland. Lower concentrations of proteolytic enzymes in the intestines of children and suckling animals lead to worsening of infection and increasing the survival of *T. gondii* forms (65).

The diagnosis of *T. gondii* infection in milk samples is usually based on serology, PCR, protein analysis, cell line culture, and bioassays (66, 67). Serological tests are mostly used as a preliminary test to detect the parasite, while molecular techniques such as PCR are the best choice for diagnosis of *T. gondii* infection because it has shown higher accuracy, sensitivity, and specificity than other diagnostic methods (68, 69). Seroconversion of this parasite is complex and may involve many factors. In the initial phase of infection, when the amount of antibody is not yet sufficient for diagnosis in serology methods, the animal may be PCR positive for milk and serologically negative (35). The presence of DNA of *T. gondii* in milk does not ensure the viability of the parasite in the sample. PCR has advantages over traditional methods such as bioassay and there is a good correlation between PCR and bioassay (70). The role of drinking cow's milk in the epidemiology of toxoplasmosis is small (65), while milk, regardless of the type of animal, especially if consumed raw, can be considered a potential source of infection (59, 71). Moreover, the long persistence of *T. gondii* cysts in infected bovine milk

and homemade fresh cheese under regular refrigeration conditions (49). Finally, raw milk may play a role in the epidemiology of *T. gondii* infection. On the other hand, various studies proposed the use of non-invasive samples such as milk as an alternative to serum samples in the diagnosis of *T. gondii* due to easier collection and storage of milk, no need for expert staff for sampling, improvement of animal welfare and reduction of productivity due to stress, and collection of sufficient quantities of the sample for analysis at the lowest cost (41, 72). Serum antibodies in an endemic area indicate a past or present invasive disease, while the presence of antibodies in milk indicates the presence of an active infection that reflected local antigenic stimuli to infection and contributes to epidemiological studies of the disease (73). About the quantity of milk, levels of antibodies in serum were higher than in other body fluids such as milk (74). There were several limitations in the present systematic review as follows: 1) a limited number of studies have examined the milk as a non-invasive and alternative sample for serum in the diagnosis of toxoplasmosis; 2) the use of English language articles due to lack of resources for translation; 3) the use of various diagnostic methods without equal specificities and sensitivities; 4) lack of evaluation of various risk factors such as type of animal, breed, and sampling season; 5) diagnosis of *T. gondii* in milk samples is possible only in female animals and in lactation seasons, especially in herbivores, and this limitation reduces the generality of the results of *T. gondii* infection with this sample; and 6) in most studies, toxoplasmosis was diagnosed in animal serum by the determination of antibodies and simultaneously in milk by serological or molecular methods; the discrepancy in the results of the original articles (in some articles the prevalence of *T. gondii* was higher in the serum sample and in others in the milk sample) is one of the limitations of this study.

Conclusion

To the best of our knowledge, this is the first systematic review providing a general view of the role of milk samples in the diagnosis of *T. gondii* infection. The data obtained in this study indicate the possibility of using milk as a substitute for serum in the diagnosis and screening of toxoplasmosis in animals. There is still a scarcity of studies investigating the role of milk as an alternative sample for serum in the diagnosis of toxoplasmosis in humans. In general, the use of milk samples instead of serum samples in the diagnosis of toxoplasmosis is debatable, and further and more comprehensive investigations, as well as the use of new methods, are needed.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Conflict of interest

The authors declare that there is no conflict of interest.

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