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Serological survey for antibodies to *Encephalitozoon cuniculi* and *Toxoplasma gondii* in pet rabbits in eastern coastal areas of China

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ABSTRACT. *Encephalitozoon cuniculi* (*E. cuniculi*) is a microsporidian parasite commonly detected in rabbits and can infect humans and cause encephalitozoonosis. And *Toxoplasma gondii* is a prevalent parasite distributed worldwide and can infect almost all warm-blooded animals, including humans. The aim of the current study was to investigate the seroprevalence of *E. cuniculi* and *Toxoplasma gondii*, and risk factors of infection in pet rabbits reared in eastern coastal areas of China (Tianjin, Shandong, Jiangsu, Zhejiang, Shanghai and Fujian). Total 222 blood samples of pet rabbits were collected from local veterinary hospitals. The seropositivity rates of *E. cuniculi* were 16.22% (36/222) according to an Enzyme-linked immunosorbent assay (ELISA). Female pet rabbits was significantly higher than that in males (P=0.002), Zhejiang were markedly higher than those in Jiangsu and Shanghai (P=0.017, P=0.022), and cross-breed rabbits were dramatically higher than those in Chinchilla, New Zealand white, Rex (P=0.02, P=0.006, P=0.008). The seroprevalence of *T. gondii* was 13.06% (29/222) by the method of ELISA. The seroprevalence in Zhejiang was significantly higher than that in Shanghai (P=0.017). No difference in seroprevalence was detected with respect to the gender, age, species, health status, or season. These findings show that *E. cuniculi* and *T. gondii* are present and spread in pet rabbits. Therefore, pet rabbits should be considered as an important reservoir of encephalitozoonosis for humans and maybe important implication for public health in eastern coastal areas of China.

KEYWORDS: *Encephalitozoon cuniculi,* Enzyme-linked immunosorbent assay, pet rabbit, public health, *Toxoplasma gondii*

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Encephalitozoon cuniculi (*E. cuniculi*) is a common causative agent of rabbits, mainly leading to renal failure, eye diseases, central nervous system disease and sudden death [21, 33]. Among these symptoms, neurological manifestations are the most important including behavior changes, depression, head tilting, ataxia, circling, rotation, seizures and paralysis [20]. This pathogen has four genotypes named I, II, III, IV, and has extensive host including rodents, herbivores, carnivores, nonhuman primates, and humans [2, 34]. These genotypes are not host specific, and humans can be infected by any one of them, so *E. cuniculi* is considered as an emerging pathogen and is receiving increasing attention [16].

Rabbits can be infected with *E. cuniculi* through placental transmission, or indraft of spores, or intake of food or water contaminated with spores [39]. Naturally infected rabbits usually do not show clinical signs, on the other hand, clinical symptoms with the exception of phacoclastic uveitis, are not sufficient to support differential diagnosis to rule out bacterial infections and injuries [8]. In addition, subclinical symptoms in infected rabbits can persist for a considerable period of time [4], however, the infection is difficult to diagnose in live rabbits [33]. In living animals, enzyme linked immunosorbent assay (ELISA) and indirect fluorescent antibody technique (IFAT) are considered as the most crucial diagnostic approach for diagnosing *E. cuniculi* infection

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[22]. Therefore, ELISA was selected in the present study. Humans are mainly infected by intake of food and water contaminated with infective spores, which are excreted through the urine and feces of rabbits [40]. People may be at risk of infecting *E. cuniculi* when they were in contact with infected pet rabbits [34]. In other words, the disease can be transmitted from infected pet rabbits to people, especially immunocompromised patients, such as recipients of transplanted organs, acquired immuno-repressed syndrome (AIDS) and cancer patients served with chemotherapy [18].

Toxoplasma gondii (*T. gondii*) is a prevalent parasite throughout the world and diffusely prevalent in both animals and humans [11]. In livestock, nearly all of the warm-blooded animals can be infected by *T. gondii* involving in domestic rabbits [10]. Rabbits are infected with *T. gondii* mainly through placenta, or by the intake of food or water contaminated with toxoplasmosis [32]. It has been confirmed that eating undercooked rabbit meat is related to human infection with *T. gondii* [5]. Rabbit meat is one of the most nutritional white meats which contain relatively low fat and cholesterol, and it has been taken as a special diet for patients with heart disease and kidney disease [14]. For humans, one of the important risk factors for infection with *T. gondii* is the consumption of undercooked or raw meat from infected intermediate hosts [11]. Thus, rabbits had been taken as potential reservoirs for the transmission of *T. gondii* [37]. Pet rabbits living with people, their risk of carrying *T. gondii* needs investigation. Rabbits and humans are often recessive after infection with toxoplasmosis and do not show any clinical symptoms, therefore, detection of antibodies are often used in epidemiology [1].

It was reported that the seroprevalence rates around the world were very high (37–68%), indicating that *E. cuniculi* infection is ubiquitous in rabbits [13]. Rabbits which are the primary host for *E. cuniculi*, are increasingly being raised as pets in China. Infected pet rabbits can excrete the spores of *E. cuniculi* fitfully, and they may cause potential risk for *E. cuniculi* infection to pet rabbit raisers [36]. Many epidemiological investigations are focused on domestic rabbits, and the *T. gondii* epidemiology of pet rabbits has not attracted much attention. However, little is known about *E. cuniculi* in pet rabbits in the eastern coastal areas of China. Similarly, few studies have been carried out on the seroprevalence of *T. gondii* in pet rabbits in China. Therefore, it is necessary to carry out an epidemiological investigation on the seroprevalence and the risk factors concerned with seropositivity of the two diseases in pet rabbits from eastern coastal areas of China.

MATERIALS AND METHODS

Samples

A total of 222 blood samples used for detecting *E. cuniculi* and *T. gondii* and of pet rabbits from local veterinary hospitals were collected from October 2018 to November 2020 in eastern coastal areas of China including Tianjin, Shandong, Jiangsu, Zhejiang, Shanghai and Fujian (Fig. 1). For each sampled pet rabbit, information including gender, age (months), location, species, health status (symptomatic/asymptomatic) and season were recorded. Serum specimens were obtained by centrifugation from whole blood and stored at −80°C for subsequent testing.

Determining E. cuniculi and T. gondii antibody

E. cuniculi antibody was detected by Rabbit *E. cuniculi* antibody ELISA Reagent test kit (Jining Industry Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Briefly, each plate should be provided with 2 wells of negative control, 2 wells of positive control, and one blank control hole. Briefly, the ELISA plate was incubated for 30 min at 37°C and washed for 5 times, then, the Enzyme standard reagent was added except for blank holes. After, the steps of incubation and washing were performed again, the color development reagent A and B were added at 37°C for 15 min under the dark. Finally, stop solution was added and the absorbance (OD value) of each well was measured at 450 nm by a spectral photometer. Determination of results: (1) Test validity: positive control well average ≥1.00, negative control mean ≤0.20; (2) Cut-off (CUT OFF) calculation: CUT OFF=mean of negative control wells + 0.15; Negative determination: rabbits with OD value <CUT OFF are negative; Positive determination: rabbits with OD value ≥CUT OFF are positive.

The detection of antibody against *T. gondii* was performed by ELISA. The operation process and result determination were determined according to the manufacturer's instructions with *T. gondii* antibody (Tox Ab) ELISA Reagent test kit (Jining Industry Co., Ltd.) similar to the operation and calculation method of the above kit. These pet rabbits were assigned as either *T. gondii* negative or *T. gondii* positive.

Statistical analyses

Data were analyzed according to Pearson's χ^2 test for independence using the statistical software package SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). The risk factors included gender, age, location, species, health status and season in the current survey. The χ^2 goodness-of-fit test was used to analyze the risk factors. Identification of a risk factor required a 95% confidence level ($P<0.05$).

Ethical statement

The animal hospital collected blood from pet rabbits for testing in accordance with the requirements of animal welfare. All the serum used in this investigation came from the remaining serum after the inspection of each animal hospital, and the pet owner's consent had been obtained.

Fig. 1. Geographic distribution of sampling locations for pet rabbits in eastern coastal areas of China (Tianjin, Shandong, Jiangsu, Shanghai, Zhejiang and Fujian).

RESULTS

The seroprevalence of *E. cuniculi* in the present survey were displayed in Table 1. Among the 222 pet rabbits, 36 (16.22%) were positive according to the ELISA assay. Seroprevalence in female pet rabbits (24.07%) was significantly higher than that in males (8.77%) (P=0.002). Seroprevalence in Zhejiang (28.13%) was markedly higher than those in Jiangsu (7.32%) (P=0.017) and Shanghai (7.69%) (P=0.022). Seroprevalence in cross-breed rabbits (31.34%) were dramatically higher than those in Chinchilla (9.68%) (P=0.02), New Zealand white (9.09%) (P=0.006), Rex (8.33%) (P=0.008).

The overall seroprevalence of *T. gondii* in pet rabbits was 13.06% (29/222) in the six provinces (Table 2). The prevalence in Zhejiang was significantly higher than that in Shanghai (P=0.017). The positive serum rates of *T. gondii* were 14.04% (16/114) in males and 12.04% (13/108) in females. *T. gondii* seropositivity of these pet rabbits were 12.90% (4/31) in Chinchilla, 13.64% (6/44) in New Zealand white, 14.29% (2/14) in Dutch, 13.33% (3/36) in Rex, 10.00% (3/30) in Lionhead and 16.42% (11/67) in Cross-breed rabbits.

Seroprevalence of single infection and co-infection in all infected rabbits was shown in Table 3. Total 48 rabbits were infected with *E. cuniculi* and *T. gondii*. The seroprevalence of *E. Cuniculi* alone and *T. gondii* alone in the infected rabbits were 39.58% (19/48) and 25.00% (12/48), separately. The seroprevalence of co-infection in *E. Cuniculi* and *T. gondii* was 35.42% (17/48).

DISCUSSION

E. cuniculi is a sort of opportunistic zoonosis, and it is a health risk for those people exposed to latent or infected animals [6]. The spores of *E. cuniculi* has strong environmental resistance and can survive on the ground for several weeks or even months [13]. The spread of encephalitozoonosis in individual animal populations is promoted by the environment contaminated by spores from the emiction of infected animals. The main host of *E. cuniculi* is rabbit [6]. Pet rabbits are primary likely to be infected by *E. cuniculi* through the intake of spore-contaminated food and water [7].

With the improvement of people's living standards, more and more people raise pets including pet rabbits. Rabbits may carry some zoonotic diseases including toxoplasmosis. It is generally believed that humans are infected with *T. gondii* mainly through ingesting food and water contaminated with feces of cats, or eating undercooked meat infected with *T. gondii* [11]. Kolbekova

Table 1. Seroprevalence of *Encephalitozoon cuniculi* (*E. cuniculi*) infection in pet rabbits in eastern coastal areas of China determined by ELISA

Factor	Category	No. examined	No. of positive	No. positive (%)	Odds ratio (95%CI)	*P*
Gender	Males	114	10	8.77	1	
	Females	108	26	24.07	0.303 (0.138–0.665)	0.002
Age (months)	<4	55	8	14.55	1	
	4–12	111	21	18.92	0.729 (0.300–1.772)	0.485
	≥12	56	7	12.50	1.191 (0.400–3.545)	0.753
Location	Tianjin	35	7	20.00	1	
	Shandong	49	9	18.37	1.633 (0.649–4.113)	0.294
	Jiangsu	41	3	7.32	3.167 (0.752–13.337)	0.103
	Shanghai	39	3	7.69	3.000 (0.711–12.660)	0.122
	Zhejiang	32	9	28.13	0.639 (0.206–1.980)	0.436
	Fujian	26	5	19.23	1.050 (0.292–3.775)	0.940
Species	Chinchilla	31	3	9.68	1	
	New Zealand white	44	4	9.09	1.071 (0.222–5.165)	0.931
	Dutch	14	1	7.14	1.393 (0.132–14.705)	0.782
	Rex	36	3	8.33	1.179 (0.220–6.309)	0.848
	Lionhead	30	4	13.33	0.696 (0.142–3.413)	0.654
	Cross-breed rabbits	67	21	31.34	0.235 (0.640–0.859)	0.020
Health status	Symptomatic*	29	5	17.24	1	
	Asymptomatic	193	31	16.06	1.089 (0.386–3.072)	0.872
Season	Spring	51	6	11.76	1	
	Summer	57	11	19.30	0.558 (0.190–1.636)	0.283
	Autumn	61	9	14.75	0.770 (0.255–2.331)	0.644
	Winter	53	10	18.87	0.573 (0.192–1.714)	0.316

*Symptomatic included diarrhea, anorexia, neurological signs, fever, renal failure, and others.

Table 2. Seroprevalence of *Toxoplasma gondii* (*T. gondii*) infection in pet rabbits in eastern coastal areas of China determined by ELISA

Factor	Category	No. examined	No. of positive	No. positive (%)	Odds ratio (95%CI)	*P*
Gender	Males	114	16	14.04	1	
	Females	108	13	12.04	1.193 (0.545–2.614)	0.695
Age (months)	<4	55	9	16.36	1	
	4–12	111	14	12.61	1.356 (0.547–3.361)	0.510
	≥12	56	6	10.71	1.630 (0.538–4.937)	0.384
Location	Tianjin	35	4	11.43	0.774 (0.208–2.879)	0.702
	Shandong	49	7	14.29	1.194 (0.276–5.619)	0.813
	Jiangsu	41	4	9.76	2.387 (0.409–13.919)	0.332
	Shanghai	39	2	5.13	0.387 (0.104–1.439)	0.148
	Zhejiang	32	8	25.00	0.710 (0.160–3.148)	0.651
	Fujian	26	4	15.38	0.774 (0.208–2.879)	0.702
Species	Chinchilla	31	4	12.90	1	
	New Zealand white	44	6	13.64	0.938 (0.241–3.648)	0.927
	Dutch	14	2	14.29	0.889 (0.143–5.533)	0.899
	Rex	36	3	8.33	1.630 (0.335–7.920)	0.542
	Lionhead	30	3	10.00	1.333 (0.272–6.533)	0.772
	Cross-breed rabbits	67	11	16.42	0.754 (0.220–2.588)	0.653
Health status	Symptomatic*	29	4	13.79		
	Asymptomatic	193	25	12.95	1.075 (0.345–3.349)	0.900
Season	Spring	51	6	11.76	1	
	Summer	57	9	15.79	0.711 (0.234–2.158)	0.546
	Autumn	61	9	14.75	0.770 (0.255–2.331)	0.644
	Winter	53	5	9.43	1.280 (0.365–4.488)	0.758

*Symptomatic included diarrhea, anorexia, neurological signs, fever, renal failure, and others.

Table 3. Seroprevalence of single infection and co-infection in all infected rabbits

Infection	No. Positive	Percentage (%)
Encephalitozoon cuniculi (*E. cuniculi*)	19	39.58
Toxoplasma gondii (*T. gondii*)	12	25.00
E. Cuniculi and *T. gondii*	17	35.42
Total	48	100

et al. revealed the correlations between raising rabbits and positive seropositivity of *T. gondii* in humans [19]. However, few epidemiological surveys of toxoplasmosis in pet rabbits have been performed in China. Because pet rabbits have been in contact with humans for a long time, they can also be considered as an essential factor for human infection with toxoplasmosis [5].

The serum antibody positive rate for *E. cuniculi* in the present study was higher than that of *T. gondii*, which was in accordance with previous surveys in China [25, 40]. This might be due to the fact that *E. cuniculi* can accelerate transmission directly through spores in the urine of infected animals [29].

The present investigation showed that the positive serum rate of *E. cuniculi* was 16.22% in pet rabbits in eastern coastal areas of China, which was similar to the results of early surveys conducted in farmed domestic rabbits in Egypt (15%) [6] and domestic rabbits in China (18.67%) [30]. However, it was higher than the values of 7.7% in New Zealand rabbits in Turkey [29], and lower than that in pet rabbits in Iran (59.6%) [34], Italy (67.2%) [9], Austria (58.5% in 118 rabbits) [12] and Korea (67.8%) [36]. These epidemiological results of various countries may root in the different detection methods, ecological factors, rabbit populations, or breeding systems or to some combination of these conditions [30, 36]. Although the infection rate was relatively low, the results suggest that *E. cuniculi* is present in pet rabbits in eastern coastal areas of China. The seropositivity rate in Zhejiang was significantly higher than those in Jiangsu and Shanghai. These results are in accordance with Malik *et al.* who declared that the prevalence of infection could dramatically vary from one region to another within the country because of differences in certain ecological factors and breeding systems in different areas [25]. These results may indicate the environmental pollution with infected *E. cuniculi* spores in Zhejiang is worse than in Shanghai and Jiangsu.

In the present study, the gender of pet rabbits was a significant risk factor. The *E. cuniculi* seroprevalence of female pet rabbits was significantly higher than male animals, which was consistent with previous reports [40]. Conversely, many other studies found that gender was not a risk factor [9, 16, 36, 39]. The breed has an impact on the seroprevalence of *E. cuniculi*. The *E. cuniculi* seroprevalence of Cross-breed rabbits (29.41%) was higher than those in Chinchilla (7.41%), New Zealand white (10.0%) and Rex (6.67%). Similarly, Pan *et al*. found that seropositive rate for *E. cuniculi* of Rex rabbit was higher than those of Japanese White Rabbit and New Zealand Rabbit [30]. These results reveal that there may be some correlation between the genetic line and the seropositivity against *E. cuniculi*.

Age was not a risk factor in our survey, which was in agreement with two surveys in Korea [36] and United Kingdom [17]. On the contrary, some previous findings found that infection rates were higher in older rabbits than in young rabbits [25, 35]. In the mammalian offspring, maternal antibodies to *E. cuniculi* from the colostrum are present until 4 weeks of age [24]. For animals from 4 to 8 weeks, they were seronegative [17]. There is no difference in seroprevalence between symptomatic and asymptomatic pet rabbits. However, the high seroprevalence of healthy pet rabbits should also be noticed because these rabbits may be an important source of *E. cuniculi* infection [36].

Pet rabbits could be regarded as a potential source of *T. gondii* infection. The overall seropositivity in the present study was similar to those in Algerian (14.6%) [15] and Spain (14.6%) [4]. However, it was significantly lower than those reported in Mexico (16.3%) [5], Poland (22%) [38], Slovakia (74%) [23], Iraq (86%) [3], and higher than those in Czech Republic (10%) [28], in Egypt (11.34%) [6] and central China (10.55%) [40]. The variation of the seroprevalence rates of *T. gondii* in different countries may be due to geographic position, ecological environment, gender, age, feeding management, different detection technologies and number of samples [27, 31, 41].

Many researchers found that the gender was not an important risk factor for the infection with *T. gondii* of rabbits [2, 37, 40]. The seroprevalence rates of *T. gondii* were 16.36% (9/55) in ≤4 month, 12.61% (14/111) in 4–12 month and 10.71% (6/56) in ≥12 month. Several previous studies reported that older rabbits had a higher prevalence than young rabbits [11, 14, 25]. It was considered the infection of toxoplasma is mainly transmitted horizontally rather than vertically [40]. The highest positive rate in the ≤4 month group in this study might be attributed to colostrally-derived antibodies and/or congenital infection [5].

Previous studies reported that seroprevalence in New Zealand rabbits was higher than in other breeds [5, 40]. The positive serum rates of *T. gondii* were 13.79% (4/29) in symptomatic pet rabbits and 12.95% (25/193) in asymptomatic pet rabbits. The seroprevalence rates of *T. gondii* were 11.76% (6/51) in spring, 15.79% (9/57) in summer, 14.75% (9/61) in autumn and 9.43% (5/53) in winter. These findings were in agreement with Meng *et al.* [25]. Although there are no significant differences between seasons, the prevalence in summer and autumn is higher than in spring and winter. This may be because the temperature and climatic conditions in summer and autumn are more conducive to the development of *T. gondii* oocysts than in spring and winter.

Furthermore, the serum positive rate of *T. gondii*-positive rabbits was significantly higher than that of *T. gondii*-negative rabbits. This result was consistent with the results of previous surveys [26, 40]. In addition, simultaneous infection was also very common. This mixed infection may be due to the increased susceptibility of rabbits after concomitant infection [26].

To sum up, these findings of the current survey showed that *E. cuniculi* and *T. gondii* were present and widespread in pet rabbits in eastern coastal areas of China. Considering zoonotic and public safety issues, we strongly advise that serological surveys for *E. cuniculi T. gondii* infection both in symptomatic and asymptomatic pet rabbits are very necessary. More importantly, it is time to take precautionary measures to avoid the spread of encephalitozoonosis.

POTENTIAL CONFLICTS OF INTEREST. The authors declare that they have no conflict of interest related to this study.

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