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* (NcSAG1t). 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 NcSAG1t 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 & Ugla 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* (NcSAG1) 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 an important disease 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 belonging 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 −20°C.

![Map of sampling areas. Serum samples were collected from 361 chickens from six regions in the Delta of Egypt](image-url)
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* Ncv1 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'-ACGAATTCATCAGAAAAATCACCT3' and 5'-ACGAATTCGACCAACATTTTCAGC3' which correspond to amino acids 65 to 333 (Chahan et al., 2003). The NcSAG1t gene was inserted into *EcoRI* 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 *Escherichia coli* (DH5a 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-NcSAG1t, 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 (NcSAG1t) and the GST protein. The cut off values were determined as the OD_{405} value for *N. caninum* negative sera plus two standard deviations; NcSAG1t: 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 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

<table>
<thead>
<tr>
<th>Regions</th>
<th>No. of sample</th>
<th>No. of positive sample</th>
<th>Seroprevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qalyoubiya</td>
<td>50</td>
<td>17</td>
<td>34.00</td>
</tr>
<tr>
<td>Minufiya</td>
<td>46</td>
<td>8</td>
<td>17.39</td>
</tr>
<tr>
<td>Gharbiya</td>
<td>105</td>
<td>15</td>
<td>14.29</td>
</tr>
<tr>
<td>Kafr EL-Shaykh</td>
<td>61</td>
<td>9</td>
<td>14.75</td>
</tr>
<tr>
<td>Dakahlia</td>
<td>49</td>
<td>6</td>
<td>12.24</td>
</tr>
<tr>
<td>Beheira</td>
<td>50</td>
<td>1</td>
<td>2.00*</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>381</strong></td>
<td><strong>56</strong></td>
<td><strong>15.51</strong></td>
</tr>
</tbody>
</table>

* 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.

<table>
<thead>
<tr>
<th>Season</th>
<th>No. of sample</th>
<th>No. of positive sample</th>
<th>Seroprevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter</td>
<td>173</td>
<td>17</td>
<td>9.83*</td>
</tr>
<tr>
<td>Spring</td>
<td>84</td>
<td>19</td>
<td>22.62</td>
</tr>
<tr>
<td>Summer</td>
<td>57</td>
<td>10</td>
<td>17.54</td>
</tr>
<tr>
<td>Autumn</td>
<td>47</td>
<td>10</td>
<td>21.28</td>
</tr>
</tbody>
</table>

* 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- *NeSAG1t* 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*...
Seroprevalence of N. caninum Antibodies in Chicken Samples from Delta Egypt

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 \textit{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 \textit{N. caninum} was not detected or isolated from the human tissues. \textit{N. caninum}–specific antibodies were detected in human sera in USA, Brazil and Egypt (Tranas \textit{et al.}, 1999; Lobato \textit{et al.}, 2006; Ibrahim \textit{et al.}, 2009). Because dogs are definitive hosts and excrete oocysts in their feces, the potential for human exposure to \textit{N. caninum} is high (McAllister \textit{et al.}, 1998). The infection of healthy individuals by \textit{N. caninum} may follow a course similar to that of \textit{Toxoplasma gondii}, where the vast majority of infections are asymptomatic (McCabe \textit{et al.}, 1985). Testing tissues and fluids from immunocompromised individuals and fetuses with suspected toxoplasmosis for \textit{N. caninum} may reveal that subpopulations of these patients are infected with \textit{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 \textit{NcSAG1t} is good diagnostic candidates for the detection of \textit{N. caninum} infection. This is the first study investigating the prevalence of \textit{Neospora caninum} antibodies in Egyptian chickens from the Delta of Egypt using a recombinant NeSAG1 protein-based ELISA. More studies are required to understand the high rates of \textit{N. caninum} infections in Egypt. This study provides additional information of the prevalence of \textit{N. caninum} infection in Delta of Egypt, and will assist in developing strategies for controlling the disease.

Acknowledgements

The author thank Associate Prof. Dr. Yoshifumi Nishikawa (National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, Japan) for supplying the recombinant \textit{NcSAG1t}, local veterinary practitioners specially Mrs. Reham Khatab for collecting blood samples and help during this work.

References


30. Sanderson MS, Gay JM, Baszler TV (2000). *Neospora caninum* seroprevalence and associated


