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First report on the seroprevalence of *Neospora caninum* in goats from the Federal State of Hesse, Germany

Erster Bericht über die Seroprävalenz von Neospora caninum in hessischen Ziegenbeständen

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Summary

A total of 415 goat serum samples from 26 flocks in Hesse, Central Germany, were analyzed for the presence of specific antibodies against the abortion-causing apicomplexan parasite *Neospora (N.) caninum* by immuno-enzyme assay (ELISA). In total, three serum samples were seropositive for *N. caninum* with two of them originating from a flock in Middle-Hesse and one sample coming from a flock in Northern Hesse. Western Blotting confirmed two of three ELISA-positive samples proving a low overall prevalence of 2/415 (0.48%) for caprine neosporosis. No clinical signs related to neosporosis were detected in any seropositive animal. Additionally, there was no familiar relationship between them and *N. caninum*-positive goats were purchased from different breeders. The low numbers of *N. caninum*-seropositive animals excluded risk factors assessment. Based on the current seroprevalence data, *N. caninum* infections appear of minor importance in German goat flocks. Nevertheless, taking into account that caprine neosporosis was detected in some European countries bordering Germany, further epidemiological and surveillance studies on caprine *N. caninum* infections are required to complement our findings regarding the current situation in goat populations from the other German Federal States.

Keywords: Survey, goat, Central Germany, epidemiology.

Zusammenfasssung

Insgesamt wurden 415 Ziegenserumproben von 26 Herden in Hessen, Deutschland, auf das Vorhandensein spezifischer Antikörper gegen den abortiven apikomplexen Parasiten *Neospora (N.) caninum* mittels Immunoenzymassay (ELISA) untersucht. Insgesamt konnten nur drei positive Proben ermittelt werden, wobei zwei aus einem Bestand in Mittelhessen und eine aus einem nordhessischen Bestand kamen. Ein anschließend durchgeführter Western Blot bestätigte zwei der drei ELISA-positiven Proben, die auf eine insgesamt niedrige Gesamtprävalenz von 2/415 (0,48 %) für Ziegenneosporose hinweisen. Keines der seropositiven Tiere wies klinische Symptome der Neosporose auf. Darüber hinaus lagen keine verwandtschaftlichen Beziehungen zwischen ihnen vor und die *N. caninum*-positiven Ziegen wurden von unterschiedlichen Züchtern erworben. Die geringe Seroprävalenz der vorliegenden Studie schloss die Möglichkeit, Risikofaktoren zu analysieren, aus. Die tatsächliche Bedeutung der *N. caninum*-Infektionen in deutschen Ziegenbeständen scheint aufgrund der gemessenen geringen Seroprävalenz vernachlässigbar. Da die Neosporose in Ziegen in einigen an Deutschland angrenzenden europäischen Ländern bereits nachgewiesen wurde, sind weitere epidemiologische Studien zu *N. caninum*-Infektionen von Ziegen erforderlich, um die aktuellen Erkenntnisse bezüglich anderer Bundesländer zu ergänzen.

Schlüsselwörter: Fragebogen, Ziege, Deutschland, Epidemiologie

Introduction

Neospora (N.) caninum is an apicomplexan intracellular parasite responsible for abortions and serious disease in ruminants and dogs worldwide (Dubey, 2003). It shows a wide range of intermediate host species and causes reproductive problems mainly in cattle (Hemphill and Gottstein, 2000), but is also associated with clinical reproductive and neurological infections in dogs, horses, goats, sheep, deer and marine mammals (Dubey and Lindsay, 1996; Anderson et al., 2000; Schares et al., 2001a, b; Dubey, 2003; Omata et al., 2006). Protozoan-induced abortions in sheep and goats have commonly been related to *Toxoplasma (T.) gondii* infections (Buxton et al., 1998; Dubey, 2003), however, *N. caninum* infections can also result in abortion and reproductive disorders in small ruminants with both, economical and clinical consequences (Dubey and Schares, 2011). Clinical manifestations occurring during the course of caprine neosporosis can include abortion and still birth (Barr et al., 1992; Lindsay et al., 1995; Dubey et al., 1996; Koyama et al., 2001; Eleni et al., 2004; Mesquita et al., 2013; Costa et al., 2014). Moreover, enhanced neonatal mortality associated with caprine *N. caninum* infections has been reported from different countries, such as the USA (Barr et al., 1992), Costa Rica (Dubey et al., 1996), Taiwan (Ooi et al., 2000), Brazil (Corbellini et al., 2001) and Spain (Moreno et al., 2012).

For the detection of specific antibodies against *N. caninum*, the following serological assays are commonly used: enzyme-linked immunosorbent assays (ELISAs), indirect fluorescent antibody tests (IFATs) and agglutination test (NAT) (Ortega-Mora et al., 2007; Alvarez-García et al., 2013).

So far, in Europe caprine *N. caninum* seroprevalences have been analyzed in France (8.9%, Chartier et al., 2000), Austria (68.7%, Edelhofer et al., 2005), Poland, (0.47%, Czopowicz et al., 2011), Slovakia (15.5%, Čobádiová et al., 2013), Czech Republic (6%, Bartova and Sedlak, 2012), Romania (2.3%, Iovu et al., 2012), Italy (5.7%, Gazzonis et al., 2016), Turkey (10.2%, Ütük et al., 2011) and Spain (5,6%, García-Bocanegra et al., 2012; 6%, Díaz et al., 2016; 1.08%, Rodríguez-Ponce et al., 2016). In Germany, seroprevalence studies have been performed in other animal species, including cattle (Schares et al., 1998; Weber et al., 2000, Bartels et al., 2006), swine (Damriyasa et al., 2004), south American camelids (Wolf et al., 2005) dogs (Klein and Müller, 2001; Schares et al., 2001a) and foxes (Schares et al., 2001b). Moreover, the clinical and pathological presence of *N. caninum* has been reported in German cattle (Söndgen et al., 2001) and dogs (Peters et al., 2000).

The main objective of this study was to determine the caprine seroprevalence of *N. caninum* in Hesse particularly because of the importance of goat husbandry in this region is strongly associated with landscape preservation, species conservation and milk or meat production through grazing activities, which also represent a possible route of horizontal transmission for this parasite.

Materials and Methods

Analyzed population

The goat flocks in Hesse had similar characteristics regarding biosecurity, production, reproduction and health management. The majority of the analyzed flocks hold a small amount of animals (less than 100 goats, 82.1%), mostly maintained under semi-intensive conditions (78.6%). Most of them (85.7%) were members of the Hessian Goat Breeders (Hessischer Ziegenzuchtverband, HZV), commercially produced milk and meat, and routinely tested their animals for caprine arthritis and encephalitis virus (CAEV) and caseous lymphadenitis (CLA). The animals were kept together with other *N. caninum*-susceptible hosts, especially cattle (10.7%), sheep (46.4%), South American camelids (7.1%) and dogs (67.9%), but also with poultry (46.4%), cats (60.7%) and horses (32.1%). All flocks were routinely controlled by the Veterinary Ambulance of the Clinic for Obstetrics, Gynecology and Andrology of the Justus Liebig University Giessen.

Sample size

The sample size was calculated according to data given by the Hessen Epizootic Fund (Hessische Tierseuchenkasse) which estimated a population of 20 247 animals distributed in 3109 goat farms. Using the formula for freedom of disease (Cameron and Baldock, 1998), with a 2.5% overall expected prevalence, at 95.0% confidence level, and assuming all the sampled animals as a unique population with an identical probability of infection within the flock and among flocks, 402 samples needed to be analyzed to accomplish the objective; however, a total of 415 samples were taken. Due to a small number of goats per flock and in order to determine the presence of *N. caninum*, the Cannon and Roe´s formula (1982) was used (5% expected prevalence inside each flock at 95.0% confidence level). The study was conducted in 26 German goat flocks randomly selected within a list of owners voluntarily willing to participate. According proportional allocation, the farms were distributed as follows: 6 were located in Northern Hesse, 11 in Middle Hesse and 9 in Southern Hesse. The sampled goat breeds were the following: White German Improved Goat (WDE) (23%), German Improved Fawn (BDE) (19.2%), Boer (BRZ) (34.6%), Thuringian Forest (TWZ) (15.4%), Peacock (3.8%) and mixes (3.8%).

Sample collection and survey

The selected animals had to be older than 8 months to participate in this research, in order to avoid false negatives due to the non-detection of the parasite by the immune system (Mesquita et al., 2013). All animals included in this study were examined clinically to detect any signs of infections. The blood sampling was performed by punctuation of the jugular vein. Tubes were transported in coolers for keeping a temperature between 5 to 10°C. In the laboratory, the samples were centrifuged for 5 min at 10000 x g, sera were isolated and frozen at –20°C until further use. Immediately after sampling and in order to assess risk factors associated to *N. caninum* serostatus, a questionnaire was applied to the farmers, to obtain information about housing conditions, goat kids husbandry, management, abortion recurrence and presence of clinical signs related to apicomplexan parasite infections.

Enzyme-linked Immunosorbent Assay (ELISA)

The IDScreen® *Neospora caninum* Indirect Multi-species ELISA from IDVet® (Montpellier, France) was used to detect *N. caninum*-specific antibodies in caprine serum

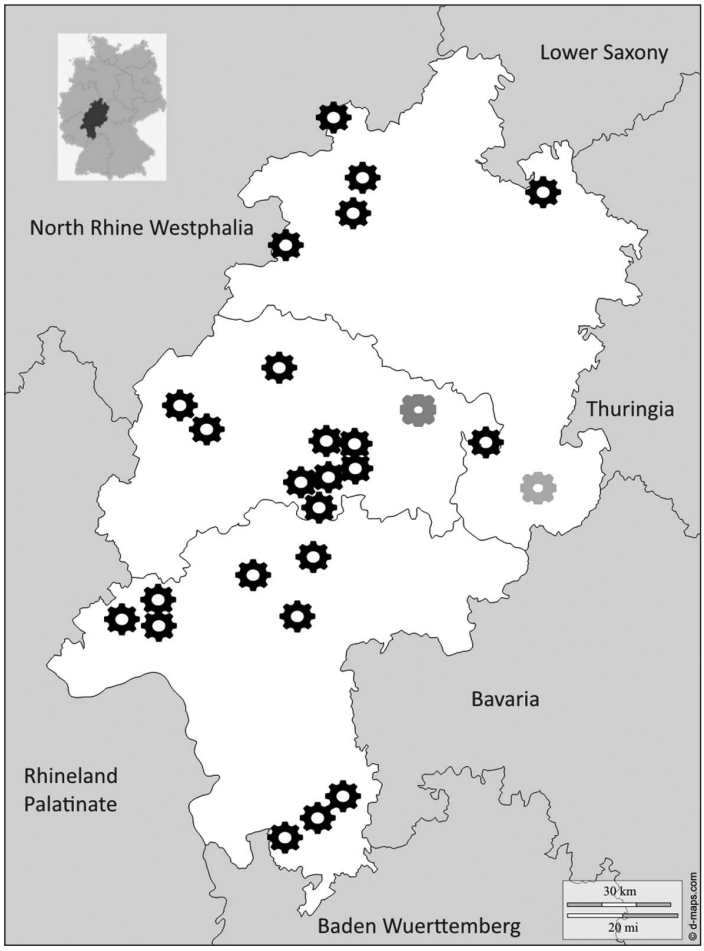

FIGURE 1: *Location of the participating seronegative farms (black gear icons) and flocks with Neospora caninum seropositive and suspect goats (dark grey and light grey gear icons, respectively) within the Northern-, Middle- and Southern-Hesse.*

samples. This assay showed a sensitivity of 99.6% and specificity of 98.9% (Álvarez-García et al., 2013). The samples were processed according to the manufacturer's protocol. For validation, the average of the optical densities (OD) of the positive controls, and the difference between averages of OD of positive and negative control sera were calculated. Applying the optical density data from the different serum samples, Serum Positive Percentages (S/P) were calculated, with respect to the average of the positive control sera, using the following formula: S/P = (OD of sample x 100): (average OD of positive control). As recommended by the manufacturer, serum samples that yielded S/P percentages less than 40% were considered as negative, samples with S/P values between 40–50% were scored as weakly positive and sera with S/P values greater than 50% were determined as positive. The seropositive samples of the current study were additionally analyzed in the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institut) using Immunblot techniques.

Statistical analysis

Frequencies of the management conditions inside each goat flock were calculated. The global and specific within-herd seroprevalences with 95% confidence intervals were assessed. At the beginning of the study, based on the hypothesis of at least 2.5% of goats yield positive results, a statistical analysis by logistic regression was planned.

Results

Two goat sera reacted positive with ELISA (S/P 84% and 62%) and the other one with a weak positive result (S/P 44%). Two seropositive animals came from the same flock in Middle Hesse and the weak positive animal was detected in a farm in Northern Hesse; the remaining 24 flocks showed seronegative results (Fig. 1). The Immunblot-based analyses confirmed seropositivity in one positive animal from the flock in Middle Hesse and the weak positive sample from Northern Hesse demonstrating a low global seroprevalence of 0.48% (2/415). As such, these two flocks from Middle and Northern Hesse accounted 5% (1/20) and 7.7% (1/13) intra-flock seroprevalence respectively, whilst all other flocks showed a within-herd *N. caninum* seroprevalence of 0% (Tab. 1).

The descriptive data of all analyzed animals are shown in Table 1. The seropositive goats were two females, between four and six years old, showing neither a history of abortion nor reproductive problems or neurological disorders. At the time of sampling, the clinical examination of these animals showed no signs of infection. According to the flock rearing registers, these goats had no familiar relationship to each other and were introduced to the farm from two different flocks 3 years ago. The weakly positive goat was also a female, more than 4 years old and presented three normal births before. No history of abortions, reproductive or neurological disorders were registered before for this animal. In addition, the animal presented no signs of infection. It was also purchased from another breeder approximately 3 years ago. Owing to the low *N. caninum* seroprevalence found in the present study, no statistical association between the seropositive animals and risk factors could be assessed.

Discussion

The German goat population was estimated 150 000 individuals (Destatis, 2010) and continues growing. This investigation focused mainly on the goat flocks registered in the HZV.

Previously, the presence of *T. gondii*-specific antibodies (a closely related apicomplexan to *N. caninum*) was reported by Lenz (2014) in goats with clinical abortions in one flock in North Hesse. Whilst the presence of *N. caninum*-specific antibodies in bovine hosts is commonly reported in Germany (Söndgen et al., 2001; Schares et al., 2003; Bartels et al., 2006); to our best knowledge this study represents the first report of the presence of caprine specific antibodies against *N. caninum* in goats from Hesse and one of the earliest reports in total Germany. However, it has to be considered that the positive results obtained in the present study could also be due to false positive reactions in the ELISA. Nevertheless, this assay was reported to exhibit a high specificity (Álvarez-García et al., 2013) causing only 1.1% false positive results. However, since it is generally recommended to use at least two different diagnostic methods, such as IFAT, IHA, ELISA, PCR or Western Blot (Czopowicz et

TABLE 1: *Number and percentage of animals analyzed and distribution of seropositive individuals according to flock, breed and regions*

Flock	Region	Animals	Animals tested (%)	Positive animals	Predominant Breed
1	Middle-Hesse	47	20 (42.50%)	2 (10.0%)	BRZ
2	Middle-Hesse	29	22 (75.90%)	0	BRZ
6	Middle-Hesse	62	15 (24.19%)	0	WDE + BDE
7	Middle-Hesse	98	25 (25.51%)	0	BRZ
12	Middle-Hesse	16	9 (56.52%)	0	BRZ
13	Middle-Hesse	14	10 (71.42%)	0	PZ
15	Middle-Hesse	19	9 (47.40%)	0	BRZ
16	Middle-Hesse	35	23 (65.71%)	0	BRZ
17	Middle-Hesse	31	13 (41.93%)	0	WDE
19	Middle-Hesse	48	22 (45.83%)	0	WDE
24	Middle-Hesse	12	6 (50.0%)	0	WDE
26	Middle-Hesse	321	30 (9.34%)	0	WDE + BDE
3	Northern-Hesse	80	26 (32.50%)	0	BDE
4	Northern-Hesse	6	4 (66.66%)	0	TWZ
9	Northern-Hesse	23	13 (56.52%)	1 (7.7%)	PZ
18	Northern-Hesse	6	6 (100.0%)	0	Omb
5	Northern-Hesse	302	31 (10.26%)	0	WDE
8	South-Hesse	4	4 (100 %)	0	Omb
10	South-Hesse	32	18 (56.25%)	0	BRZ
11	South-Hesse	168	28 (16.67%)	0	TWZ
14	South-Hesse	15	9 (60.0%)	0	BRZ
20	South-Hesse	17	9 (52.94%)	0	TWZ
21	South-Hesse	16	9 (56.52%)	0	BDE
22	South Hesse	17	10 (58.82%)	0	TWZ
23	South Hesse	223	28 (12.55%)	0	BDE
25	South-Hesse	31	16 (51.61%)	0	BRZ
	TOTAL	1672 (100 %)	415 (24.82%)	3 (0.72%)	

White German Improved Goat (WDE), German Improved Fawn (BDE), Boer (BRZ), Thuringian (TWZ) Peacock (PZ), and other mixed breeds (Omb)

al., 2011; Bartova and Sedlak, 2012; Gazzonis et al., 2016) to exclude false positive results, the positive samples obtained in ELISA were additionally analyzed via immunoblotting technique. Here, two out of three positive samples were confirmed in their seropositivity.

Overall, the low global seroprevalence determined in this study (0.48%) was consistent to data published for other European countries (Chartier et al., 2000; Czopowicz et al., 2011; Bartova and Sedlak, 2012; Gazzonis et al., 2016), in which *N. caninum* prevalences in goats remained below 10%. Thus, the prevalence data in this study was lower than those obtained in other European studies such as from Slovakia and Turkey (Čobádiová et al., 2013; Ütük et al., 2011). The intra-flock prevalences (5 and 7.7%) showed that the presence of *N. caninum* in Hesse was lower than expected, which is in agreement with other recently published data in Poland (Czopowicz et al., 2011). However, these authors recommended, that although *N. caninum* prevalences were low, caprine neosporosis should not be underestimated, since higher flock-level seroprevalences with serious repercussions could be emerging in the future. Thus, we also would recommend further serological and surveillance studies to be performed within caprine flocks, particularly in those that did not subscribe to HZV, in order to better understand the actual situation of this abortive disease.

Most of the owners maintained their animals in an intensive and semi-intensive management, grazing in fields close to dense forests or urban areas, where domestic dogs as potential oocyst shedders were commonly observed. As determined in personal interviews with the farmers, they noticed the canids mainly close to water sources and fences, where canid faeces were also frequently found. These conditions may favor the spread of *N. caninum* within the flocks as postulated elsewhere (Czopowicz et al., 2011; Čobádiová et al., 2013; Topazio et al., 2014; Gazzonis et al., 2016). Additionally, the owners described that this situation happened with a higher frequency during summer and autumn than in other seasons, when humidity is higher allowing the oocyst sporulation, which has concordance with the data described by Čobádiová et al. (2013) in Slovakia, Luo et al. (2016) in China and Díaz et al. (2016) in Spain.

Despite the number of positive animals was extremely low and risk factors could not be measured, the survey revealed that most of the caprine flocks in Hesse maintained a semi-intensive system with local breeds, which are environmentally better adapted inside the flock and probably reduced the seroprevalence of *N. caninum*, contributing to the absence of clinical signs associated with neosporosis, as mentioned in other European studies (Díaz et al., 2016; Gazzonis et al., 2016). The differences between the current data and the seroprevalences in goats from other European countries (e.g. Spain or Slovakia) may also depend on different climate conditions, farm management and sensitivity or (and) specificity of the diagnostic assays (Álvarez-García et al.,2013; Cobádiová et al., 2013; Díaz et al., 2016).

To sum up, we conclude that *N. caninum* infections indeed occur in goat flocks from Hesse but at a very low prevalence. Nonetheless, further studies are required to obtain more information on the current situation in small ruminant populations in other German Federal States. To improve the data sets, a higher number of goat flocks not having subscribed to HZV or to the Veterinary Ambulance Service should also be analyzed.

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Conflict of interest statement

The authors ratified that they have no competing interests in the present study.

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