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Epidemiological survey on the occurrence of *Anaplasma phagocytophilum* infection in sheep reared in central Italy

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Highlights

- Sheep are a natural domestic reservoir of *Anaplasma phagocytophilum*.
- A moderate prevalence (8%) of *A. phagocytophilum* in sheep was registered in Grosseto area, central Italy.
- Domestic animals are epidemiological sentinels for infection by *A. phagocytophilum.*
- Seroprevalence of *A. phagocytophilum* is higher in small flocks.

Abstract

Anaplasma phagocytophilum is a tick-borne pathogen of sheep and other ruminants, humans, horses and dogs. Current data on the prevalence and diffusion of *A. phagocytophilum* among domestic animals in Italy are fragmentary, especially those related to sheep, considered a natural domestic reservoir.

We investigated the prevalence of *A. phagocytophilum* infection in dairy sheep from Tuscany (Italy), by testing 624 sera with the immunofluorescent antibody (IFA) test.

The sera belonged to 33 different flocks distributed throughout the province of Grosseto (Tuscany). Positive results were found in 21 out of the 33 flocks tested (63.63%, 95% IC 47.22-80.05%) with a prevalence rate within flocks ranging from 3.33% to 59.26%. The seroprevalence against *A. phagocytophilum* within the sheep population was 8.01% (95% IC 6.13-10.41%), and most of the positive cases showed low antibody titres (1/40-1/80). The multivariate analysis identified "small flock" (< 300 animals) as the factor most closely related to serological positivity.

Our results reveal a moderate circulation of *A. phagocytophilum* among sheep flocks in central Italy.

Keywords: *Anaplasma phagocytophilum*; sheep; Italy

1. Introduction

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is an obligate intracellular gram-negative bacterium that selectively targets peripheral blood neutrophils, and occasionally eosinophils and monocytes (Dumler et al., 2001). Based on the genetic analysis of 16S rRNA-encoding genes, many variants differing from each other in terms of biological and ecological aspects, including variations in host pathogenicity, vectors, and geographical distribution were delineated among *A. phagocytophilum* complex species. These may be the causative agents of the tick-borne fever (TBF) of sheep and cattle, equine granulocytic anaplasmosis (EGA), canine granulocytic anaplasmosis (CGA), and human granulocytic anaplasmosis (HGA) (Dumler et al., 2001).

The epidemiology of *A. phagocytophilum* infection is closely related to the presence of hard ticks (Family Ixodidae), which act as competent vectors, and their specific biotopes. *Ixodes ricinus* is considered the most important vector of *A. phagocytophilum* in western Europe (Ogden et al., 1998, Lillini et al., 2006), but the potential of a transovarial further than the transstadial transmission has not be demonstrated yet. *A. phagocytophilum* persist in enzootic cycles due to the presence of "carrier" mammalian hosts, such as wildlife ruminants (mainly roe deer, *Capreolus capreolus*) and rodents (Stuen et al., 2010; Overzier et al., 2013). However, the pathogenic role of *A. phagocytophilum* in such mammalian hosts has not yet been defined (Milner and van Beest, 2013).

Among domestic species, sheep show an high susceptibility to *A. phagocytophilum* infection, and have been used to study the pathogenesis of infection as an experimental model. Sheep may also act as a domestic natural reservoir because they carry the organism for prolonged periods after infection (up to 25 months after primary infection), showing variable bacteraemia (Foggie, 1951; Stuen et al., 1998).

A. phagocytophilum infection in sheep is well known due to the significant economic losses associated with the clinical implications in the sheep industry, especially in Scandinavian countries

(above all Norway) (Stuen, 2003; Stuen et al., 2011). The disease is associated with a self-limiting febrile illness, followed by a range of clinical signs that are attributable to immunosupression due to the prolonged defects in neutrophils and lymphocytes, thus leading to pneumonia and septicaemia caused by *Mannheimia haemolytica* and *Biberstenia trehalosi* (Boyce et al., 2004; Woldehiwet, 2006; Grandi et al., 2018). Furthermore, lambs may develop "tick pyaemia", a crippling lameness and paralysis due to infection with concurrent bacteria such as *Staphylococcus aureus* (Stuen et al., 2009). Significant indirect losses (e.g. reduced growth, drop in milk yield, reduction in growth indices and fertility of rams) and occasionally miscarriages are also found in sheep farming (Stuen et al., 2011).

To date, data on the prevalence and diffusion of *A. phagocytophilum* among domestic animals in Italy are limited, especially those related to sheep (Lillini et al., 2006; Ebani et al., 2008; Diaferia et al., 2008).

The aim of the present work was to conduct a seroprevalence survey of *A. phagocytophilum* on sheep reared in Tuscany, a region in central Italy with the fourth most abundant sheep population in Italy, after Sardinia, Sicily, and neighbouring Latium.

2. Material and methods

2.1. Study population and questionnaire survey

In the tick-season from May to June 2011, 624 blood samples were collected from adult sheep of the Sarda breed in 33 dairy flocks located in a mainly hilly area of Grosseto (Tuscany, central Italy), extending from latitude 42.851078°N to 42.51902°N and from longitude 11.129635°E to 11.531657°E. The altitude of the farms ranged from 13 to 574 m above sea level. Grosseto has approximately 730 sheep flocks and 122,000 sheep, and sheep are sympatric with wild ungulates, including roe deer (*Capreolus capreolus*).

The sample size was calculated using the formula $n = Z^2 * p * (1-p)/CI^2$, where Z is the Z-value (e.g., 1.96 for a 95% confidence level), p is the expected prevalence, expressed as a decimal, and CI is the confidence interval, expressed as a decimal (Mariano et al., 2009). With an expected prevalence of 50% (0.5), a desired precision estimate of 0.04 and a confidence level of 0.95 a sample size of 600 animals is then required.

With approximately 122,000 sheep and 730 flocks in the district, the sample size provides a 95% confidence level (CL) for sheep-level prevalence, with a confidence interval (CI) of 3.93, and a 95% CL for a herd-level prevalence with a CI of 17.24 (Cenci Goga et al., 2013). The flocks were randomly selected using premise-identification dairy database numbers at the beginning of the study to ensure a representative sample of farms from the district (specifically all numbers for a given area were printed, cut out and drawn from a "hat"). Between 8 and 45 animals were selected per flock, approximately proportional to the total number of adult sheep in the flock. A questionnaire was prepared to include questions on rearing system (extensive: daily grazing in favourable weather conditions and returning to fold at night or daily grazing with possibility of shelter in bad weather; intensive: sheep housed day and night). The questionnaire was administered by the attendant veterinarian at the time of blood sample collection.

2.2. Sample collection and serological test

Venous blood samples were collected in tubes without anticoagulant from each selected animal and transferred to the laboratory on ice. After centrifugation (1,000 x g for 10 minutes), the sera were stored at -20°C until being tested for IgG antibody class against *A. phagocytophilum*, using an indirect immunofluorescence antibody assay (IFA). The IFA assay was performed using a commercial equine variant of *A. phagocytophilum* (formerly *Ehrlichia equi*) (Megascreen® FLUOANAPLASMA ph. Horbranz, Austria) and a commercial rabbit fluorescein isothiocyanate-conjugated anti-sheep IgG (Sigma-Aldrich, St Luis, MO, USA), diluted 1/40 (Stuen et al., 2013) in

Blue Evans solution. Sera were screened for antibodies at a dilution of 1/40 (cut off), and if positive, the sera were further diluted and retested to determine the final titre.

2.3. Statistical analysis

To identify risk factors associated with infection of *A. phagocytophilum*, first a univariate analysis of the variables of interest was conducted with binary logistic regression followed by multiple logistic regression performed with StatView 5 for Mac OS (SAS Inst. Inc., Cary, NC, USA).

3. Results

Fifty out of the 624 sera analyzed (8.01%, 95% IC 6.13 - 10.41%) were positive for antibodies (IgG) against *A. phagocytophilum.* Positive results were found in 21 out of the 33 flocks tested (63.63%, 95% IC 47.22-80.05%), with a rate of prevalence within flocks ranging from 3.33% to 59.26%. Positive sera showed antibody titres ranging from 1/40 to 1/320; in particular, 21 out of the positive sera (42% ± 6.98) had an antibody titre of 1/40, twenty-two (44% ± 7.02) had a titre of 1/80, five (10% ± 4.24) a titre of 1/160 and two (4% ± 2.77) showed a titre of ≥ 1/320.

The multivariate analysis identified the variable "small flock" as associated to serological positivity (OR 2.45, CI = 1.23 – 4.88; Table 1). We did not observe any confounding effect of the rearing system on the relationship between the size of flocks and serum positivity.

4. Discussion

Our results show a moderate presence of *A. phagocytophilum* among flocks from the province of Grosseto (central Italy). The overall prevalence of *A. phagocytophilum* (8%) among sheep was slightly lower than earlier observations conducted in central Italy, showing values ranging from 12 to 18% (Lillini et al., 2006; Ebani et al., 2008; Diaferia et al., 2008), and much lower than the rate reported by Torina et al. (2010) in Sicily (98%). However, the prevalence rate detected in Sicily may

be strongly overestimated because antibody detection was conducted using an ELISA test that does not discriminate between *A. phagocytophilum* and *Anaplasma ovis*, which represents the predominant species of *Anaplasma* occurring in sheep (Scoles et al., 2008).

Since IgG antibodies against *A. phagocytophilum* are expected to last a lifetime, cross-sectional serosurveys might have the limitation to provide only a partial account of past infections. However, the present study should give a good indication of a recent exposition to *A. phagocytophilum* infection and, thus of the infective pressure on the sampled population, due to the fact that the sampling period overlaps with one of the peak in abundance of the vector *I. ricinus* in the studied areas (Bisanzio et al., 2005).

Most of the antibody titres detected in our study were fairly low. Earlier studies indicate frequent cross-reactions between different variants of *A. phagocytophilum* (Dumler et al., 1995). However, the antibody titre to heterologous strains of *Anaplasma* may be lower than a homologous strain, which might also affect the risk of false negatives or low titres (Walls et al., 1999). Since in the present work a commercial IFA test using a heterologous horse variant of *A. phagocytophilum* was used to determine the antibody titre, because no antigen from a sheep variant of *A. phagocytophilum* was commercially available, it is possible to speculate that higher titres may have been found using a more appropriate antigen. Moreover, several other factors might have an impact on the low antibody response e.g. the tick infection rates and the level of bacteremia that depends on the time of the primary infection and on the *A. phagocytophilum* variant involved (Thomas et al., 2012).

The number of seropositive animals in the flock was negatively associated with the size of the flock. In fact, seroprevalence was higher in smaller flocks (< 300 sheep). The negative association between seroprevalence rate and flock size may be correlated to the breeding system and thus to the different tick-pressure by the exophilic tick *I. ricinus*. In fact, in the present study area, the farms with higher numbers of sheep tended to be more intensive with animals having less grazing activity and thus a

lower exposition to the tick-bites, compared to animals reared in small flocks where the prevalent breeding system was extensive.

We found no evidence of clinical disease on sheep flocks investigated. Although *A. phagocytophilum* infection based on serological and biomolecular methods has been reported in association both with domestic and wild species including sheep, to date the only documented clinical cases in Italy have been described in dogs (De la Fuente et al., 2005; Dondi et al., 2014), horses (Alberti et al., 2005), and humans (Ruscio and Cinco, 2003). Several 16S rDNA sequence variants of *A. phagocytophilum* have been identified in sheep (Stuen et al., 2006; Granquist et al., 2010), and several studies have suggested that these variants lead to different immunological reactions and clinical symptoms (Stuen, 2003). It should thus not be ruled out that variants circulating in Italy among domestic ruminants, unlike those spread in northern Europe, have a scant pathogenicity.

The genetic heterogeneity of *A. phagocytophilum* seems to impact further on the clinical features observed also on the zoonotic potential of the strains. Several studies suggest that the variants of *A. phagocytophilum* from ruminants differ from those associated with human ones (De la Fuente et al., 2005; Rar and Golovljova, 2011), which can, however, be shared with small rodents, wild boars, horses and dogs (Massung et al., 2002; Petrovec et al., 2006; Scharf et al., 2011). Recent genotyping based on additional loci compared to the traditional 16S rDNA (e.g. *msp*2, *msp*4, groESL) have again raised the issue of a possible zoonotic risk from the circulation of variants harboured by domestic and wild ruminants (Jahfari et al., 2014). To support the use of sheep as epidemiological sentinels for infection by *A. phagocytophilum*, recently recognized as an emerging human tick-borne pathogen in the U.S. and Europe (Goodman, 2005; Thomas et al., 2009), future studies investigating the genetic variation within the *A. phagocytophilum* strains isolated from humans and sheep flocks from the same geographic area should be conducted.

5. Conclusions

In conclusion, the results of the present study reveal a moderate circulation of *A. phagocytophilum* among sheep flocks in Tuscany (central Italy), with a seroprevalence rate of 8.01% and low antibody titres (between 1/40 and 1/80). Moreover the multivariate analysis identified "small flock" (< 300 animals) as the only risk factor associated with *A. phagocytophilum* infection.

These findings provide valuable information on the epidemiology of this pathogen in the country and documents the presence of the bacteria in one of its natural domestic reservoir.

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Table 1: Farm-level factors associated with serological positivity to *Anaplasma phagocytophilum*: results of multiple logistic regression.

Size of flock	**Odds ratio (95% CI)**	**P-value**
Big ($\geq$ 400)	1*	
Medium (300 $\leq$ x < 400)	1.23 (0.53 - 2.85)	0.62
Small (< 300)	2.45 (1.23– 4.88)	**0.0104****
Production system		
Intensive	1*	
Extensive	0.98 (0.35 – 2.69)	0.97

*: Reference level, **: P< 0.05