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High seroprevalence of *Toxoplasma gondii* and probability of detecting tissue cysts in backyard laying hens compared with hens from large free-range farms

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ABSTRACT

Serological assays are commonly used to determine the prevalence of *Toxoplasma gondii* infection in livestock, but the predictive value of seropositivity with respect to the presence of infective tissue cysts is less clear. The present study aimed at the identification of seropositive and seronegative free-range laying hens from organic and backyard farms, and the relationship with the presence of viable tissue cysts. In addition, potential risk and protective factors on the selected farms were investigated. An in-house *T. gondii* surface antigen (TgSAG1, p30, SRS29B) ELISA was validated with sera from experimentally infected chickens and used to examine 470 serum samples collected from laying hens from organic and backyard farms at the end of their laying period. A total of 11.7% (55/470) of all chickens tested positive, and another 18.9% (89/470) of test results were inconclusive. The highest seroprevalences were observed on small backyard farms with 47.7% (41/86) of chickens being seropositive while another 20.9% (18/86) of test results were inconclusive. Twenty-nine seropositive, 20 seronegative and 12 laying hens which yielded inconclusive ELISA results, were selected for further examination. Hearts and limb muscles of these hens were examined for *T. gondii* tissue cysts in a bioassay with IFNγ-knockout or IFNγ-receptor-knockout mice. Viable *T. gondii* was isolated from 75.9% (22/29) of the seropositive, 25.0% of the inconclusive (3/12), and 5.0% (1/20) of the seronegative chickens. All 26 chickens tested positive in heart samples, while drumstick muscles (i.e. limb muscles) tested positive only in three. Data on putative risk and protective factors were collected on the farms using a standard questionnaire. Generalised multilevel modelling revealed farm size, cat related factors ('cats on the premise', 'cats used for rodent control'), hen house/hall related factors ('size category of hen house/hall', 'frequency category of cleaning hen house/hall', 'service period') as significantly associated with seropositivity to *T. gondii* in hens. The final model, which included the age of the birds as an effect modifier and farm as a random effect variable, revealed that the use of cats for rodent control and an area available per hen in the chicken run of >10 sqm were statistically significant risk factors for *T. gondii* seropositivity. Overall this study showed that exposure to *T. gondii* is common in small backyard farms but is rare on large organic farms with a high density of free-range hens, even when cats were present on the premises.

1. Introduction

Toxoplasma gondii infections in humans are prevalent in many countries of the world (Pappas et al., 2009). Human toxoplasmosis includes congenitally and postnatally acquired toxoplasmosis (Schlüter et al., 2014). Congenital toxoplasmosis is transmitted from the recently infected mother to the fetus. Placental or fetal infection may cause abortion, the birth of severely affected children (e.g. hydrocephalus, seizures, retardation) or children developing symptoms of toxoplasmosis in later life (e.g. ocular toxoplasmosis). In most cases, postnatally acquired *T. gondii* infections have no severe consequences. It is assumed, however, that a large number of ocular uveitis cases in humans are caused by postnatal

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T. gondii infection (Maenz et al., 2014). Toxoplasmosis in immunocompromised patients (e.g. transplant patients) is of growing importance. This form of toxoplasmosis can result either from a reactivated, persistent infection or from a recently acquired new infection. In some cases, the infection has been transmitted from the infected donor to the uninfected recipient patient (Patrat-Delon et al., 2010).

Risk factor studies suggest that the consumption of raw or undercooked meat is a major source of human *T. gondii* infections in Europe (Cook et al., 2000; Kapperud et al., 1996) and in one study the consumption of raw or undercooked poultry was also observed as a risk factor (Kapperud et al., 1996). The environmentally resistant *T. gondii* oocysts shed by felids, i.e. domestic cats, contribute to a significant proportion of human infections. Cats are definitive hosts of *T. gondii* and oocysts may cause infection, e.g. directly via cleaning the cat litter box (Kapperud et al., 1996), by soil contamination (Cook et al., 2000), via eating unwashed vegetables or fruits (Kapperud et al., 1996) or drinking surface water (Kapperud et al., 1996). Elevated risks of infection were also associated with locally produced cured, dried or smoked meat, raw oysters, clams or mussels, working with meat and drinking goat milk (Jones et al., 2009), but the relative importance of these individual risk factors needs to be determined.

To gain more insight into the role of chicken meat as a source of human infection with *T. gondii*, data on the prevalence of infective tissue cysts in the meat are needed. Serological assays are commonly used to determine the prevalence but the predictive value of seropositivity with respect to the presence of infective tissue cysts in various livestock species is largely unknown. Viable *T. gondii* has been frequently isolated from chickens found positive by IFAT, in a modified agglutination test (MAT) or in an ELISA (Beltrame et al., 2012; Casartelli-Alves et al., 2014; da Silva et al., 2003; Dubey et al., 2016.

Chickens are known to be highly susceptible to *T. gondii* infections (reviewed by Dubey, 2010); however, industrialised husbandry has decreased *T. gondii* exposure of poultry and it was impossible to find viable *T. gondii* in a large-scale study of chicken breast samples from retail meat stores in the United States (Dubey et al., 2005). Only a few from a population of more than 2000 chickens (1.3%) were positive by ELISA (Dubey et al., 2005). With the increasing demand for organic meat, the risk of human infection from poultry meat may increase, because viable *T. gondii* have been very frequently isolated from backyard chickens (reviewed in Dubey, 2010). Furthermore, there is a rising market share of chicken meat preparations such as chicken burgers and sausages, increasing the risk of consuming undercooked products. Although laying hens represented only 5% of chickens slaughtered in Germany in 2015 (Statistisches Bundesamt, https://www.destatis.de/DE/ZahlenFakten/Wirtschaftsbereiche/LandForstwirtschaftFischerei/TiereundtierischeErzeugung/Tabellen/Gefluegelfleisch.html; last accessed 17.03.2017), they are, due to their age, a possible source of *T. gondii* infection, at least in rural areas where chickens are often slaughtered on-farm and consumed locally.

Toxoplasma gondii seems to have predilection sites in chickens, i.e. different organs or body parts seem to harbour varying numbers of viable tissue cysts. A recent review revealed that brain, heart, and meat or muscle scored relatively highly with regard to positive *T. gondii*-findings (Opsteegh et al., 2016a). We therefore sampled two types of tissue, i.e. the heart as a predilection site and the lower limb musculature (drumstick musculature) representing tissue frequently used for human consumption.

Free-range chickens are thought to play an important role in the epidemiology of *T. gondii* in rural areas, perhaps more than rodents (Dubey, 2010). There are several reasons for this assumption: (i) chickens are clinically resistant to *T. gondii*, (ii) they live longer than rodents, and (iii) they are often slaughtered on farms, so that slaughtering remnants and offal may be eaten by cats if inadequately disposed of. In addition, free-range chickens represent a good indicator for soil contamination with *T. gondii* oocysts as they feed from the ground, which exposes them to infection with oocysts (Dubey, 2010). Cats fed naturally infected chicken tissues can shed millions of oocysts (Dubey et al., 2002).

In general, free-range chickens have a much higher risk of acquiring a *T. gondii* infection than caged chickens (Salant et al., 2016; Yan et al., 2009; Zhu et al., 2008); not only backyard farms, but also free-ranging chickens kept on large farms were shown to have a high *T. gondii* seroprevalence (Chumpolbanchorn et al., 2013; Salant et al., 2016). Relatively few studies have investigated risk factors for *T. gondii* infections in chickens. The presence of cats, the number of domestic cats (>3 cats) or the presence of feral cats have been reported as risk factors (Magalhaes et al., 2016; Millar et al., 2012). In addition, the use of water from a reservoir created by a dam was associated with risk (Magalhaes et al., 2016). In the study by Millar and colleagues, laying hens had a higher risk of testing seropositive for *T. gondii* compared with broiler chickens and it was hypothesised that this was due to the fact that laying hens are usually older than broiler chickens and the cumulative time of exposure to *T. gondii* is therefore greater (Millar et al., 2012). Further studies are needed to improve the knowledge on risk and protective factors for *T. gondii* infection in chickens.

One of the objectives of this study was therefore to identify potential risk and protective factors for *T. gondii* infections in hens kept in the organic and backyard farms included in this study. To examine laying hens and to identify viable infections in them, an ELISA was established based on *T. gondii* surface antigen 1 (TgSAG1, p30, SRS29B), one of the major surface antigens of *T. gondii* tachyzoites. A similar approach had been used in a previous epidemiological study on ducks and geese (Maksimov et al., 2011). The ability of this ELISA to identify infected chickens was further confirmed by bioassay in selected seropositive and seronegative hens. The risk factor study revealed the age of the hens as an important variable that needs to be included as an effect modifying variable in statistical analyses. Using this approach, it was possible to identify further risk factors in addition to those reported in previous studies.

2. Materials and methods

2.1. In-vitro cultivation of *T. gondii* tachyzoites

The *T. gondii* strains RH (Sabin, 1941), ME49 (Lunde and Jacobs, 1983), and NED (Howe and Sibley, 1995), i.e. representatives of the three main clonal lineages of *T. gondii* (Types I, II, and III, respectively), were cultivated at 37 °C, 5% CO_2 in Vero or MARC-145 cells for 3–5 days with DMEM supplemented with 1% glutamine, 2% FCS and 1% antibiotic solution (10,000 i.u. of penicillin and 10,000 μg of streptomycin/ml of solution). To harvest *T. gondii* tachyzoites, the cells were scraped from the flask and tachyzoites were purified by filtration using 5 μm filters (Millipore, Eschborn, Germany), washed five times by centrifugation at 700*g* (4 °C, 8 min) and resuspended in ice-cold PBS.

For the experimental infection of chickens, the tachyzoites of all three strains were counted using a Neubauer chamber, checked by Trypan Blue exclusion for viability and used immediately after harvest. One day before parasites were harvested for antigen production, the FCS-supplemented medium was removed and infected cells were further cultivated under FCS-free conditions.

RH strain tachyzoites were washed with PBS by centrifugation (700*g*) when used for antigen preparation and dispensed onto IFAT slides or stored as a pellet at −80 °C until used for purification of the TgSAG1 as described previously (Maksimov et al., 2011).

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