Seroprevalence of *Neospora caninum* Antibodies in Chicken Samples from Delta Egypt Using a Recombinant NcSAG1 Protein-Based ELISA

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Neospora caninum **is an obligate intracellular protozoan that causes abortion and economic loss in the cattle industry. This study aimed to estimate the prevalence of anti-*N. caninum* antibodies in chicken using ELISA methods based on the surface antigen 1 of *N. caninum* (*Nc*SAG1t). The overall prevalence of *N. caninum* in chicken was 15.51%. The seroprevalence was high in Qalyoubiya, Minufiya, Kafr EL-Shaykh, Gharbiya, Dakahlia Provinces; (34%, 17.39%, 14.75%, 14.29%, and 12.25% respectively). In contrast, the seropervalence was low in Beheira Province only 2%. The lowest prevalence was recorded in the winter. On contrary, the prevalence was higher in the spring autumn and summer. The risk of infection with *N. caninum* was 1.4 times higher for females than for males. Finally, antibodies to *N. caninum* showed significant increase in free-range chickens compared to caged chickens. The high prevalence of neosporosis in chicken indicated that neosporosis may be widely distributed in Delta of Egypt. Recombinant *Nc*SAG1t is good diagnostic candidates for the detection of *N. caninum* infection.**

Neospora caninum is a protozoan parasite that was first described in a litter of dogs in Norway in 1984 (Bjerkas *et al.,* 1984). *N. caninum* is an obligate intracellular protozoan that causes abortion and economic loss in the cattle industry. It has been implicated as one of the major causes of infectious abortion of cattle worldwide (Hattel *et al.,* 1998; Dubey, 1999; Trees *et al.,* 1999). Abortion due to neosporosis can occur at any stage of pregnancy but is most likely to occur at 5–6 months of gestation (Dubey, 1999). Cows that aborted in a previous pregnancy due to neosporosis can abort again (Obendorf *et al.,* 1995). Sheep, goat, deer, horses, water buffalo, and camel, which have been identified as intermediate hosts, have also infrequently been reported to be naturally infected (Dubey & Lindsay 1996). Previous reports extends the list of intermediate hosts of *N. caninum* to include birds and may have important epidemiological consequences (Costa *et al.,* 2008; Mineo *et al.,* 2011). The dog, coyotes and red fox have been identified as definitive hosts for *N. caninum* (McAllister *et al.*, 1998; Gondim *et al.,* 2004; Wapenaar *et al.,* 2006).

Serological testing is an important method for detecting *N. caninum* infection, and includes the indirect fluorescent antibody test (IFAT), *Neospora* agglutination test (NAT), immunoblot analysis, and enzyme linked immunosorbent assay (ELISA) (Bjorkman & Uggla 1999; Jenkins *et al.,* 2002, Dubey 2003; Von Blumroder *et al.,* 2004). The ELISA was sensitive and specific, while the results were quantifiable and reproducible.

However, the use of whole tachyzoites or tachyzoite-derived antigens may result in false-positives due to cross-reaction with other closely related parasites (Chahan *et al.,* 2003). Therefore, it is necessary to develop a reliable, sensitive, and specific diagnostic test using parasite specific antigens. The molecular search for diagnostic antigens for *N. caninum* infection has been focused on the identification of immunodominant antigens that are recognized by sera from animals infected with geographically distant isolates

and from both acute and chronically infected animals. The surface antigen 1 of *N. caninum* (*Nc*SAG1) is an important candidate for developing a diagnostic reagent for neosporosis (Hemphill *et al.,* 1997; Howe *et al.,* 1998).

In previous surveys from Egypt, antibodies to *N. caninum* were detected in human samples (7.92%) (Ibrahim *et al.,* 2009). *N. caninum* antibodies were also detected in 3.6% of 166 camels, 20.43% of 93 cattle and 1.85% of 54 rabbits (Hilali *et al.,* 1998; Ibrahim *et al.,* 2009). A total of 51 out of 75 (68%) water buffalo sera had antibodies to *N. caninum* (Dubey *et al.,* 1998).

Economically, neosporosis is considered important diseases in animals. Hence, the aim of this study was to estimate the epidemiology and seroprevalence of *N. caninum* antibodies in free range and caged chickens from different regions in the delta of Egypt as an indicator of soil contamination due to *N. caninum* oocysts.

Materials and Methods

Serum samples

During year 2011, a total of 361 chickens (*Gallus domesticus*) during summer, winter, autumn and spring from different Provinces of the Delta of Egypt were obtained for the present study. See a map of sampling area (Fig. 1). Free-range chickens were purchased (with consideration on sex) from 4 villages belong to four Provinces (Qalyoubiya, Minufiya, Gharbiya and Beheira). The chickens were kept free ranging in the framer fields belong to the previously mentioned four Provinces without fencing and only housed at night. Approximately three ml venous blood was withdrawn from each chicken and serum was collected by centrifugation of the clogged blood. A total of 154 serum samples from free-range chickens were obtained. 207 serum samples (with consideration on sex) from caged chickens were obtained by collecting chicken blood in bird slaughterhouses in Gharbiya, Beheira, Kafr EL-Shaykh and Dakahlia where only from chicken raising in the farms. Blood was collected from the brachial wing vein of individual chicken, incubated at room temperature for 1h, and then centrifuged at 1000×*g* for 10 min, and the serum was collected and stored at −20ºC.

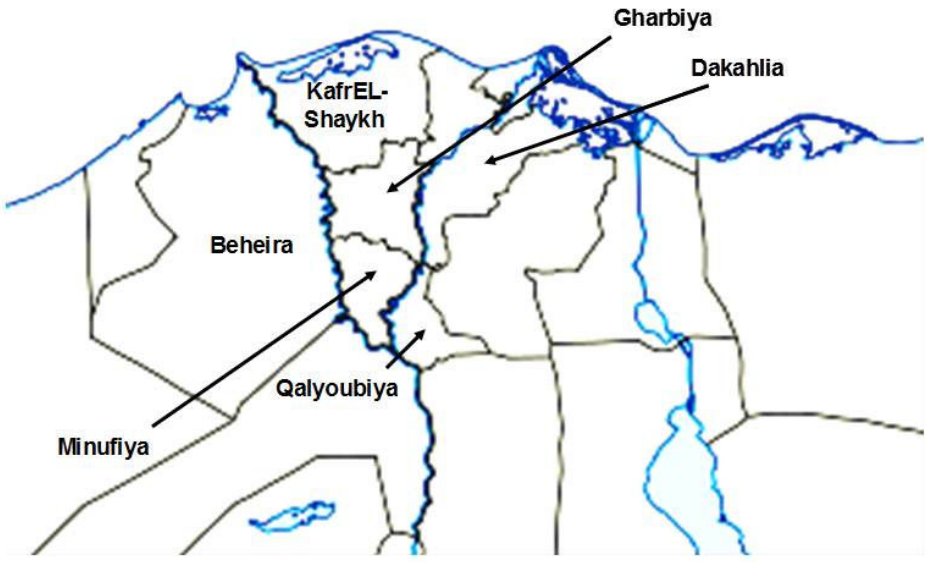

Figure 1. Map of sampling areas. Serum samples were collected from 361 chickens from six regions in the Delta of Egypt

Antigens

The antigen NcSAG1t was a gift from Associate Prof. Dr. Yoshifumi Nishikawa (National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, Japan). Briefly, the template DNA for polymerase chain reaction (PCR) was extracted from tachyzoites of *N. caninum* Nc-1 strain (Dubey, 2003; Chahan *et al.,* 2003; Ibrahim *et al.,* 2009). The truncated NcSAG1 (NcSAG1t) gene, without sequence encoding a hydrophobic signal peptide and a C-terminus, was amplified by PCR with two primers 5`-ACGAATTCATCAGAAAAATCACCT-3` and 5`-ACGAATTCGACCAACATTTTCAGC-3` which correspond to amino acids 65 to 333 (Chahan *et al.,* 2003). The NcSAG1t gene was inserted into *Eco*RI site of the bacterial expression vector, pGEX-4T-3 (Promega, Madison, WI). The resulting plasmid was designated as pGEX/NcSAG1t. pGEX/NcSAG1t was expressed as glutathione S-transferase (GST) fusion protein (GST-NcSAG1t) in *Esherichia coli* (DH5α strain) and the proteins were purified by glutathione sepharose 4B (Amersham Pharmacia Biotech) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Ibrahim *et al.,* 2009).

ELISA

ELISA was performed according to modified procedure described previously (Ibrahim *et al.,* 2009). The plates were coated using the recombinant antigens (GST-*Nc*SAG1t, or GST, 5µg/ml), produced as described earlier, in a coating buffer (50 mM carbonate) and incubated overnight at 4°C. After washing once with washing buffer (phosphate buffer saline (PBS) containing 0.05% Tween 20), the plates were blocked with blocking solution (PBS containing 3% skim milk) at 37°C for 2 hrs. After washing once with washing buffer, 50 µl of serum diluted (1:100) in blocking solution was added to duplicate wells for each sample and then incubated at 37°C for 1 hr. After washing six times with washing buffer, the plates were incubated with 50 µl of horseradish peroxidase (HRPO)-conjugated rabbit anti-chicken Immunoglobulin G (Invitrogen, Camarillo, CA), diluted in blocking solution (1:4000) per well at 37°C for 1 hr. After washing six times with washing buffer, the plates were incubated with 100 µl substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS)) in an ABTS buffer (0.1 M citric acid, 0.2 M sodium phosphate) per well at room temperature for 1 hr. The absorbance at 405 nm was measured using a microplate reader (Seac, Radim Company, Italy). The ELISA results were determined by the difference in mean optical densities at a value of 405nm (OD_{405}) between the recombinant antigen (*Nc*SAG1t) and the GST protein. The cut off values were determined as the OD_{405} value for *N. caninum* negative sera plus two standard deviations; *Nc*SAG1t: 0.02 in chicken (n=20). The negative sera from sera stock were tested and confirmed negative by direct agglutination test (DAT) and indirect fluorescent antibody test (IFAT).

Statistical Analysis

The chi-square test was used to evaluate significant differences ($P < 0.05$) of infection rate in animals of different seasons, habitat, sex and locations.

Results

A total of 361 chicken blood samples were randomly collected from different Provinces s in the Delta of Egypt and simultaneously assayed using a recombinant NcSAG1t protein-based ELISA to determine the serological prevalence of *N. caninum*. The overall prevalence of *N. caninum* was 15.51%.

According to the region, the prevalence of *N. caninum* in chicken from Delta region was summarized in table 1. The seroprevalence was very high in Qalyoubiya Province; 17 of 50 (34%). Moreover, the prevalence was high in other Provinces: Minufiya 8 of 46 (17.39%), Kafr EL-Shaykh 9 of 61 (14.75%), Gharbiya 15 of 105 (14.29%), and Dakahlia 6 of 49 (12.25%). On the other hand, the seropervalence was very low in Beheira Province; 1 of 50 (2%).

Table 1. Seroprevalence of *N. caninum* infection in chicken from different regions of Delta of Egypt during 2011

Regions	No. of sample	No. of positive sample	Seroprevalence (%)
Qalyoubiya	50	17	34.00
Minufiya	46	8	17.39
Gharbiya	105	15	14.29
Kafr EL-Shaykh	61	9	14.75
Dakahlia	49	6	12.24
Beheira	50	1	2.00*
Total	361	56	15.51

* Prevalence of antibody to *N. caninum* is significantly different compared to other regions (P<0.05, chi-square test).

According to the season, the seroprevalence of *N. caninum* in chicken was summarized in table 2. The seasonal prevalence was highest in the spring 22.62%, followed by autumn and summer 21.28% and 17.54% respectively. During winter the prevalence was significantly reduced 9.83% compared to the other seasons.

Table 2. Seasonal seroprevalence of *N. caninum* infection in chicken from different regions of Delta of Egypt during 2011.j

Season	No. of sample	No. of positive sample	Seroprevalence (%)
Winter	173	17	9.83*
Spring	84	19	22.62
Summer	57	10	17.54
Autumn	47	10	21.28

* Prevalence of antibody to *N. caninum* is significantly different compared to other seasons (P<0.05, chi-square test).

Furthermore, the prevalence of *N. caninum* in chicken populations was compared on the basis of sex and habitat. Chi square value indicated that the difference recorded between female and male animals for *N. caninum* was not statistically significant (Fig. 2). In females, antibodies were found in 47 out of 284 (16.55%). Anti- *Nc*SAG1t were detected in 9 out of 77 (11.69%) in male chickens. Finally, antibodies to *N. caninum* showed significant increase in free-range chickens 33 of 154 (21.43%) compared to caged chickens 23 of 207 (11.11%) (Fig. 3).

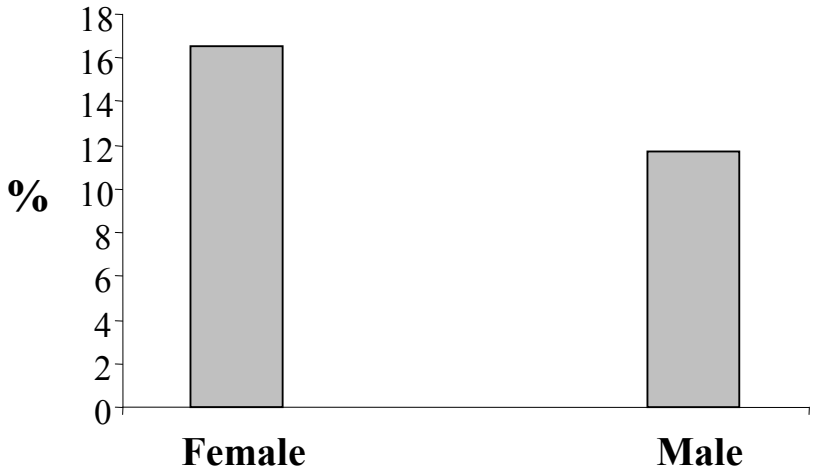

Figure 2. Seroprevalence of *N. caninum* infection among male and female chicken from different regions of Delta of Egypt during 2011

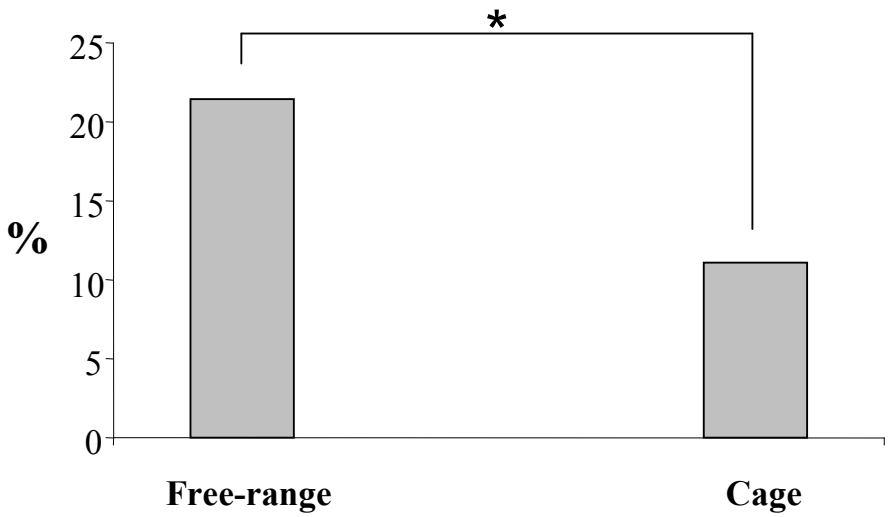

Figure 3. Seroprevalence of *N. caninum* infection among free range and caged chicken from different region of Delta of Egypt during 2011. * Prevalence of antibody to *N. caninum* significantly different (P<0.05, chi-square test).

Discussion

Diagnosis of neosporosis in cattle is usually based on histopathology and immunohistochemistry (IHC) on the aborted fetus. However, in many cases, fetal tissues are not available. The alternative diagnosis is by detection of *N. caninum* specific antibodies in bovine serum since the presence of antibodies in an animal indicates that the animal is, or has recently been, infected with the parasite (Bjorkman & Uggla, 1999). The development of specific, sensitive and inexpensive serological tests for *N. caninum* is critical in studying the epidemiology of this parasite. Many serological diagnostic methods have been developed to diagnose *N. caninum*

infection. ELISA with purified recombinant antigen was thought to be an effective way to diagnose *N. Caninum* infection (Jenkins *et al.,* 1997). Compared with the native antigens, recombinant antigens have an additional benefit: they are easily produced in large quantities and can be readily standardized for diagnostic assays. The diagnostic potential of surface antigen 1 of *N. caninum* (NcSAG1) expressed in *Escherichia coli* was previously evaluated, and the result indicated that the recombinant NcSAG1 could be a reliable reagent for use as an antigen in an ELISA for the serodiagnosis of *N. caninum* infection.

The prevalence of *N. caninum* in chicken in the Delta of Egypt was detected by ELISA with *Nc*SAG1t as coated recombinant antigens. Anti- *N. caninum* antibodies were detected in chicken (*Nc*SAG1t: 15.51%). The seroprevalence was high in Qalyoubiya, Minufiya, Kafr EL-Shaykh, Gharbiya, Dakahlia Provinces; (34%, 17.39%, 14.75%, 14.29%, and 12.25% respectively). On the other hand, the seropervalence was low in Beheira Province only 2%. The presented data were consistent with previous studies conducted in Egypt, *N. caninum* have been reported in water buffalo camels, cattle and rabbits (Dubey *et al.,* 1998; Hilali *et al.,* 1998; Ibrahim *et al.,* 2009). High seroprevalence of *N. caninum* in Israeli dairy herd dams and aborted fetuses has been previously reported (Shkap *et al.,* 2002; Fish *et al.,* 2004). The seroprevalence of *N. caninum* anti bodies in dairy cattle was 17.9% in Senegal (Kamga-Waladjo *et al.,* 2010).

Generally several risk factors have been associated with *N. caninum* seroprevalence; these include presence and number of dogs (Bartels *et al.,* 1999), feeding pooled colostrum to calves (Mainar-Jaime *et al.,* 1999), rabbits and poultry on the farm (Hemphill & Gottstein, 2000), stocking density (Sanderson *et al.,* 2000), and seasonality (Thurmond *et al.,* 1995).

In this study the lowest prevalence of *N. caninum* was recorded in the winter season. In contrast the seasonal prevalence was higher in the spring autumn and summer. Similar results were obtained by Nasir *et al.,* (2012) who demonstrated that the pattern of prevalence of *Neospora caninum* in dairy buffaloes (*Bubalus bubalis*), that was assessed in Pakistan, was closely associated with the season as reflected by the highest prevalence in summer and the lowest in winter. Dubey *et al.,* 2007 suggested that higher temperature may favor a faster sporulation of *N. caninum* oocysts in the environment surrounding the cattle.

Although no significant association was found to occur between the presence of *N. caninum* and sex in chicken in the Delta of Egypt, the risk of infection with *N. caninum* was 1.4 times higher for females than for males. The author of this study did not find literature on the association of sex and antibodies against *N. caninum*. However it could be associated to difference in the behaviour of the male and females chicken or to management differences. Much more studies are required in order to corroborate this finding.

Specific antibodies to *N. caninum* were found in 21.43% free-range chickens and only 11.11% in caged chickens indicating that free-range chickens have more chance to get infected by *N. caninum*. Infection rate to the common related parasite *Toxoplasma gondii* in free-range chicken is comparable to that found in other countries (Dubey *et al.,* 2008; Zhu *et al.,* 2008; Yan *et al.,* 2009). Evidence is accumulating that transmission by oocysts may be more prevalent than initially realized, at least in some parts of the world (Dubey, 2003). In Egypt, the main risk factor associated with chicken seropositivity is the contact with soil-harboring oocysts from wild homeless dogs. If the infections of these parasites increase and spread among domestic

animals, contamination of the water and the soil will increase also. The high prevalence of neosporosis in chicken indicated soil contamination due *N. caninum* oocysts because free range chickens feed from the ground, and suggested that the meat from poultry might be an important source for human infection.

Human neosporosis is a controversial question now because *N. caninum* was not detected or isolated from the human tissues. *N. caninum*–specific antibodies were detected in human sera in USA, Brazil and Egypt (Tranas *et al.,* 1999; Lobato *et al.,* 2006; Ibrahim *et al.,* 2009). Because dogs are definitive hosts and excrete oocysts in their feces, the potential for human exposure to *N. caninum* is high (McAllister *et al.,* 1998). The infection of healthy individuals by *N. caninum* may follow a course similar to that of *Toxoplasma gondii*, where the vast majority of infections are asymptomatic (McCabe *et al.,* 1985). Testing tissues and fluids from immunocompromised individuals and fetuses with suspected toxoplasmosis for *N. caninum* may reveal that subpopulations of these patients are infected with *N. caninum*. Further study is needed to determine the extent and significance of exposure in human.

In conclusion, the current data indicated that neosporosis may be widely distributed and present the threat of an epidemic in Delta of Egypt, with high seropositivity in chickens. Recombinant *Nc*SAG1t is good diagnostic candidates for the detection of *N. caninum* infection. This is the first study investigating the prevalence of *Neospora caninum* antibodies in Egyptian chickens from the Delta of Egypt using a recombinant NcSAG1 protein-based ELISA. More studies are required to understand the high rates of *N. caninum* infections in Egypt. This study provides additional information of the prevalence of *N. caninum* infection in Delta of Egypt, and will assist in developing strategies for controlling the disease.

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