Molecular Epidemiological Investigation and Analysis of Toxoplasma Gondii Infection in Livestock, Poultry, and Pets in Henan Province, Central China

Xiao-Li Zhang
Xinxiang Medical University
Rong-Hui Zhu
Xinxiang Medical University
Jia-Qi Huang
Xinxiang Medical University
Meng-Yuan Qiao
Xinxiang Medical University
xinxin Xu
Xinxiang Medical University
Peng-Fei Bo
Xinxiang Medical University
Shuai Wang
Xinxiang Medical University
Shiguo Liu (lsg1963@163.com)
Xinxiang Medical University

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Abstract

Background: *Toxoplasma gondii (T.gondii*) is a protozoan parasite spread around the world and is concerned as one of the most important foodborne zoonotic parasites in the global world. This study aimed to determine the prevalence of *T.gondii* in foodborne animals in Henan Province, China.

Methods: During 2012 to 2015, a total of 1879 samples of foodborne animal tissues or peripheral blood were collected from 12 slaughterhouses, eight pastures and 434 farmers in different areas of Henan province using systematic random sampling method**.** The genomic DNA was extracted by the phenol-chloroform method. Moreover, then a One-tube nested polymerase chain reaction (PCR) assay was performed to amplify the 529bp repetitive sequence of *T.gondii* in these samples. *T.gondii* infections in diverse animals is different in the study.

Results: Of the 1879 samples,cat had the highest *T.gondii* infection(51.9%,28/54),followed by goose(24.3%,44/181), chicken(19.7%,91/462), cattle(12.6%,23/182), sheep(11.5%,22/191), duck(11.3%,24/212), dog(10.5%,13/124), pigeon(5.4%,16/297) and pig(4.6%,8/176). The total infection of *T. gondii* in these animals was 269/1879, and the positive rate was 14.3%.

Conclusions: The results revealed that all the livestock, poultry, and pets in close contact with humans had toxoplasma infection, which suggest that the consumption of meat products in Henan may pose a potential threat to human health. Therefore, to strengthen the prevention and control of *T.gondii* infection in animals is an important measure to develop animal husbandry, support food safety, and protect human health.

Background

Toxoplasmosis is a zoonotic disease caused by *T.gondii*, a protozoan parasite belonging to Coccidia and Acopmplexa phylum. *T.gondii* is one of the most prevalent parasites in humans and warm-blooded animal species, infecting up to a third of the population[1]. Cat is the definitive host of *T.gondii* and all warm-blooded animals, including humans, are intermediate hosts, which can lead to toxoplasmosis[2]. Oocysts are infectious one to five days after excretion, are spread through surface water, and can survive for more than a year[3]. Infection in humans or animals is primarily acquired by ingestion of food or water contaminated by oocysts or by consumption of raw or undercooked meat containing tissue cysts[1]. Infection in humans is mostly asymptomatic or mild, but immunocompromised hosts and children infected congenitally may develop serious complications[13], causing behavioral changes, neuropsychiatric disorders, abortions, stillbirth or fetal malformations, infertility and even death in humans and other mammals[15].

The detection of *T.gondii* infection with sensitive and individual strategies is pivotal progress to control and prevent toxoplasmosis. At present, there are several methods to detect *T.gondii*, such as serological analyses[11], etiological detection, and bioassay in mice. The etiological detection method is time-consuming and has low sensitivity. Also, serological assays results are prone to be false-negative or

false-positive[12]. Therefore, various PCR methods are considered as the most effective alternatives, especially nested PCR, has been used to increase the sensitivity of DNA detection in many infections[3]. In molecular biology diagnosis, conventional PCR has low sensitivity, while nested PCR has both higher specificity and sensitivity. However, compared with conventional PCR, the operation is complicated and more prone to contaminate. In order to reduce contamination as much as possible and simplify test procedures, this study established a One-tube nested PCR targeting 529bp repetitive sequence and applied it to detect the tissue and peripheral blood samples of foodborne animals in this study. The 529bp fragment is a 200- to 300-fold repetitive fragment of the *T.gondii* genome and can discriminate *T.gondii* from that of other parasites[5]. Location of *T.gondii* infection with delicate and particular methods is an essential progress in the prevention and protection of toxoplasmosis in humans and animals. Therefore, nested PCR can prove the degree of contamination of meat or meat products, thus strengthening the prevention of foodborne diseases.

Henan Province, located in the heartland of China, belongs to transition climate between North Asia and warm temperate zone, which extend the survival time of oocysts, so animals are susceptible to be infected with *T.gondii*. Meat products, milk, and eggs from infected animals may also threaten the health of other animals and people. Meat habits in Henan are mainly livestock meat and chickens. Undercooked barbecue and hot pot are prevalent in people. With the improvement of people's living standards, the popularity of pets makes people have closer contact with cats. All of these may become the sources of foodborne toxoplasmosis.

Approximately 50% of all human toxoplasmosis cases are associated with foodborne infection, and consuming undercooked meat products containing the parasite in tissue cysts is considered as the main risk of human infection[13]. Learning the biological and molecular aspects of the parasite is essential for the epidemiology of toxoplasmosis disease. Through detection and analysis, this study identified the *T. gondii* prevalence of foodborne animals in Henan Province. The results showed that widespread toxoplasmosis is a public health problem that should not be ignored, which is necessary to conduct investigations, strengthen quarantine, and control spread. Therefore, to strengthen the prevention and control of *T.gondii* infection in animals is an important measure to develop animal husbandry, support food safety, and protect human health.

Methods

Reagents

Primers were synthesized by Shanghai Sangon Bioengineering Technology Co., Ltd. The Proteinase K Buffer was purchased from Merck Drugs & Biotechnology, Germany. Tris- Phenol and PCR amplification reagents were purchased from China Beijing ComWin Biotech Co., Ltd. *T.gondii* RH strains were provided by Department of Human Parasitology in Xinxiang Medical University. Other reagents were domestic analytical reagents.

Sample collection and processing

From 2012 to 2015, 1879 tissue and peripheral blood samples were collected randomly from 12 slaughterhouses, eight pastures and 434 farmers in four regions (Zhengzhou, Zhoukou, Xinxiang, and Kaifeng) in Henan(Fig. 1). The samples were stored at −20 °C.

DNA extraction

Using the phenol-chloroform method to extract DNA[4]. When the liquid nitrogen is gone, ground muscle tissue up to a fine powder. Then the powder tissue was mixed with proteinase K buffer and vortexed until digesting completely. The suspension was left for a while and then centrifuged. Sediments were removed, and the supernatant was extracted. Blood samples centrifuged and removed the supernatant. Protease K buffer was then used to digest leukocyte sediment. So then, the DNA of tissue and blood samples were extracted by conventional methods. What is more, DNA of *T. gondii* RH strains was extracted by phenol-chloroform method, too.

One-tube Nested PCR amplification

Primer Premier5.0 software was used to design primers for nested PCR, based on the 529bp repetitive sequence of *T.gondii* RH strains(Log into GenBank viaAF146527.1). Nested PCR has two pairs of primers. External primers were: F: 5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3' and R:5'-CGCTGCAGA CACAGTGCATCTGGATT-3',and internal primers were: F: 5'-AGAAGGGACAGA AGTCGAAG-3' and R: 5'-CTCCACTCTTCAATTCTCTCC-3'. PCR reactions were initiated at 95℃ for an initial denaturation for 2 min and then cycled for 15 cycles with denaturation at 95℃ 30s, followed by annealing at 68℃ for 30 s and finally an extension step at 72℃ for 50s. Moreover, then, cycled for 35 cycles with denaturation at 95℃ 30 s, followed by annealing at 58℃ for the 30s and finally an extension step at 72℃ for 50s followed by a 5 min final extension at 72℃. Both PCR reactions were carried out in one tube. Then the amplified PCR products were fractionated on a 2% agarose gel. Trophozoite DNA of RH strain was used as a positive control, normal serum or tissue DNA was used as a negative control, and ddH2O was used as blank control.

Statistical analysis

Data was analyzed using SPSS 24.0（SPSS Inc.,New York, USA）. X2-test calculated the sensitivity and specificity of nested PCR assays. Significance was indicated when P < O. 05 for all statistical analyses.

Results

The positive rate of T. gondii in livestock, poultry, and pets in Henan

Of the 1879 samples, 269 were detected to be *T. gondii* positive using nested PCR assay, showing a total positive rate of 14.3%(269/1879). *T. gondii* infection ranged from 4.55–51.85% of examined animals in Henan with significant differences between the different species of animals ($\chi^2 = 125.337$, $P < 0.05$). (Fig. 2).

T.gondii infection in livestock in Henan Province

Reported *T. gondii* infection in livestock in Henan Province is summarized in Table 2. The highest *T. gondii* positive rate was found in cattle(12.6%), followed by sheep(11.5%) and pig(4.6%), with significant differences between different species ($\chi^2 = 7.885$ and $p < 0.05$). The comparison between pig and cattle shows $\chi^2 = 7.407$ and $P < 0.05$. The comparison between pig and sheep shows $\chi^2 = 5.933$ and $P < 0.05$. However, there were no significant differences between cattle and sheep ($\chi^2 = 0.11$ and $P > 0.05$) (Table.1).

Table 1
T.gondii infection of livestock in Henan Province

Variety	Sample size	Number of PCR positive	Number of PCR negative	Positive rate
Pig	176	8	168	4.6%
Cattle	182	23	159	12.6%
Sheep	191	22	169	11.5%
Total	549	53	496	9.7%

Table 2
T.gondii infection of **poultry** in Henan Province

Variety	Sample size	Number of PCR positive	Number of PCR negative	Positive rate
Chicken	462	91	371	19.7%
Duck	212	24	188	11.3%
Goose	181	44	137	24.3%
Pigeon	297	16	281	5.4%
Total	1152	175	977	15.2%

T. gondii infection in poultry in Henan Province

Detection of *T. gondii* infection in poultry in Henan Province is shown in Table 3. The highest *T. gondii* positive rate was found in goose(24.3%),followed by chicken(19.7%),duck(11.3%)and pigeon(5.4%), with significant differences between different species (χ^2 = 43.584 and P < 0.05). Making a comparison between chicken and duck, χ^2 = 7.205, and P < 0.05. Making a comparison between chicken and goose, χ^2 = 1.668, and P < 0.05. Making a comparison between chicken and pigeon, χ^2 = 30.568, and P < 0.05. Making a comparison between duck and goose, χ^2 = 11.512, and P < 0.05. Making a comparison between duck and pigeon, χ^2 = 6.015, and P < 0.05. Making a comparison between goose and pigeon, χ^2 = 36.684, and P < 0.05(Table.2).

Table 3
T.gondii infection of pets in Henan Province

Variety	Sample size	Number of PCR positive	Number of PCR negative	Positive rate
Dog	124	13	111	10.5%
Cat	54	28	26	51.9%
Total	178	41	137	23.0%

T. gondii infection in pets in Henan Province

Samples were collected in cats and dogs, and the positive rate was 51.9% (28/54) and 10.5% (13/124), respectively, with significant differences between the different types of markets (χ^2 = 36.313 and P < 0.05) (Table.3).

Comparison of T. gondii infection in foodborne animals in Henan Province.

The highest positive rate was found in pets (23.0%, 41/178), followed by poultry (15.2%, 175/1152), and livestock (9.7%, 53/549) (Table 4). According to the results of the chi-square analysis, it shows χ^2 = 9.821 and P > 0.05 when comparing livestock with poultry. It shows χ^2 = 21.374 and P < 0.05 when making a comparison between livestock and pets. The comparison between poultry and pets shows χ^2 = 6.971 and P < 0.05.

Table 4
T.gondii infection of livestock, poultry, and pets

Variety	Sample size	Number of PCR positive	Number of PCR negative	Positive rate
Livestock	549	53	496	9.7%
Poultry	1152	175	977	15.2%
Pets	178	41	137	23.0%
Total	1879	269	1610	14.3%

Discussion

Mammals and birds, including humans, can acquire *T. gondii* infection by ingesting oocysts, which have been shed in the unsporulated form in infected felids[10]. Consumption of raw or undercooked meat is the leading way to be infected. Different animals have a different infection of *T. gondii*, which may be attributed to the dissimilarities in susceptibility to *T. gondii*. Risk factors associated with the increased toxoplasma infection have been surveyed, including farm type and management, feeding source, the presence of stray cats, methods of rodent and bird control, carcasses handling and disposal, and water quality[8]. Besides, *T.gondii* infection in these animals varies due to different countries and regions[6]. (Fig. 3)

Among the livestock in Henan Province, the positive rate of *T.gondii* is lower in pigs, while cattle and sheep are higher. The lower positive rate in pigs could be attributed to the improvements in farm management, including strict confinement and biosafety regulations, better rodent control, and proper carcasses disposal. Cattle and sheep are raised extensively and semi-intensively. Due to feeding habits and sanitary conditions, cattle and sheep are more likely to be infected with oocysts in feces. These results suggest that these animals have been repeatedly infected due to high levels of environmental pollution, as a large number of infected stray cats have excreted oocysts on poorly managed farms. What is more, high levels of environmental pollution from felines oocysts and outdoor management system of animals can also be explained, as demonstrated by Gebremedhin et al[7].

For food safety, pigs are important hosts for *T. gondii*, because contaminated pork is one of the primary sources of human infection, and per capita consumption of pork exceeds that of any other meat[13]. It is worth noting that the positive rate of pigs in this study is also lower than that in other areas,which suggests that with the standardization of pig breeding and the improvement of food safety awareness, *T. gondii* infection in pork gradually decreases and tends to be stable. Although mutton makes up only a small proportion of total meat consumption, it is popular among some ethnic groups in Henan. Also, with the improvement of people's living quality, beef and mutton gradually become an essential part of people's diet, so it should also be valued.

Among the prevalence of *T. gondii* in poultry, the highest toxoplasma infection was found in goose, which is in keeping with the finding of Yeeling et al[9]. One reason may in part be due to the goose is exposed to

soil and water, which are both potential sources of Toxoplasma infection. Another is that the growth period of goose is more prolonged, whereas duck has a shorter one so that goose presented higher toxoplasma infection than the duck. Although chicken is mainly caged, it is fed with grass and vegetable, in Henan Province, which will increase toxoplasma infection of chicken. Moreover, free-range chickens habitually scratch the soil in search of food and are therefore susceptible to be infected in an environment that could be contaminated with oocysts[14]. Among poultry, pigeons have the lowest rate of toxoplasma infection because they are mostly caged and mainly fed with fodders and grains.

T. gondii infection not only affects the development and reproduction of animals but also causes a certain amount of economic losses to the farms. Raw or half-raw eggs are one of the risk factors for human infection with *T. gondii*. Compared to south China, Henan Province has fewer lakes and smaller freshwater area. Therefore, livestock meat and chicken are primary meat sources of local people, and duck and goose are consumed less than that in south China. The toxoplasma prevalence in livestock in Henan province is lower compared to South China, and it is unknown whether this is related to the meat diet. Pigeon meat may occasionally be a source of *T. gondii* infection for hunters and other animals so that it could be a risk factor in humans or other animals.

The significant difference of toxoplasma infection rate was observed between cats and dogs. Cats have a high toxoplasma prevalence, while dogs are relatively low. The possible reason is that cats are the definitive hosts of T. gondii and play a role in the transmission of T. gondii to humans. In rural areas, domestic cats mostly wander outside and eat animals such as rodents and birds so that it may be the result of high positive rate. On the contrary, dogs are usually caged and have fewer outdoor activities. As we know, as the definitive host species with the largest population in the world, domestic cats have become the most important hosts of parasites, both evolutionally and epidemiologically[10].

Among the three foodborne animal categories, the positive rate of *T. gondii* is highest in pets, poultry is second, and the lowest is livestock, which suggests that free-range animals are more susceptible to be infected with *T. gondii* because they are more often exposed to materials contaminated with *T. gondii* oocysts (e.g., soil, fodder, and water) or tissue cysts (e.g., carcasses of small rodents or insectivores). Another reason may be attributed to Henan's climate. Located in the central of China, Henan Province belongs to transition climate between North Asia and warm temperate zone, with four seasons, abundant rainfalls, adequate sunlight, which increases oocysts' persistence and dispersion. Besides, based on geographical characteristics of Henan province, it is of considerable significance to understand the status of toxoplasma infection in livestock, poultry, and pets in Henan province for the control of Toxoplasma infection in China.

In recent years, with the increase of pets and the changes in the human diet, toxoplasmosis in human and animal has been on the rise worldwide. This study indicates that free-range animals are more susceptible to be infected by *T.gondii* from the environment and provide relevant information about the exposure of humans to *T. gondii* through the consumption of raw or undercooked meat. Our results are essential to improve the risk assessment of human contamination through foodborne meat manipulation and

consumption. Potential strategies include improving labeling of meat sources and processing types (e.g., bred indoors or frozen) and measures to reduce foodborne animal infections (e.g., improvement of farm hygiene).

Conclusion

Approximately 50% of all human toxoplasmosis cases are associated with foodborne infection. Location of *T. gondii* infection with delicate and particular methods is an essential progress in the prevention and protection of toxoplasmosis in humans and animals. In this study, Onetube-nested PCR was used to detect the epidemiological status of *T. gondii* in foodborne animals in Henan Province, China, to strengthen the prevention of foodborne diseases and develop animal husbandry.

Declarations

Ethics approval and consent to participate

This study was carried out in strict accordance with the National Natural Science Foundation of China ethical guidelines for biomedical research.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors contributed to this artitle. SW,XYZ,CKS and SGL conceived and designed the experiments.XXX,CZJ and PFB performed PCR assay. XLZ, RHZ,JQH and MYQ did data analysis and wrote manuscript. The author(s) read and approved the final manuscript.

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Figures

Figure 1

The map of sampling regions in Henan in this study. The sampling locations are marked in shadow. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

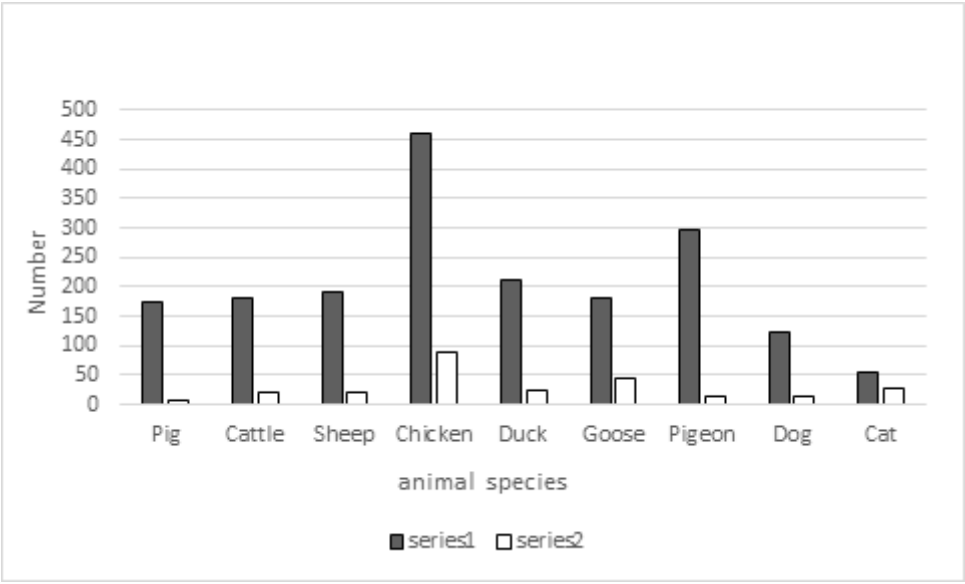

Figure 2

T.gondii infection in foodborne animals in Henan Province Series1 is the total number of samples. Series 2 is a positive number of samples

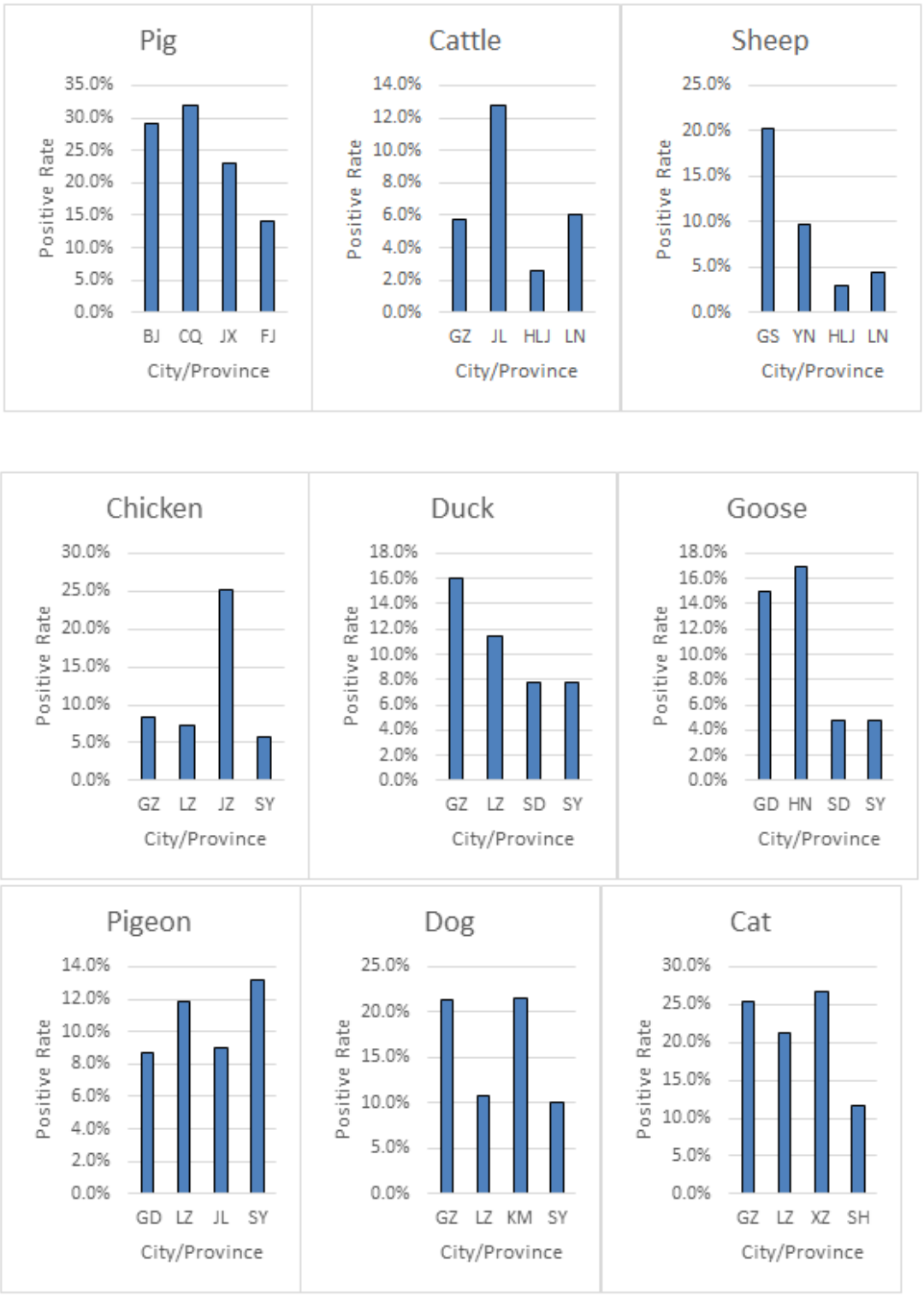

Figure 3

T.gondii positive rates in different provinces or cities.
BJ:Beijing,CQ:Chongqing,JX:Jiangxi,FJ:Fujian,GZ:Guangzhou,JL:Jilin,HLJ:Heilongjiang,LN:Liaoning,
GS:Gansu,YN:Yunnan,LZ:Lanzhou,JZ:Jingzhou,SY:Shenyang,SD:Shandong,HN:Hainan,
GD:Guangdong,KM:Kunming,XZ:Xuzhou,SH:Shanghai