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Full Length Article

Seroprevalence of *Toxoplasma gondii* in the Backyard Chickens of the Rural Areas of Faisalabad, Punjab, Pakistan

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Abstract

This study reports the seroprevalence of *Toxoplasma (T.) gondii* in backyard chickens of rural areas of district Faisalabad, Punjab, Pakistan. Backyard chickens (n=300) were selected randomly from five different villages of district Faisalabad, Pakistan. Blood samples were collected randomly and subjected to Latex agglutination test for screening of *T. gondii*. Seropositive chickens were sacrificed to collect blood. Vital organs including heart and brain were also collected for histopathological examination and mouse bioassay. Overall seroprevalence of *T. gondii* was 36.33%. Area- and sex-wise seroprevalence was detected as non-significant (P>0.05). Age-wise analysis showed highest seroprevalence rate (57.14%) in chickens of age group ranging from 1.5-2 years (P=0.00). The chickens kept along with pet cats showed higher seroprevalence (53.89%; 95% CI=0.401, 1.375) as compared to those kept without pet cats. Feeding and watering patterns showed non-significant (P= 0.085; OR=0.643) impact on the seroprevalence of *T. gondii*. In mouse bioassay, toxoplasmosis was reproduced only in 40% of the mice population being infected. Histopathological studies revealed congestion, necrosed areas and inflammatory cells in brain and heart. Findings of the present study concluded that infection of backyard chickens with *T. gondii* is prevalent in Faisalabad, Pakistan which may have significant public health concerns and implications for prevention and control of toxoplamosis in this area.

Keywords: *Toxoplasma gondii*; Seroprevalence; Backyard chickens; Rural areas; Pakistan

Introduction

Toxoplasmosis, caused by *Toxoplasma (T.) gondii*, is one of the most important zoonotic diseases with a worldwide geographical distribution (Dubey and Beattie, 1988; Amin *et al.,* 2013). All warm blooded animals and human beings can be affected by this organism. According to an estimate, approximately 25% of the world population is carrying this parasite (Petersen, 2007; Ahmad *et al.*, 2013). Ingestion of contaminated food or water with oocysts shed by the cats or by accidental ingestion of raw or undercooked meat containing the tissue cysts could be the reason of transmission of the parasite to human beings (Tenter *et al*., 2000). Among human population, toxoplasmosis is very common and its frequency usually differs depending upon the geographical area and almost every third person is infected with toxoplasmosis in Iran (Hamzavi *et al.,* 2007). Intermediate hosts for this parasite are all mammals and birds including the human beings; whereas, felids act as both definitive and intermediate host for the parasite (Waree, 2008).

Backyard poultry is one of the important sources of transmission of this infection due to their habit of choosy feeding from the ground which contained the oocysts shed by the cats. Man acquires infection with *T. gondii* by consumption of raw and undercooked infected meat of intermediate hosts like poultry birds, or by ingestion of sporulated oocysts via consumption of contaminated food and water (Dubey and Jones, 2008). Like other infections in mammals, poultry birds do not show specific clinical signs and follow a sub clinical course. However, some clinical disorders like lethargy, feed refusal, dyspnea, mild fever and rarely diarrhea may be observed (Olivier *et al.,* 2007). Due to the difficulty in the diagnosis, different serological tests are being used for the diagnosis of toxoplasmosis (Bueno *et al.,* 2010). Previous studies showed that a small proportion of affected individuals acquired the infection but the

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majority got exposure to *T. gondii* by ingestion of undercooked or raw meat containing tissue cysts, ingestion of oocysts shed by infected cats or consumption of contaminated drinking water or fresh vegetables (Ghazaei, 2006). *T. gondii* has been demonstrated in backyard poultry in different countries including Egypt (El-Massry *et al*., 2000), Brazil (de Silva *et al*., 2003) and India (Devada *et al*., 1998) but no data in this regard is available in Pakistan. Information on the parasite pervasiveness and environment is essential to understand the transmission pathways between man and animals; and to design strategic measures to control the toxoplasmosis.

Keeping in view, the present study was designed to find out the seroprevalence of *T. gondii* in backyard poultry of rural areas of district Faisalabad, Punjab, Pakistan. Attempts were also made to demonstrate the organism from serologically positive chickens by Mouse Bioassay. Results of the present study will be helpful to develop appropriate countermeasures against toxoplasmosis.

Materials and Methods

The Study Period and Area

The field studies were carried out during a period of 1 year (July 2011 to June 2012) on backyard chickens kept under free range conditions located in five different villages of district Faisalabad (31°21′52″N, 72°59′40″E), which extends about 16,000 km^2 in northeast Punjab, Pakistan. The villages were selected by random sampling using lottery method. The selected villages included Chak No. 97RB (Johal), Chak No. 210RB (Lakhuana), Chak No. 213RB (Manawala), Chak No. 225 RB (Malkhanwala) and Chak No. 74 JB (Thikriwala). The average annual temperature of study area is 17.4-21.6°C and annual precipitation is 300 mm. The climatic conditions of the study area feature it as an arid climatic zone (the information was obtained from Faisalabad district Government website: http://www.faisalabad.gov.pk/default.aspx).

Sampling and Data Collection

A total of 300 blood samples (n=60, from each village) were collected from five selected villages through random sampling to get the sera samples. All the samples were properly labeled and brought to Immunoparasitology Laboratory, Department of Parasitology, University of Agriculture, Faisalabad (UAF), Pakistan under cold conditions by placing them in ice packs. All the sampled chickens were marked and labeled with plastic rings to trace back the seropositive cases. The necessary data of each and every chicken was collected on a questionnaire, developed to record the information regarding area, age, sex, general body conditions, feeding pattern, watering pattern and presence of pet animals kept, if any.

Serological Diagnosis of *Toxoplasma gondii*

The collected sera samples were screened for anti-*Toxoplasma gondii* antibodies by Latex agglutination test using commercially available kit (Global invitro® LLP, UK). The assay was performed according to the manufacturer's instructions.

Procurement of Seropositive Chickens and Bioassay in Mice

The seropositive chickens were traced back and purchased followed by collection of their vital organs including hearts and brains. Half of the hearts and brains were preserved in 10% neutral buffered formalin for histopathological examination (Bancroft and Gamble, 2008) and the remaining half were stored at 4ºC for use in the Mouse Bioassay (Garcia *et al*., 2006). Briefly, hearts and brains from seropositive chickens were pooled and homogenized in five volumes of physiological saline (0.89% NaCl; pH 7.2). Suspension thus obtained was mixed with peptic digestive fluid (1.3 g pepsin (Avonchem®, UK) + 2.5 g NaCl (Merck®, Germany) + 3.5 mL conc. HCl (Merck®, Germany) + 500 mL distilled water). Digesta was homogenized and homogenate was incubated for 60 min in a vortex shaker at 37ºC followed by centrifugation at 3000×g for 20 min. The supernatant from the homogenate after adding Pencillin (100 IU/mL) and Streptomycin (100 µg/mL) was inoculated subcutaneously into six experimental mice. Mice were monitored up to seven days for illness, clinical signs and/or mortality. Dead mice were subjected to postmortem examinations and necropsy findings were recorded. From dead mice, tissue impression smears were prepared and stained with Geimsa stain (Sigma-Aldrich®, USA) for examination of *T. gondii* tachyzoites or tissue cysts. The survived mice were bled on day 5th post-inoculation and were tested for *T. gondii* antibodies with Latex agglutination test as described in previous section.

Statistical Analysis

Data thus collected was analyzed statistically through odds ratios and confidence interval at a level f 95%; whereas, chi-square test was also applied on percent prevalence.

Results

Overall, Area, Sex, Age and Pets Associated Seroprevalence of *Toxoplasma gondii*

One hundred and nine out of 300 sera samples (36.33%) were positive for antibodies against *T. gondii* by Latex agglutination test. Results of the area-wise analysis of seropositivity of *T. gondii* showed the highest prevalence (45%) in Chak No. 210 RB (Lakhuana) followed by those in Chak No. 213 RB (Manawala), Chak No. 74 JB (Thikriwala), Chak No. 225 RB (Malkhanwala) and Chak

No. 97 RB (Johal), respectively. Although apparently higher seroprevalence was recorded in Chak No. 210 RB (Lakhuana) but statistical analysis revealed a non-significant difference (P=0.485; χ^2= 2.448) in the rate of area-wise seroprevalence of *T. gondii* in selected villages (Fig. 1).

Sex-wise study on the distribution of toxoplasmosis revealed that out of total sera samples (n=60) collected from male birds, 21 samples were positive for *T. gondii* antibodies with a seroprevalence rate of 35%. On the other hand, a bit higher seroprevalence rate (36.67%; n=88/240) was detected in female birds; whereas, statistical analysis demonstrated a non-significant difference between the sexes (OR= 0.930; 95% CI= -0.664, 0.519) (Table 1).

The data collected from the serological analysis was arranged into four different groups (<1 year; >1 year but <1.5 year; >1.5 year but < 2 years and > 2 years) based upon the age groups recorded for each bird at the time of sampling. Chickens of age group (>1.5 but < 2 years) showed the highest rate of seroprevalence (57.14%) followed by those in age groups > 2 years; >1 year but <1.5 year; and <1 year, respectively. The statistical analysis showed that age wise distribution of *T. gondii* differ significantly (P=0.000; χ^2= 43.83) in different age groups (Table 1).

Out of 300 samples, 154 samples were taken from where pet cats were kept along with chickens. The association of pet cats and toxoplasmosis in rural poultry showed that seroprevalence of *T. gondii* was significantly higher (53.89%; n= 71/154) (P= 0.00; χ^2= 13.059) in the sera samples of chickens where cats were kept as pet animals as compared to those without cats (26.02%; n=38/146) (OR= 2.431; 95% CI= 0.401, 1.375) (Table 1).

Table 1: Seroprevalence of *Toxoplasma gondii* in relation to sex, age and pets in the backyard poultry of rural areas in district Faisalabad, Pakistan

Factors	Positive/Total samples	Sero-prevalence (%)	P-Value	χ^2-Value	Odds ratio (95% CI)
Sex wise sero-prevalence					
Male	21/60	35.00	0.810	0.058	0.930 (-0.664, 0.519)
Female	88/240	36.67			
Age wise seroprevalence					
<1 year	08/90	8.88	0.00	43.83	-
>1 but <1.5 year	55/122	45.08			
>1.5 but <2 year	24/42	57.14			
>2 years	22/46	47.82			
Pet wise seroprevalence					
With cats	71/154	53.89	0.00	13.059	2.431 (0.401, 1.375)
Without cats	38/146	26.02			

At P<0.05, statistical difference is significant

Table 2: Seroprevalence of *Toxoplasma gondii* in relation to feeding and watering pattern in the backyard poultry of rural areas in district Faisalabad, Pakistan

Factors	Positive/Total samples	Sero-prevalence (%)	P-Value	χ^2-Value	Odds ratio (95% CI)
Feeding pattern wise seroprevalence					
Ground feeding	54/148	36.48	0.957	0.003	1.013 (-0.458, 0.484)
In feeders	55/152	36.66			
Watering pattern wise seroprevalence					
Natural source	32/107	29.90	0.085	2.97	2.431 (-0.946, 0.062)
Drinkers	77/193	39.89			

At P<0.05, statistical difference is significant

Feeding and Watering Pattern Wise Distribution of *Toxoplasma gondii* in the Backyard Poultry

Results of relationship between feeding pattern and seroprevalence of *T. gondii* showed that seroprevalence was higher (36.48%; n=54/148) in chickens reared on ground feeding as compared to those being offered feed in specialized feeders (36.18%; n=55/152); although the difference was statistically non-significant (χ^2= 0.003; P= 0.957; OR=1.013; 95% CI= -0.458, 0.484) (Table 2).

Moreover, seroprevalence of *T. gondii* was higher (39.89%; n=77/193) in birds being offered clean drinking water in specialized drinkers as compared to those drinking water from sewerage channels and/or stagnant water etc. Statistical relationship of drinking pattern of water in birds and seroprevalence of *T. gondii* showed a non-significant difference (χ^2= 2.97; P= 0.085; OR=0.643; 95% CI= -0.946, 0.062) (Table 2).

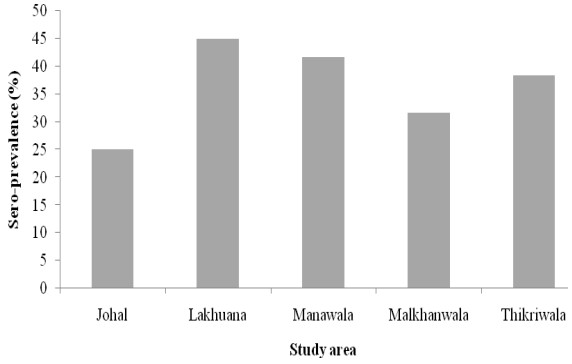

Fig. 1: Area-wise seroprevalence of *Toxoplasma gondii* in the backyard poultry of rural areas of district Faisalabad, Pakistan. All values are statistically similar (P=0.485; χ^2= 2.448)

Mouse Bioassay

Sixteen hours post-inoculation of heart and brain suspension, two mice (M1 and M3) were found dead. Post mortem findings of dead mice revealed multiple hemorrhages on the skin and sub-cutaneous tissue, pulmonary congestion and hemorrhages and paler kidneys in M1; whereas, in M3, skin and lungs were normal but kidneys were swollen. Moreover, streaks on the liver and

congestion of heart with clotted blood were also observed in M3 mice. On 2nd day, decreased feed intake was observed in all the survived mice and on 60 h post inoculation nostril muscles along with eyes of one mice (M4) were found swollen. On 3rd day, all the mice showed progressive weight loss and somewhat abnormal behavioral signs including reduced feed intake. On day 4th, survived mice became emaciated. Moreover, left hind legs of all three mice were swollen. On day 5th, survived mice were sacrificed for serological testing to detect the anti-*T. gondii* antibodies and histopathological studies.

Screening of Experimentally Infected Mice for Anti-*Toxoplasma gondii* Antibodies

Results of Latex agglutination test revealed that two mice M2 and M4 were positive for *T. gondii*; whereas, serum of M5 mice did not showed a positive reaction to *T. gondii* antigen. Moreover, tissue impression smears from all the mice were prepared using vital organs such as brain, heart, liver, kidneys. Brain smears of M2 and M4 showed the presence of tissue cysts in the brain.

Histopathological Findings

Chicken: In brain tissue, mild to moderate degree of congestion in the thalamus region indicating the inflammation was observed. Microglia and individual cell necrosis was also recorded. At few places, inflammatory cells were also present around the blood vessels indicative of perivascular cuffing. Chromatolysis of neurons was present at few places. At few places, small necrotic zones were present while most of the places were normal. In heart tissue, mild to moderate degree of congestion was also present in some myocardial fibers. Myocardial infarction was present as indicated by the necrotic changes. Inflammatory cells were also present in the heart. Coagulative type of necrosis was also present at some places that was indicative of severe inflammatory reaction.

Mice: In brain, inflammatory zones were present in the thalamus region. Individual cell necrosis was also present. At few places, zones of necrotic cells were seen. Mild to moderate degree of congestion and zones of inflammatory cells were present. In heart, individual myocardial fibers necrosis was noted, indicative of mild to moderate congestion. Myocardial infarction was indicated by the necrotic changes. Inflammatory cells and coagulative necrosis due to severe inflammatory reaction was also observed.

Renal tissues showed mild degree of congestion in renal parenchyma. Urinary spaces in glomeruli were clear. At few places nuclei of tubular epithelial cells were condensed and pyknotic, while on other places these were normal in appearance. In liver, mild degree of vacuolar degeneration was present. Sinusoidal places were dilated at few places. Cell swelling and individual cell necrosis of hepatocytes was also present. Plasma cells were observed at the site of inflammation. Bile duct hyperplasia was also recorded in the portal area.

Discussion

Toxoplasmosis is a widespread zoonotic disease caused by the coccidian protozoan, *T. gondii.* It is an important cause of abortion, still birth and certain other reproductive disorders in different animal species including human beings (Dubey and Beattie, 1988). Cats serve as definitive hosts for *T. gondii*; whereas, human beings may acquire *Toxoplasma* infection either by ingestion of sporulated oocysts or via ingestion of bradyzoites in the tissues of food animals (Dubey and Jones, 2008; Esteban *et al.,* 1995). Backyard poultry is considered to be a potential source for spread of this disease as people keep them for egg and meat purpose especially in the rural areas. The diagnosis of toxoplasmosis is conventionally made by the direct demonstration and isolation of the parasite from autopsy or biopsy samples, but such techniques are unsuitable for use in large scale surveys. Therefore, several serological tests are being employed to detect *T. gondii* antibodies in mammalian sera including indirect haemagglutinaion, modified agglutination test, latex agglutination test, indirect fluorescent antibody technique, enzyme linked immunosorbent assay, Sabin Feldman dye test and complement fixation test (Karaca *et al.,* 2007; Sevgili *et al.,* 2005; van der Puije *et al.,* 2000; Nieto and Melendez, 1998).

Several attempts have been made around the globe to detect the prevalence rate of toxoplasmosis in different animal species including lambs (Dubey *et al*., 2008), goats (Tasawar *et al.,* 2011), wild avian species (Darwich *et al*., 2012), pigeons (Lima *et al*., 2011), chickens (Dubey *et al.,* 2003a; 2004a; 2007a) etc. which are considered to be important source of transmission of this disease to human beings. In Pakistan, a few prevalence surveys have been conducted in different animal species (Ramzan *et al*., 2009; Tasawar *et al*., 2011, 2012) but so far no work has been done on the prevalence of toxoplasmosis in backyard poultry that may serve as an important source of the transmission of disease in human beings.

In current study, the overall seroprevalence of *T. gondii* in backyard poultry of district Faisalabad vicinity was found 36.33%; whereas, some previous studies showed a much higher prevalence rate of *T. gondii* in different countries of the world i.e. 66% in Amazon, Brazil (Dubey *et al*., 2006a); 64% in Ghana (El-Massry *et al*., 2000); 55% in Chile (Dubey *et al.,* 2006b); 53% in Argentina (More *et al.,* 2012); 44.4% in Colombia (Dubey *et al.,* 2005c) and 39% in Sri lanka (Dubey *et al*., 2005d) . On the other hand, a lower prevalence rate has also been reported in some regions including China (36%) (Zhao *et al.,* 2012), Egypt (28%) (Dubey *et al.,* 2003b), China (27%) (Yan *et al.,* 2009), Indonesia (26.6%) (Dubey *et al*., 2008); India (17.9%)

(Sreekumar *et al.,* 2003) and Israel (18%) (Dubey *et al.,* 2004b).

In the current study, variation in area-wise seropositivity of *T. gondii* might be correlated with customs, traditions, life style of the inhabitants, age of the animals and husbandry practice (Smith, 1991). Apart from this, prevalence rate may also be associated with the presence of cats that excrete oocysts which after sporulation become infectious to man and animals (Ghorbani *et al.,* 1990). In present study, the area-wise difference in seropositivity of *T. gondii* in backyard poultry may also be attributed to the differences in animal husbandry practices, geographical conditions and animal welfare (Yun *et al.,* 2011).

In various earlier reports, wide variation in the seropositivity of *T. gondii* in chickens had also been reported in different regions of a country including Argentina (Dubey *et al.,* 2003d), Brazil (Dubey *et al.,* 2003c; 2006a; 2007b; de Silva *et al.,* 2003; de Oliveria *et al.,* 2009), India (Devada *et al.,* 1998; Sreekumar *et al.,* 2003) and United States of America (Dubey *et al.,* 2003a; 2007c).

Relationship of toxoplasmosis with sex of the birds revealed a non-significantly higher seroprevalence of *T. gondii* antibodies in females as compared to males. Generally, female animals are reported to be more susceptible to protozoan parasites as compared to male (Alexander and Stinson, 1988). Furthermore, in mice model female mice reported to be more sensitive to pathogenic symptoms of toxoplasmosis than male (Roberts *et al*., 1996). Some other previous reports had also shown higher seroprevalence rates of toxoplasmosis in females as compared to males of dogs (Bharathi *et al.,* 2011), goats (Ramzan *et al*., 2009; Tasawar *et al*., 2011) and human beings (Haldar *et al*., 1993); whereas, Tasawar *et al.* (2012) reported a higher prevalence rate in males as compared to females. The differences in the hormonal profiles of males and females may play an important role in determining the susceptibility to parasitic infections (Miller, 1990). It is widely accepted that certain hormones including the sex-associated hormones directly influence the immune system (Roberts *et al*., 2001). It has been reported that estrogen enhances antibody production and androgen suppress both T- and B- cell immune responses (da Silva, 1999), but immunity in females can be broken down due to various factors including nutrition, age, reproductive and certain environmental factors (Tasawar *et al*., 2012).

In this study, a significant difference (P= 0.00) was detected in different age groups in seropositivity to *T. gondii*. The highest seroprevalence (54.14%) was detected in older birds (>1.5 years but < 2 years); whereas, the group of youngest birds revealed lowest prevalence rate (8.8%). A direct correlation of seroprevalence of *T. gondii* antibody with age of the animals might be related to the fact that as animal became older, its cumulative likelihood for exposure increased or older birds had more opportunities to get infection than the younger ones (Zhao *et al*., 2012). A significantly higher seropositivity rate was detected in chickens which were kept along with cats that might be correlated with the fact that cats shed the oocysts, contaminated the soil with *T. gondii* and chickens due to their habits of scratching the earth and feeding, facilitated the greater access to the hidden feces of cats (Dubey *et al*., 2008). Role of cats, as definitive host of *T. gondii,* in the transmission of disease to different animals including chicken had also been reported in some previous studies (Zhao *et al*., 2012; Yan *et al*., 2009).

A non-significant relationship between the seroprevalence of *T. gondii* and feeding/watering patterns in backyard chickens was detected. Although, a positive correlation between the prevalence of *T. gondii* and feeding pattern in free range chickens had been reported by Dubey *et al.* (2002); whereas, in the present study independence of the prevalence of *T. gondii* to feeding habit was observed which may be attributed to the fact that in the study area domesticated backyard poultry, birds are mostly offered kitchen wastes (fruit and vegetables peals) and are therefore less exposed to ground for feeding purpose. Ground feeding is an important risk factor in the transmission of *T. gondii* in free range chickens (Yan *et al.,* 2009; Dubey *et al.,* 2003a).

Hearts and brains from seropositive chickens were used in the Mouse bioassay to demonstrate the virulence of isolates as highly virulent (Type I) and mildly virulent/avirulent (Type II and Type III) (Howe and Sibley, 1995). In the current study, no mortality in the inoculated mice was observed due to toxoplasmosis; although mice became sluggish in movements, decreased their feed and water intake leading to emaciation, dehydration and progressive weight loss.

Nostril muscles along with eyes were swollen in one of the experimental mice on day 2nd post-infection; although nostril and eyes of remaining mice were inflamed but their eyes developed redness. Similar findings in mice bioassay had been reported by Lindsay *et al*. (1995) and Hrda *et al*. (2000). On day 5th post inoculation, all the survived mice were killed humanely which showed multiple hemorrhages on the skin and sub-cutaneous tissue; congestion and hemorrhages on the lungs; swollen kidneys; and streaks on the liver. Congestion of heart with clotted blood was also observed. Similar to the findings of the present study, low pathogenicity of *T. gondii* isolates from chickens to mice had been demonstrated in previous studies reported by Dubey *et al.* (2005a, b).

Histopathological examinations of seropositive chicken's brain revealed mild to moderate degree of inflammation in terms of congestion in the thalamus region. At few places, presence of inflammatory cells around the blood vessels as an indicative of perivascular cuffing was seen similar to that reported in some previous mouse model studies (Kittas *et al*., 1984). The small zones of inflammatory cells and microglial lesions were also seen. These observations were consistent to the previous findings by Kittas *et al*. (1984). Individual cell necrosis was also obvious that might be due to the release of toxin(s) by the

parasites, lymphokines by the inflammatory cells or small infarcts due to localized blood vessel occlusions at sites of parasite invasion (Ferguson *et al*., 1991). Histopathological examinations of heart in seropositive chickens revealed mild to moderate degree of congestion at few places in the myocardial fibers. Inflammatory cells and coagulative necrosis at few places indicated severe inflammatory reaction. No such information on pathological changes induced by *T. gondii* in chicken's heart is reported.

Histopathological findings of mice brain samples indicated mild congestion along with inflammatory zones in the thalamus region, individual cell necrosis and perivascular cuffing. Similarly, focal necrosis, perivascular cuffing and inflammatory reactions in mouse model of *T. gondii* had been reported in some previous studies (Nicoll *et al*., 1997; Ferguson *et al*., 1991).

Based upon findings of present study, it was concluded that backyard poultry of rural areas of district Faisalabad, Punjab, Pakistan is infected with *T. gondii*. The seroprevalence was associated with age group of birds and pet cats kept along with these birds that may raise significant public health concerns and has implications for the prevention and control of toxoplamosis in this district of Pakistan. It is suggested that public health authorities should pay attention to monitor the problem.

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