

Comparison of Giemsa Staining, Intraperitoneal Injection and Oral Administration Methods in Rat Brain Infected with *Toxoplasma Gondii*

Sajad Rashidi,*¹ Javid Sadraei¹ Mohamad Jafari-Modrek²

1. Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

2. Department of Parasitology, Infectious Diseases and Tropical Medicine Research Center, Zahedan University of Medical Sciences, Zahedan, Iran

Article information	Abstract
Article history: Received: 11 Aug 2012 Accepted: 20 Oct 2012 Available online: 17 Apr 2013 ZJRMS 2014; 16(2): 45-49 Keywords: Toxoplasma Souri mice Giemsa *Corresponding author at: Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, IR Iran E-mail: sajaderashidi@yahoo.com	Background: <i>Toxoplasma gondii</i> is one of the most common protozoan parasites in humans and animals in all countries of the world. The aim of this study was to detect <i>Toxoplasma</i> parasite in the brain of wild rats in Tehran using smear preparation, Giemsa staining, Intraperitoneal injection and oral administration to Souri mice. Materials and Methods: Forty rats were collected from different areas of Tehran. Smears were prepared from rat brains on glass slides and stained using Giemsa. In the second method, a cell suspension was prepared from rat brain and was given orally and injected intraperitoneally into Souri mice. In peritoneal method, peritoneum of the mice was examined for parasites. In oral method, the titer of <i>Toxoplasma</i> antibody in sera of Souri mice was determined using <i>Toxoplasma</i> IgG antibody kit and anti-mouse conjugate of Sigma company. Results: All results were negative in Giemsa staining method. In the second method, the results were negative and no parasites were observed in peritoneum of Souri mice. In oral administration method, after ingestion of suspensions by Souri mice and measuring the IgG titer, 50% of them showed a positive titer after one month. Conclusion: In detection of <i>Toxoplasma gondii</i>, the method of smear preparation on glass slides followed by Giemsa staining, and intraperitoneal injection of brain suspensions to Souri mice are of less value in comparison with oral administration of suspensions and determining the titer of IgG in sera of Souri mice. Copyright © 2014 Zahedan University of Medical Sciences. All rights reserved.

Introduction

Toxoplasma gondii is one of the most common protozoan parasites in the world capable of reproduction within the cells. This parasite can cause toxoplasmosis in all warm-blooded animals. There are plenty of animal hosts of this parasite resulting in high prevalence of it. Felidae, as the only final host of the parasite, excrete the oocyst form of the parasites in their feces. Asexual forms of the parasite such as tachyzoite and bradyzoite are seen in intermediate hosts [1].

The rate of infection in humans and other animal hosts has been reported differently in various parts of the world. *Toxoplasma* infection in humans, especially in people with defective immune system, pregnant women, children and those with underlying disease could entail serious damage. Thus, detection of this parasite in humans and other organisms, especially animals closely related with humans, seems to be necessary.

There are many diagnostic procedures in the diagnosis of *Toxoplasma* in humans and animals such as microscopic methods, oral administration and intraperitoneal injection of infected tissues to susceptible laboratory animals, serological methods (ELISA, etc.) and molecular techniques, each with their own sensitivity and specificity in detecting the parasite [2-5]. Although molecular methods are currently the most accurate

method in the diagnosis, they are too expensive. There are different statistics of *Toxoplasma* infection in Iran and other countries, some of which are presented below. In previous studies in Iran using serological tests, the incidence of the parasite has been determined in some hosts, so that the highest incidence (51%) in animal hosts has been observed in domestic poultry.

In a research similar to our study by Zia-Ali, the incidence of infection in some hosts such as sheep and goats was reported to be 22.8% and 14.3%, respectively [6]. In the study of Hashemi et al on sheep and goats in Qazvin, Kerman and Azerbaijan, the infection rate was reported to be 24.3% and 20%, respectively [7].

In the study of Rahbari et al. the prevalence of toxoplasmosis in sheep in three regions of Mazandaran using direct agglutination method was reported to be respectively 64.35%, 54.5% and 49% [8]. In the research on prevalence of toxoplasmosis in sheep and goats in Khuzestan Province by Hoghooghi et al, incidence rates of 13.8% and 13.1% were reported, respectively [9]. There have been few studies on birds in our country in this regard.

In a survey conducted by Ghorbani et al. in Tehran on domestic poultry using IHA method, the prevalence of *Toxoplasma* was reported to be 20.5% and in Mazandaran

30.3% [10]. In a study by Keshavarz et al. on a number of birds in Kerman, the prevalence of *Toxoplasma* was reported 10.5% [11]. Regarding the considerable presence of wild rats in Tehran and their close association with cats and human ambient, likely resulting in human infestation [12, 13], in this study we decided to survey the incidence of *Toxoplasma* parasite in the brain of these animals using three methods: smear preparation followed by Giemsa staining, Intraperitoneal injection of brain suspensions to Souri mice and oral administration of the suspensions to Souri mice followed by measurement of *Toxoplasma* IgG in sera of Souri mice.

The above-mentioned methods were used in this study for diagnosis, and the accuracy of these methods in *Toxoplasma* detection was determined to some extent. One advantage of using such methods as tissue smear staining, oral administration and intraperitoneal injection relative to molecular methods is their low cost and availability. Although studies on *Toxoplasma* parasite in rat brain are pretty rare in Iran, many studies have been done on this species in other countries, and various rates of infection have been reported [14].

For example, in a survey in 2005, 308 rats were caught, and their brains and hearts were evaluated for presence of *Toxoplasma*. The incidence rate of *Toxoplasma* was reported to be 0.8% using serological tests, i.e. only two rats showed a positive titer. The heart and brain of infected rats were injected to mice, and cysts were formed in their brains after a while. This study showed that in Grenada and West Indies, the rats have no significant role in prevalence of *Toxoplasma gondii* [15].

In another study similar to ours in Memphis and Tennessee, a number of rats were collected, then anesthetized and killed using chloroform. After that, a suspension was prepared from their brains and injected into the peritoneal cavity of mice. After 4 weeks, various parts of mouse body were searched for the parasite including peritoneal fluid. Infection rate was reported 8.7% in these parts [16].

In another study in England, the prevalence of *Toxoplasma gondii* in rats in Britain has been found to be 35%. In this study, it was stated that Toxoplasmosis in rats, just like cats, shows no specific symptoms, and more importantly, the predominant form of Toxoplasmosis in rats is the congenital form. It also showed that rat plays an essential role in the wild cycle of *Toxoplasma* as the intermediate or reservoir host [17].

In another study in 2003, a given number of parasitic oocysts were orally administered to Wistar rats. The rats were killed after two months, and their brains were extracted. The rate of cyst formation in the brain of rats was reported 82%. In this study, it was found that formation of cysts in the brain of rats is dependent upon various factors such as the quantity of oocysts and resistance of the body of rats [18].

In a study in the Philippines, the rate of infection was reported 55.5% [19]. In another study in Croatia in 2005, the brains of 142 rats and 86 mice were removed, and a suspension was prepared from them. The suspensions were injected subcutaneously into mice, and the sera of

Souri mice were evaluated for a positive titer of antibody against *Toxoplasma* parasite, showing positive results [20].

Materials and Methods

This study was performed in Faculty of Parasitology, Medical College of Tarbiat Modares University. The first step in the study was ensnaring live wild rats collected from different parts of Tehran. The sample in this study consisted of wild street rats, the collection of which was a difficult task. A number of cages were prepared for this study to catch live rats.

It should be noted that the cages were placed in most areas of Tehran. As far as possible, we attempted not to put the cages too close to each other, and to install the cages in the areas of North, South, East and West of Tehran. Thus, 40 wild rats were collected from different areas of Tehran. Given the fact that this study was descriptive, only descriptive statistics including frequency and frequency tables were used. The sampling method was stratified random, and similar studies were used as reference to determine the sample size.

It should be noted that in the early stages of this research, 60-70 wild rats were caught from different areas of metropolitan Tehran. However, due to a variety of reasons including improper extraction of brain from some of the rats, bacterial and fungal infection of some samples after extraction of and a number of other problems, only the brain of 40 rats were used for this research. On the other hand, trapping for hunting the rats in a large crowded metropolitan like Tehran and their anesthetizing, autopsy and brain extraction was a difficult task, and it was not possible to evaluate more than 40 rats. After collecting the rats and determining the geographical location of each sample, each and every rat was anesthetized using ether.

For this purpose, the cage containing the rat was placed in a plastic bag, a few milliliters of ether were added and the plastic bag was sealed up to complete unconsciousness of the rats. After anesthetizing the rats, the plastic bag was opened; the rats were out of the cage and placed on a special tray. To avoid recovery of the rats during the work and for not bothering the rats, the rat spinal cord was dissected, and immediately after disinfecting the scalp, the skull was split and the brain was removed and put in normal saline. It should be noted that because of parasitic and microbial contamination, the safety precautions should be strictly observed while working with wild rats, and the proper gown, mask, gloves and special glasses must be used.

To do this research, it was necessary to obtain a cell suspension from the brain of each rat. For this purpose, the brain was first placed in a specific container, and was completely crushed using a syringe plunger. In doing this, the veins and large extra tissues in the brain were removed. Then, 10 ml of normal saline was added to the crushed brain and was perfectly homogenized. The suspension was then poured into a tube, and was again mixed by Shaker, and was incubated in -20°C for subsequent steps.

Preparation of brain smears on glass slides and Giemsa staining

For this purpose, first for each of the samples, a small piece of brain particles in suspension was removed using a forceps and placed on glass slides, preparing a thin smear. The slides were then placed in room temperature to dry completely, and were then fixed using methanol. After evaporation of methanol and drying the smears, the slides were stained using Giemsa stain diluted in 1:20 ratio with water for 10 minutes, washed using tap water, and placed in room temperature to dry. Using a light microscope and the $\times 100$ lens and immersion oil, the slides were examined. The slides were thoroughly examined in this way.

Intraperitoneal suspension injection to Souri mice

For this purpose, 40 Souri mice were obtained (for each rat brain, a Souri mouse); the mice were then numbered in accordance with the number of caught rats. For injection of suspension into the peritoneum of Souri mice, the suspensions were prepared for injection in two ways. In the first method, one milliliter of the suspension was injected by syringe directly into the peritoneal cavity of Souri mice.

In the second method, which was performed immediately after the first procedure, a solution containing the following ingredients was prepared and mixed in equal proportions by volume with brain suspension, and was injected into the peritoneal cavity of Souri mice (0.5 ml suspension and 0.5 ml the prepared solution). To make the desired solution, 1 gr of NaCl, 1.4 ml of HCL, 1 mg pepsin (1:60,000 Assay Activity) was dissolved in 100 ml of distilled water [21].

This solution was prepared to rupture the lining of the likely brain cysts and release the bradyzoites. After suspension injection into the peritoneum of mice, we put the mice in a cage for further study and kept them in animal house.

Oral administration of suspension to Souri mice

In this stage, 40 new Souri mice were selected (one Souri mouse for each infected rat brain). To ensure the absence of infection in mice, before administration of suspensions to them, 1 ml of their blood was drawn (from the corner of their eye), and the serum was separated for measurement of Toxoplasma IgG antibody, and was kept in -20°C freezer. Then, 1 ml of pure suspension solution and after a while another 1ml of suspension (0.5 ml pure suspension+0.5ml prepared solution to rupture the cysts) was orally administered to Souri mice, and the mice were kept in animal house for 4-6 weeks for further studies. Then, blood was again drawn from the mice (from the corner of their eyes).

After separation of the serum, it was kept in -20°C freezer. Then, using ELISA and IgG antibody kit, IgG antibody titer was measured in sera of Souri mice. In this method, for increasing the accuracy and specificity of the responses, anti rat IgG conjugate of Sigma company was used instead of the conjugate in the kit. Finally, Toxoplasma IgG titer was measured in sera of Souri mice before and after oral administration [22].

Results

Results from this survey include: 1- Results of preparing direct smear from the brain of wild rats and Giemsa staining 2- Results of intraperitoneal injection of suspensions to Souri mice 3- Results of oral administration of suspensions to Souri mice.

1-Results of direct smear preparation from the brain of wild rats and Giemsa staining

In examination of the slides under a light microscope using $\times 100$ lens, none of the 40 samples were positive for brain cyst. There were cells similar to Toxoplasma gondii cysts, but they were in fact brain cells. Such cells had no conspicuous wall, but they were full of particles similar to bradyzoites. Since these cells were also observed when a smear was prepared from the brain of healthy laboratory rats (negative control), they could not be cysts.

2-Results of intraperitoneal injection of suspensions to Souri mice

In this method, the brain suspension of wild rats was injected to peritoneum of Souri mice, but after a month, no parasite was found in the peritoneum of Souri mice. Except for two mice, which fell victim to bacterial infection, there was no mortality in Souri mice, and no disease symptoms were observed (In this way, every 3 days the peritoneum of Souri mice was examined for parasites).

3-Results of oral suspension administration to Souri mice

The results of this method showed that serum titer of Toxoplasma gondii IgG antibody was negative in Souri mice before oral administration of brain suspensions, but the sera separated after oral inoculation showed a positive toxoplasma IgG antibody titer. The results in the table below show 50% rate of infection to toxoplasma parasite in brain of wild rats in different neighborhoods in Tehran.

In this method, the concentration of antibody in positive samples was reported between 0.5 to 0.9. The titer of negative control samples and minimum level of test (the Cut Off value) were determined using four serum samples from laboratory rats which were negative for Toxoplasma using Dye Test [23]. Sera from four wild rats infected with Toxoplasma were designated as positive titer of control sample. It should be noted that the serum titer of negative control samples was 0.40 and positive control sample titer was 1.3 (Table 1).

Discussion

Considering the fact that no cyst was observed in brain smear of these 40 samples, direct brain smear preparation and Giemsa staining appear not to be an accurate method to detect Toxoplasma parasites. This is because the parasite may not form any cysts in the brain, the cysts may be dispersed in brain parenchyma, the cysts may be tiny and not detectable or be in sites from which the smear has not been taken. In some of the studies conducted on laboratory white rats, a large number of

Table 1. Results of oral suspension administration to Sourì mice (*Toxoplasma gondii* IgG antibody titers)

Specimen number	Areas	Results	Specimen number	Areas	Results
1 33.3	Hashemi	-	21 35.3	Mehr abad	+
2 31.1	Rah ahan	+	22 24.1	Alghadir	-
3 23.1	Shohada	+	23 32.2	Jvanmarde ghasab	+
4 37.1	Khalij	+	24 32.1	Jvanmarde ghasab	+
5 19.4	Tehranpars	-	25 13.2	Sadeghieh	+
6 30.1	Resalat	+	26 19.1	Tehranpars	+
7 13.4	Sadeghieh	+	27 23.4	Shohada	+
8 21.2	Emam hosein	+	28 13.3	Sadeghieh	+
9 18.1	Zafar	-	29 24.2	Alghadir	+
10 33.1	Hashemi	+	30 18.2	Zafar	+
11 22.3	Pole chobi	+	31 13.1	Sadeghieh	-
12 28.1	Evin	+	32 34.2	Satar khan	-
13 36.2	Darband	-	33 31.2	Rah ahan	-
14 28.5	Evin	-	34 21.1	Emam hosein	-
15 19.2	Tehranpars	-	35 37.2	Khalij	-
16 28.2	Evin	+	36 23.3	Shohada	-
17 28.4	Evin	-	37 33.2	Hashemi	-
18 26.1	Darakeh	+	38 22.1	Pole chobi	-
19 32.4	Javanmarde ghasab	-	39 34.1	Satar khan	-
20 19.3	Tehranpars	-	40 22.2	Pole chobi	-

tachyzoites were injected into the peritoneal cavity of laboratory rats, but after a month or two, no cystic formation in the rat brain was found. This was while the immune system of the rats was suppressed using immunosuppressive drugs [24]. The results of this research largely confirm accuracy of our smear preparation and Giemsa staining method. Perhaps using histological methods, tissue section and Histochemical staining is more effective in diagnosis. On the other hand, it appears that the Intraperitoneal injection of infected brains to peritoneal cavity of Sourì mice and finding the parasites in Intraperitoneal fluid is not effective for diagnosis.

Perhaps one of the reasons for this is low level of parasites in brain of wild rats or mouse immune system domination over the injected parasites. As stated, oral inoculation of brain suspensions to Sourì mice and determination of toxoplasma IgG antibody titers in sera of Sourì mice showed 50% infection rate of them. It seems that assessment of IgG antibody titer in sera of Sourì mice after oral administration is more accurate in detection of toxoplasma parasite. In a study by Tabaei on the infection rate of street cats in Tehran, the incidence of this parasite was reported to be 89%, and there appears to be a significant correlation of its results with the results of our study due to close association between street rats and cats in Tehran [25]. Another point considered in this study was non-pathogenic strain of toxoplasma parasite present in the brains of these rats, as despite the Intraperitoneal injection and oral administration of suspensions to Sourì mice, no sign of illness and death in them was observed, and it looks like the strains of toxoplasma parasite in the

brain of wild rats in Tehran are not pathogen, and cannot cause any symptoms in Sourì mice. In a survey by Zia Ali on pathogenic strains of toxoplasma on the brain of ducks in Tehran, it was observed that the strain in these ducks failed to cause disease or symptoms in Sourì mice after oral inoculation, while the strains isolated from brain of ducks in Egypt were pathogenic and caused symptoms in Sourì mice [6]. It seems that this is also true in rats, i.e. even though the strains of toxoplasma in the brain of rats in Tehran were not pathogenic, they may cause disease or symptoms in Sourì mice in other countries. In studying the *Toxoplasma* parasite, it is recommended to use several methods simultaneously, and if possible, molecular techniques should be used along with serological and histological methods

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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