Toxoplasma gondii and Neospora caninum in Free-Range Chickens in Henan Province of China

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Background. Chickens serve as an intermediate host for Toxoplasma gondii and Neospora caninum; infection of free-range (FR) chickens with these organisms is a useful indicator of soil and environmental contamination with oocysts. A total of 700 FR chicken serum samples and 300 heart samples were collected from Henan province from March to July 2015. Antibodies to T. gondii were found in 18.86% (132/700) of the chickens by modified agglutination test (cut-off 1:25), while 23.14% (162/700) were positive for N. caninum by indirect fluorescent antibody test (cut-off 1:25). T. gondii DNA was detected in the myocardium digestion liquids of 4/25 (16%) FR chickens. The PCR results of N. caninum DNA from FR chicken myocardium digestion liquids (𝑛=25) were all negative. Attempts to isolate viable T. gondii were unsuccessful. The results showed that there were antibodies to T. gondii and N. caninum in FR chickens from Henan province. Accordingly, effective control of feces from cats and dogs and improved pet hygiene habits were needed. To the author’s knowledge, this is the first report of the presence of N. caninum antibody in chickens from China.

1. Background

Many studies have evaluated Toxoplasma gondii and Neospora caninum infections in birds worldwide, including chickens [1, 2]. Chickens contribute to the T. gondii and N. caninum life cycle by acting as intermediate hosts or mechanical vectors [3, 4]. The main route of infection for chickens is assumed to be through ingestion of oocysts from soil. Limited reports of natural neosporosis in birds indicate that they do not show any significant histological lesions [4, 5]. Chickens are resistant to toxoplasmosis, and most T. gondii positive chickens remain asymptomatic. However, investigation of T. gondii and N. caninum infection in the free-range (FR) chickens is a useful indicator for assessment of soil and environmental contamination with oocysts.

Despite the high body temperature of avians (41°C), they could be infected by toxoplasmosis. Further, T. gondii tachyzoites have been observed in avian red blood cells [1]. Meat from chickens is consumed widely and is known to be the primary source of toxoplasmosis infection for humans. Viable T. gondii have been widely isolated from chicken heart, brain, and muscle in North America and Europe by bioassay in mice or cats [2]. China has the highest number of chickens in the world (approximately 4 billion, https://top5ofanything.com/list/0cffba91/Countries-With-the-Most-Chickens) and an unknown number of wild birds. However, only two viable T. gondii have been isolated from chickens in China [6, 7]. Reports of the genotype and pathogenicity of T. gondii revealed significant differences among populations in Asia and other parts of the world [2, 8]. Accordingly, further studies to develop methods of isolating T. gondii from China are warranted.

Few English papers have investigated the seroprevalence of T. gondii in birds from China. Yan et al. summarized the literature (1985–2008) pertaining to T. gondii prevalence in chickens from China [9]. Although chickens, sparrows, and pigeons have been shown to be intermediate hosts of N. caninum [4], there is little information regarding the N. caninum seroprevalence in chickens from China. The present study was conducted to investigate the prevalence of N. caninum
and *T. gondii* infections in FR chickens from central China and to isolate *T. gondii*.

## 2. Methods

### 2.1. Sera Samples

The study area was Henan province (33.54° N, 113.30° E), which is located in central China. A total of 700 FR chicken sera and 300 fresh hearts from individual farms in Henan province were collected (Figure 1 and Table 1). Clinical information (age, gender) regarding all chickens except batch 5 and batch 6 was obtained.

### 2.2. Examination of Chickens Sera for *T. gondii* and *N. caninum* Antibodies

Sera samples from 700 FR chickens were tested for antibodies to *T. gondii* using the modified agglutination test (MAT) [2]. *T. gondii* positive serum and *N. caninum* positive serum from mice were provided by Dr. J. P. Dubey (ARS, USDA) as reference sera. Whole formalin-treated *T. gondii* tachyzoites antigens were obtained from Kerfast Company (catalog number EH2002). Additionally, 700 serum samples were tested for *N. caninum* antibodies by the indirect fluorescent antibody test (IFAT). The 96-well IFAT plates (whole formalin fixed *N. caninum* tachyzoites) were kindly provided by Dr. Q. Liu (China Agricultural University, China). The IFAT secondary antibody was goat anti-chicken IgY H&L labeled with Alexa Fluor 488 (Abcam Company, ab150169). Positive control, negative control, and blank were performed on each plate. A titer of 1:25 was considered to indicate exposure to *T. gondii* in chickens [2, 3]. In addition, all serum samples were tested for *T. gondii* parasites at 1:50, 1:100, and 1:200 dilutions. IFAT was used to test chicken serum *N. caninum* antibody (cut-off: 1:25), and only bright fluorescence of the whole tachyzoite surface was considered as a positive result [10].

### 2.3. Isolation of Viable *T. gondii* from Chicken Tissues by Bioassay of Kunming Mice

Chicken myocardia (10 g) from the samples with MAT of *T. gondii* seropositive (MAT, ≥50) chickens (*n* = 25) were digested individually in pepsin and bioassayed in Kunming (KM) outbred mice as previously described [2]. Each heart homogenate was inoculated subcutaneously into two mice. All inoculated mice were observed daily for illness. Dead mice were examined for *T. gondii* by making impression smears from the lung or brain, which were inoculated into a new group of mice regardless of whether they were negative or positive. Survivors were bled on 42 days postinoculation (DPI) and 1:25, 1:200 dilution of serum from the mouse was tested for *T. gondii* antibodies using the MAT. Mice were killed after 49 DPI, and brains of all mice were examined for tissue cysts and inoculated into new groups of mice. The sheep hearts containing *T. gondii* cysts were used as protocol control.

### 2.4. DNA Isolation and Polymerase Chain Reaction (PCR) Identification of *T. gondii* and *N. caninum*

Chickens myocardium digestion liquids from chicken hearts were also used to detect *T. gondii* DNA. The DNA isolated from *T. gondii* (CTI strain) or *N. caninum* (NCI strain) was used as a reference for PCR. CTI strain *T. gondii* was kindly provided by Dr. J. P. Dubey (ARS, USDA). NCI strain *N. caninum* was kindly provided by Dr. Q. Liu (China Agricultural University, China). The DNA of digestion heart samples was extracted.
Table 1: Prevalence of *T. gondii* in free-range chickens from Henan of China.

| Batch number | Location | City/sample reception date | Number of samples received | Number of seropositive samples (cut-off: 1:25, %) | Isolates obtained by mice from hearts a |
|--------------|----------|----------------------------|----------------------------|-----------------------------------------------|----------------------------------------|
| 1            | I        | Xinyang/9 March 2015       | 201                        | 56 (27.9)                                    | —                                      |
| 2            | III      | Xinxiang/9 March 2015      | 40                         | 3 (7.5)                                       | —                                      |
| 3            | IV       | Jiyuan/9 March 2015        | 68                         | 10 (14.7)                                    | —                                      |
| 4            | II       | Nanyang/9 March 2015       | 14                         | 6 (42.9)                                     | —                                      |
| 5            | I        | Xinyang/8 May 2015         | 7                         | 2 (28.6)                                     | 0/2                                    |
| 6            | I        | Xinyang/10 May 2015        | 51                        | 12 (23.5)                                    | 0/8                                    |
| 7            | V        | Zhoukou/14 May 2015        | 62                         | 29 (46.8)                                    | 0/15                                   |
| 8            | I        | Xinyang/20 May 2015        | 13                         | 0                                             | —                                      |
| 9            | I        | Xinyang/24 May 2015        | 14                         | 1 (7.1)                                       | —                                      |
| 10           | I        | Xinyang/2 June 2015        | 15                         | 0                                             | —                                      |
| 11           | I        | Xinyang/9 June 2015        | 17                         | 0                                             | —                                      |
| 12           | I        | Xinyang/1 July 2015        | 7                          | 0                                             | —                                      |
| 13           | I        | Xinyang/8 July 2015        | 19                         | 13 (6.8)                                     | —                                      |
| **Total**    |          |                            | 700                        | 132 (18.86)                                  | 0/25                                   |

aNumber of positive groups/number of inoculated groups.
bNo heart tissue available.
cNo detailed information regarding chickens.
dSampling city in Figure 1.

using a commercial DNA extraction kit (Tiangen Biotec Company, DP304). PCR assays for *T. gondii* and *N. caninum* were performed using the specific primer pairs TOX5/TOX8 (5'-CGCTGACAGCATGCTGGAT3' and 5'-CCCAGCTCGCTCTGGCGAT-3') and NP6/NP21 (5'-CAGTCACCTACGTCTTCT-3' and 5'-GTGCGTCCAATCCTGTAAC-3') [11, 12]. The products from *T. gondii* were expected to be 450 bp. *N. caninum* expected products were 328 bp. Briefly, *T. gondii* reaction cycle was as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of amplification (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min) and then final extension at 72°C for 10 min. *N. caninum* reaction cycle was as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of amplification (94°C for 1 min, 53°C for 1 min, and 72°C for 2 min) and then final extension at 72°C for 10 min.

2.5. Statistical Analysis. Statistical analysis was performed by GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed by the Chi-square test or Fisher's exact test to determine the association between infection with each parasite and independent risk factors: gender (male and female) and age (≤30 days, >30 days). *p* < 0.05 was considered statistically significant.

3. Results and Discussion

Antibodies to *T. gondii* were found in 18.86% (132/700) of FR chickens from Henan province of China (Table 1). Seropositivity rates varied with respect to gender, with antibodies being found in 33.59% of males and 14.48% of females (*p* < 0.01) (Tables 2 and 3). This was probably due to a greater probability of exposure to *T. gondii* oocysts in males because of the active characteristic of roosters. Many studies indicated that gender was not a risk factor for toxoplasmosis [1, 13]. However, *T. gondii* change the concentration of steroid hormones in the host and enhance the susceptibility of males or females to toxoplasmosis; it was decided by the stains *T. gondii* [14, 15]. A high percentage of male sheep were also positive for *T. gondii* antibody based on serological survey of China (*p* > 0.05) (Feng et al., unpublished data).

The seroprevalence of *T. gondii* is age related, with higher seroprevalence being observed in older chickens (>30 days, 18.75%) than in younger chickens (≤30 days, 15.85%); however, this difference was not significant (Tables 2 and 3). It is worth noting that 15.85% of 82 young chickens were positive for *T. gondii* antibody, indicating maybe vertical transmission from embryos.

Among the 700 FR chickens tested for *N. caninum* antibodies, 23.14% (162/700) were positive (Tables 2 and 3). The PCR results of *N. caninum* DNA from FR chicken myocardium digestion liquids (n = 25) were all negative. Age and gender showed no association with seroprevalence (*p* > 0.05). Additionally, 3.57% (25/700) FR chickens mix was exposed to the *T. gondii* and *N. caninum*. Moreover, 25.61% (21/82) of younger chickens (<30 days) were seropositive for *N. caninum*. An epidemiological survey of cattle and dogs supported the postnatal transmission of *N. caninum* [16, 17]. The high seropositive rate in younger chickens indicated that *N. caninum* transmission possibly occurs via eggs in birds. However, vertical transmission by *N. caninum* in chickens was not detected by microscopy, PCR, or BALB/c mice bioassays [18]. The methods of microscopy and PCR were specific and sensitive, but there were limitations for only
Table 2: Seroprevalence of T. gondii and N. caninum infection in free-range chickens.

| Characteristics          | Number tested | Positive number in different titers | Seroprevalence % (positive number) | 95% CI |
|-------------------------|---------------|-------------------------------------|------------------------------------|--------|
|                         |               | 1:25  | 1:50 | 1:100 | Above 1:200 |               |                     |
| T. gondii (MAT, cut-off: 1:25) |
| Gender: female          | 511           | 46    | 10   | 4     | 14          | 14.48 (74)    | 11.68–17.81        |
| Gender: male            | 131           | 37    | 2    | 2     | 3           | 33.59 (44)    | 26.06–42.05        |
| Age (days) ≤ 30         | 82            | 10    | 0    | 0     | 3           | 15.85 (13)    | 9.37–25.40         |
| Age (days) > 30         | 560           | 73    | 12   | 6     | 14          | 18.75 (105)   | 15.73–22.20        |
| No information*         | 58            | 9     | 0    | 0     | 5           | 24.14 (14)    | 14.85–36.64        |
| Total                   | 700           | 92    | 12   | 6     | 22          | 18.86 (132)   | 16.13–21.93        |
| N. caninum (IFAT, cut-off: 1:25) |
| Gender: female          | 511           | 123   | 3    | 3     | 3           | 24.07 (123)   | 20.56–27.97        |
| Gender: male            | 131           | 27    | 3    | 3     | 3           | 20.61 (27)    | 14.52–28.38        |
| Age (days) ≤ 30         | 82            | 21    | 2    | 2     | 2           | 25.61 (21)    | 17.34–36.06        |
| Age (days) > 30         | 560           | 129   | 12   | 12    | 12          | 23.04 (129)   | 19.73–26.70        |
| No information*         | 58            | 12    | 12   | 6     | 6           | 20.69 (12)    | 12.10–32.92        |
| Total                   | 700           | 162   | 6    | 0     | 0           | 23.14 (162)   | 20.17–26.41        |

*No detailed information (gender, age) regarding chickens.

Table 3: Odds ratio of gender and age of chickens as risk factors for T. gondii and N. caninum.

| Factor          | Category | OR     | 95% CI       | p value |
|-----------------|----------|--------|--------------|---------|
|                 |          | T. gondii |              |         |
| Gender          | Female   | 2.987  | 1.926–4.630 | < 0.0001*|
|                 | Male     |        |              |         |
| Age (days) ≤ 30 |          | 1.225  | 0.653–2.299 | 0.6471  |
| Age (days) > 30 |          | 1.150  | 0.675–1.961 | 0.5795  |
|                 | N. caninum |          |              |         |
| Gender          | Female   | 1.221  | 0.764–1.953 | 0.4874  |
|                 | Male     |        |              |         |
| Age (days) ≤ 30 |          | 1.650  | 0.933–2.890 | 0.0846  |
| Age (days) > 30 |          | 1.350  | 0.798–2.301 | 0.2786  |

OR: odds ratio; "*" indicates significant difference.

checking few samples. It was temporal tissue distribution and parasite loads during N. caninum infection in BALB/c murine model [19]. Gerbils, immunodeficient mice, and cell cultures were the successful model for isolating N. caninum from tissues [20]. The exact evidence for N. caninum infection from hen to egg needs to be further investigated and proved.

T. gondii was not isolated from the mice inoculated with tissues of any chickens hearts in this study. The sheep hearts containing T. gondii cysts were used as protocol control. This excludes the errors of the protocol, such as pH of the solutions, enzymatic activity, and period of digestion. It has been shown that the density of T. gondii cysts in hearts is higher than in brain or muscle in chicken, and the heart is the ideal choice for isolation of T. gondii [2,21]. T. gondii bioassays in cats are more sensitive than in mice. The use of gamma interferon gene knockout mice or immunosuppressed mice facilitates early detection of T. gondii because most strains of T. gondii are asymptomatic in outbred mice. Sreekumar did not isolate any viable T. gondii from 186 (133 MAT seropositive) chicken tissues from India by mouse bioassay [22]. However, Qian successfully isolated T. gondii (11 isolations from 23 cat tissues) using immunosuppressed Kunming mice [23]. T. gondii could grow efficiently in macrophages of rats treated with glucocorticoids [24]. The unsuccessful isolation may be related to the low density of T. gondii cysts burden in chickens of different regions or the natural resistance to T. gondii infection in some strains of mice. PCR indicated that the presence of T. gondii DNA was 16% (4/25) in FR chicken myocardium digestion liquids, which yielded PCR product of approximately 450 bp. The low infection rate observed upon molecular analysis verified the light burden of T. gondii cysts in chickens from central China.

4. Conclusions

Our results showed that there were antibodies to T. gondii and N. caninum in FR chickens from Henan province of China. The contamination environment was the source of infection with toxoplasmosis for humans and other animals. Accordingly, effective control of feces from cats and dogs and improved pets hygiene habits were needed.

Ethical Approval

This study was approved by the institutional animal use protocol committee of the Henan Agricultural University, China.

Competing Interests

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.
Authors’ Contributions

Yongjie Feng and Yaoyao Lu performed the laboratory tests and data analysis and drafted the paper. Yinghua Wang participated in the collection of samples and the laboratory tests. Jing Liu helped in the study design. Longxian Zhang helped in the writing of the paper. Yurong Yang designed the study protocol, analyzed the results, and wrote the paper. All authors read and approved the final version of the paper. Yongjie Feng and Yaoyao Lu are equal contributors.

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References

[1] J. P. Dubey, “A review of toxoplasmosis in wild birds,” Veterinary Parasitology, vol. 106, no. 2, pp. 121–153, 2002.
[2] J. P. Dubey, Toxoplasmosis of Animals and Humans, CRC Press, Taylor & Francis, Boca Raton, Fla, USA, 2nd edition, 2010.
[3] J. P. Dubey, “Toxoplasma gondii infections in chickens (Gallus domesticus): prevalence, clinical disease, diagnosis and public health significance,” Zooneses and Public Health, vol. 57, no. 1, pp. 60–73, 2010.
[4] S. L. Donahoe, S. A. Lindsay, M. Krockenberger, D. Phalen, and J. Šlapeta, “A review of neosporosis and pathologic findings of Neospora caninum infection in wildlife,” International Journal for Parasitology: Parasites and Wildlife, vol. 4, no. 2, pp. 216–238, 2015.
[5] T. W. P. Mineo, A. O. T. Carrasco, T. F. Rasó, K. Werther, A. A. Pinto, and R. Z. Machado, “Survey for natural Neospora caninum infection in wild and captive birds,” Veterinary Parasitology, vol. 182, no. 2–4, pp. 352–355, 2011.
[6] L. Wang, H.-W. Cheng, K.-Q. Huang et al., “Toxoplasma gondii prevalence in food animals and rodents in different regions of China: isolation, genotyping and mouse pathogenicity,” Parasites and Vectors, vol. 6, no. 1, article 273, 2013.
[7] G.-W. Zhao, B. Shen, Q. Xie et al., “Isolation and Molecular Characterization of Toxoplasma gondii from chickens in China,” Journal of Integrative Agriculture, vol. 11, no. 8, pp. 1347–1353, 2012.
[8] E. K. Shwab, X.-Q. Zhu, D. Majumdar et al., “Geographical patterns of Toxoplasma gondii genetic diversity revealed by multilocus PCR-RFLP genotyping,” Parasitology, vol. 141, no. 4, pp. 453–461, 2014.
[9] C. Yan, C. L. Yue, Z. G. Yuan et al., “Toxoplasma gondii infection in domestic ducks, free-range and caged chickens in southern China,” Veterinary Parasitology, vol. 165, no. 3–4, pp. 337–340, 2009.
[10] J. Martins, O. C. H. Kwok, and J. P. Dubey, “Seroprevalence of Neospora caninum in free-range chickens (Gallus domesticus) from the Americas,” Veterinary Parasitology, vol. 182, no. 2–4, pp. 349–351, 2011.
[11] G. Scharas, D. C. Herrmann, A. Beckert et al., “Characterization of a repetitive DNA fragment in Hammondia hammondi and its utility for the specific differentiation of H. hammondi from Toxoplasma gondii by PCR,” Molecular and Cellular Probes, vol. 22, no. 4, pp. 244–251, 2008.