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Co-exposure to *Anaplasma* spp., *Coxiella burnetii* and tick-borne encephalitis virus in sheep in southern Germany

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Abstract

The intracellular bacteria *Anaplasma* spp. and *Coxiella burnetii* and the tick-borne encephalitis virus (TBEV) are tick-transmitted pathogens circulating in the southern German sheep population. Knowledge of interaction among *Anaplasma* spp., *C. burnetii* and TBEV in sheep is lacking, but together they might promote and reinforce disease progression. The current study aimed to identify co-exposure of sheep to *Anaplasma* spp., *C. burnetii* and TBEV. For this purpose, 1,406 serum samples from 36 sheep flocks located in both southern German federal states, Baden-Wuerttemberg and Bavaria, were analysed by ELISAs to determine the antibody levels of the three pathogens. Inconclusive and positive results from the TBEV ELISA were additionally confirmed by a serum neutralisation assay. The proportion of sheep with antibodies against *Anaplasma* spp. (47.2%), *C. burnetii* (3.7%) and TBEV (4.7%) differed significantly. Significantly more flocks with *Anaplasma* spp. seropositive sheep (91.7%) were detected than flocks with antibodies against TBEV (58.3%) and *C. burnetii* (41.7%), but there was no significant difference between the number of flocks which contained TBEV and *C. burnetii* seropositive sheep. Seropositivity against at least two pathogens was detected in 4.7% of sheep from 20 flocks. Most co-exposed sheep had antibodies against *Anaplasma* spp./TBEV (n = 36), followed by *Anaplasma* spp./*C. burnetii* (n = 27) and *Anaplasma* spp./*C. burnetii*/TBEV (n = 2). Only one sheep showed an immune response against *C. burnetii* and TBEV. Flocks with sheep being positive against more than one pathogen were widely distributed throughout southern Germany. The descriptive analysis revealed no association between the antibody response of the three pathogens at animal level. Taking the flocks as a cluster variable into account, the exposure to TBEV reduced the probability of identifying *C. burnetii* antibodies in sheep significantly (odds ratio 0.46; 95% confidence interval 0.24–0.85), but the reason for this is unknown. The presence of *Anaplasma* spp. antibodies did not influence the detection of antibodies against *C. burnetii* and TBEV. Studies under controlled conditions are necessary to evaluate any possible adverse impact of co-exposure to tick-borne pathogens on sheep health. This can help to clarify rare disease patterns. Research in this field may also support the One Health approach due to the zoonotic potential of *Anaplasma* spp., *C. burnetii* and TBEV.

Keywords *Anaplasma phagocytophilum*, *Anaplasma ovis*, *Dermacentor marginatus*, *Flaviviridae*, *Ixodes ricinus*, Tick-borne encephalitis, Tick-borne fever, Q fever, Zoonosis

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Findings

The intracellular bacteria *Coxiella burnetii, Anaplasma phagocytophilum* and *Anaplasma ovis*, and the tick-borne encephalitis virus (TBEV, *Flaviviridae*) are tick-transmitted pathogens and circulate in sheep flocks in the southern German federal states, Baden-Wuerttemberg (BW) and Bavaria (BAV) [1–3]. These pathogens also have a zoonotic potential and can cause illness in humans, such as flu-like symptoms and neurological disorders [4–7]. The main vector of *A. phagocytophilum* and TBEV is *Ixodes ricinus* and this tick species is widely distributed throughout Germany [4, 8, 9]. *C. burnetii* has also been found in *I. ricinus* [10], but *Dermacentor marginatus* is considered to transmit this pathogen to sheep [11, 12]. The existence of *D. marginatus* is limited to certain areas in southern Germany [8]. Recently, *A. ovis* was identified in engorged *D. marginatus* from Bavarian sheep, but this does not prove its vector competence, and solid data about *A. ovis* vectors are still lacking [3]. The clinical signs of these tick-borne pathogens are diverse in sheep. An infection with *C. burnetii* can result in reproductive disorders [5]. Haemolytic anaemia is caused by *A. ovis*, whereas an *A. phagocytophilum* infection results in tick-borne fever [13]. Moreover, *A. phagocytophilum* is an immunosuppressive agent and negatively affects the function of neutrophils, resulting in a higher susceptibility to secondary infections [14]. TBEV infection seems to be asymptomatic, but neurological signs in sheep have been reported [2, 15]. Concurrent infections of the louping ill virus (LIV, *Flaviviridae*) with *A. phagocytophilum* promote the onset of severe LIV-associated neurological disorders [16]. Furthermore, a dual infection of *A. phagocytophilum* and TBEV resulted in a significantly higher TBEV antibody response compared to a consecutive infection [15]. However, knowledge of interaction among *Anaplasma* spp., *C. burnetii* and TBEV in sheep is lacking, but together they might promote and reinforce disease progression.

The current study aimed to identify to which extent grazing sheep had antibodies against *Anaplasma* spp., *C. burnetii* and TBEV. For this purpose, 1,406 serum samples from 36 sheep flocks located in BW and BAV were analysed to detect antibodies against the three tick-borne pathogens. Initially, the blood samples were collected for a Q fever study, and the number of specimens required from each flock to estimate the positivity rate was calculated on the assumption of 3% expected prevalence, 95% confidence interval, 80% power and 5% precision [1]. A maximum of 44 animals per flock were sampled between November 2017 and June 2018. The blood sampling was performed in accordance with high ethical standards and approved by the federal state governments. The locations of the flocks are presented in Fig. 1.

Antibodies against *Anaplasma* spp., *C. burnetii* and TBEV were determined by three different commercial ELISAs in accordance with the manufacturers' instructions and described in detail elsewhere [1, 17, 18]. An inhibition of $\geq 30\%$ was assessed as positive for the *Anaplasma* spp. assay (Anaplasma Antibody Test Kit, cELISA v2, VMRD, Inc., Pullman, WA, USA), but this ELISA does not differentiate between antibodies against *A. phagocytophilum* and *A. ovis*. A sensitivity of 91.9% and a specificity of 86.9% were assumed according to Shabana et al. [19]. A S/P (%) > 40 for the *C. burnetii* ELISA was considered positive (Q Fever Antibody Test Kit, IDEXX Switzerland AG, Liebefeld, Switzerland), in accordance with the sensitivity and specificity of 100% each stated by the manufacturer. Regarding the TBEV ELISA (Immunozym® FSME IgG all Species, PROGEN Biotechnik GmbH, Heidelberg, Germany), the manufacturer specified samples with > 126 Vienna Units (VIEU)/mL as positive; values between 63 and 126 VIEU/mL were classified as inconclusive. A sensitivity of 97% and a specificity of 99% were assumed in accordance with the product information. The inconclusive and positive samples were confirmed with a serum neutralisation assay as recently described, and antibody titres of $\geq$ 1:40 were counted as positive [17].

The test results and their agreement were evaluated in descriptive tables. To determine the true prevalence at animal and flock level, the apparent prevalence was corrected for misclassification probabilities (sensitivity and specificity of the diagnostic tests) using the Rogan-Gladen estimator [20]. The prevalence of antibodies against more than one pathogen in the same individual or flock was also adjusted by correcting the test accuracies for parallel testing [21]. In addition, the proportion of positive antibody results of the three pathogens at animal and flock level was compared by Fisher's exact test. Subsequently, a logistic regression that considered the antibody result of one pathogen as the outcome and the other pathogen as the risk factor as well as the flocks as a cluster variable, was performed for the binary test results at animal level. The results of the two antibody tests were analysed in a logistic regression model to detect a significant association between pathogen exposure. Odds ratios were calculated to determine the strength and direction of a possible association. The association of the test results at flock level was analysed using Fisher's exact test. A P-value of < 0.05 was considered significant. For all calculations, the statistical software SAS (SAS Institute Inc., Cary, NC, USA) was used.

There was a significant difference among the results of true seroprevalence between *Anaplasma* spp., *C. burnetii* and TBEV at animal level ($p < 0.05$). Most sheep had antibodies against *Anaplasma* spp. (47.2%),

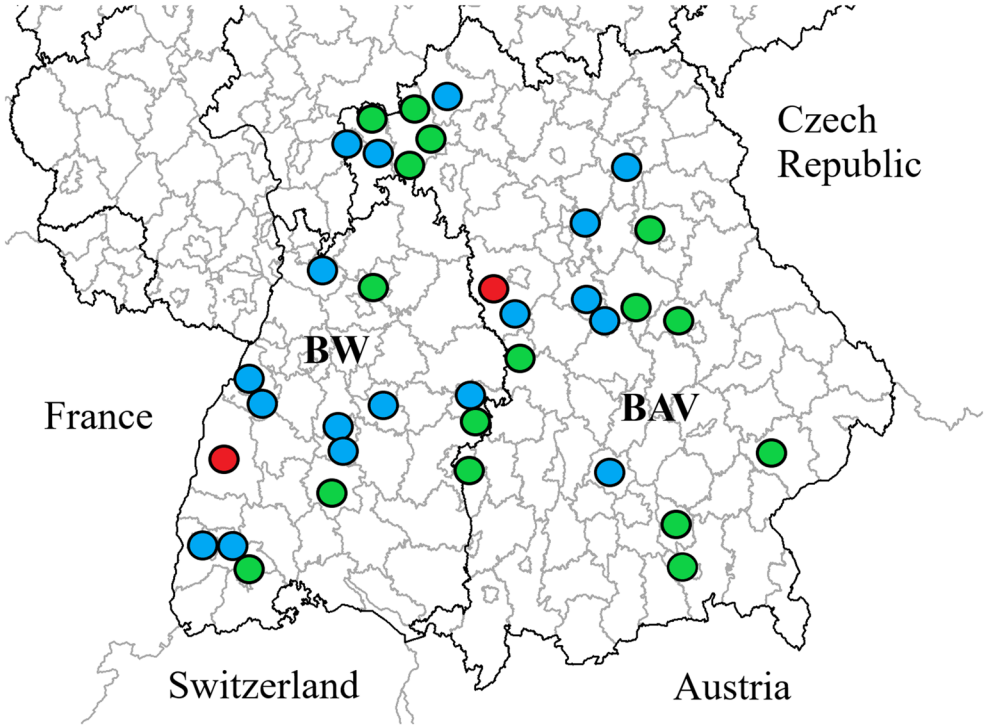

Fig. 1 Location of 36 examined sheep flocks in southern Germany. Concurrent positive antibody levels against *Anaplasma* spp., *Coxiella burnetii* and tick-borne encephalitis virus were determined in individual sheep in two flocks (red). Co-exposure to two pathogens at animal level were identified in 18 sheep flocks (blue), whereas no co-exposed sheep were detected in 16 flocks (green). BW: Baden-Wuerttemberg, BAV: Bavaria

followed by TBEV (4.7%) and *C. burnetii* (3.7%). Significantly more flocks with *Anaplasma* spp. seropositive sheep were detected (n = 33; 91.7%) compared to flocks being seropositive for TBEV (n = 21; 58.3%) and *C. burnetii* (n = 15; 41.7%) ($p < 0.05$), but there was no significant different between flocks which contained TBEV and *C. burnetii* seropositive sheep. Seropositivity against at least two pathogens was detected in 66 (4.7%) sheep from 20 flocks (55.6%). The flocks with co-exposed sheep were widely distributed throughout southern Germany. Details of sheep and flocks with antibodies against more than one of the pathogens are presented in Table 1; Fig. 1, respectively. The descriptive analysis revealed no association of the three pathogens at animal level (Table 2). Taking the flocks as a cluster variable into account, there was a significant association between presence of *C. burnetii* and TBEV antibodies. The exposure to TBEV reduced the probability of identifying *C. burnetii* antibodies in sheep by half (Table 3). The presence of *Anaplasma* spp. antibodies did not influence the antibody detection of *C. burnetii* and TBEV. Based on current knowledge, *A. ovis* appeared only locally in one sheep flock from northern Bavaria, but this flock did not participate in the current study [3]. Therefore, most *Anaplasma* spp. antibodies

Table 1 Seropositivity of sheep against at least two tick-borne pathogens determined by serological assays

Co-exposure of tick-borne pathogens	Number of antibody positive sheep
Anaplasma spp./*C. burnetii*	27
C. burnetii/TBEV	1
Anaplasma spp./TBEV	36
Anaplasma spp./*C. burnetii*/TBEV	2

In total, 66 sheep (n = 1,406) in 20 flocks (n = 36) from southern Germany had antibodies against at least two tick-borne pathogens. The corrected seroprevalence at animal level was considered. *C. burnetii: Coxiella burnetii*, TBEV: tick-borne encephalitis virus

Table 2 Association between antibodies against three tick-borne pathogens in sheep at animal level

Pathogen 1	Pathogen 2	Cohen's Kappa	*p*-value
C. burnetii	*Anaplasma* spp.	0.0081	0.48
C. burnetii	TBEV	0.0025	0.76
Anaplasma spp.	TBEV	0.0036	0.81

Results were evaluated by descriptive analysis and the *p*-values referred to the Fisher's exact test. ($p < 0.05$). *C. burnetii: Coxiella burnetii*, TBEV: tick-borne encephalitis virus

Table 3 Association between antibodies against three tick-borne pathogens in sheep at animal level with flock as a cluster variable

Dependent variable	Independent variable	Odds ratio [95% confidence interval]	*p*-value
C. burnetii	*Anaplasma* spp.	0.93 [0.53–1.64]	0.81
C. burnetii	TBEV	0.46 [0.24–0.85]	0.01
Anaplasma spp.	TBEV	1.25 [0.90–1.74]	0.17

Results were evaluated by logistic regression analysis with flock as a cluster variable, and the *p*-values referred to the corresponding Chi square test ($p < 0.05$). *C. burnetii*: *Coxiella burnetii*, TBEV: tick-borne encephalitis virus

were possibly induced by *A. phagocythophilum* due to the wide dissemination in the German sheep population [3, 22, 23]. This was taken into account while interpreting the presented findings.

The significant differences among antibody rates of *Anaplasma* spp., TBEV and *C. burnetii* at sheep level might correlate with the presence of the pathogens in ticks collected in southern Germany. Up to 8.3% of questing *I. ricinus* contained *A. phagocytophilum* [24], whereas the detection rate of TBEV ranged from 0 to 5.3% [25], and *C. burnetii* has been determined only in one engorged *D. marginatus* so far [12, 26]. Information about natural co-exposure with *Anaplasma* spp., *C. burnetii* and TBEV in sheep is extremely rare [27]. Significantly fewer sheep with *C. burnetii* antibodies were detected in flocks which also had sheep with antibodies against TBEV. Knowledge of interaction between both pathogens is missing, and we can only speculate about the possible reciprocal influence of both pathogens in sheep flocks. This observation needs further investigation in the future. The humoral immune response against *Anaplasma* spp., *C. burnetii* and TBEV lasts for several months in sheep [17, 28, 29]. Furthermore, co-infection with various pathogens were described for different tick species, but findings on the coincidental infection with *Anaplasma* spp., *C. burnetii* or TBEV are seldom in ticks [30–32]. Considering both these circumstances, we assume that natural exposure of sheep occurs through infestation of different ticks infected with one of the above-mentioned agents. In addition, infections with the pathogens might occur consecutively rather than at the same time. This is supported by previous findings of Paulsen et al. [15], who demonstrated that only a simultaneous infection of *A. phagocytophilum* and TBEV in lambs resulted in a significantly higher TBEV antibody response, but a consecutive infection had no influence on the antibody response of both pathogens. In the current study, the presence of *Anaplasma* spp. antibodies also did not influence the TBEV antibody detection at animal and flock level. Nevertheless, the number of lambs suffering from tick-borne encephalitis (TBE) seems to be on the increase in southern Germany [33], and the immunosuppressive impact of *A. phagocytophilum* should be investigated in lambs naturally infected with TBEV. Despite the fact that a detrimental influence was not confirmed after experimental infection with TBEV, the artificially infected lambs did not develop clinical signs of TBE [15]. Therefore, the outbreak of TBE in sheep appears to depend on as yet unknown factors.

The authors are aware of the limitations of the present study. The antibody response against the three tick-borne pathogens is the result of a natural exposure in the field in the past. Therefore, it is impossible to determine the exact time of infection and the possibly related clinical impact. Moreover, we cannot rule out that false-seropositive sheep were included in the evaluation because of an estimated specificity of 86.9% [19] of the *Anaplasma* spp. ELISA, and a possibly reduced specificity of the *C. burnetii* ELISA [34]. Only the inconclusive and positive results from the TBEV ELISA were confirmed by a serum neutralisation assay, which is considered as gold standard for TBEV antibody detection [17]. This minimises the risk of sheep being tested false-seropositive for TBEV antibodies. All in all, the low numbers of co-exposures could also be the consequence of imperfect specificity of the diagnostic tests used. In the future, co-infection has to be determined by further tests such as molecular assays to detect pathogen DNA. Nevertheless, our findings contribute to the complex issue of tick-borne pathogens in sheep. Co-exposure to *Anaplasma* spp., *C. burnetii* and TBEV seems to be sporadic among grazing sheep flocks in southern Germany. The further spread of TBEV and the emerging onset of *A. ovis* in Germany might increase cases of co-infection [3, 4]. More targeted investigations are needed to evaluate an adverse impact of co-exposure to tick-borne pathogens on sheep health due to the fact that *A. phagocytophilum* influences the immune response and disease progression of concurrent flavivirus infections [15, 16]. In addition, sheep flocks can be implemented as sentinels to identify potential new risk areas of emerging zoonotic pathogens such as TBEV [17]. Therefore, further research in this field may also support the One Health approach.

Abbreviations

BAV	Bavaria
BW	Baden-Wuerttemberg
cELISA	Competitive enzyme-linked immunosorbent assay
ELISA	Enzyme-linked immunosorbent assay
LIV	Louping ill virus
S/P (%)	Sample/positive percentage
TBE	Tick-borne encephalitis

TBEV	Tick-borne encephalitis virus
VIEU	Vienna units

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Author contributions
Study conception and design were carried out by BUB, MR, MG and CS. Manuscript preparation was carried out by BUB and CS. Laboratory work was performed by MS, LK, IS and WR. Samples were collected by BUB. CS performed the statistical analysis. All authors have read and approved the final version of the manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with German Animal Welfare Legislation and the EU Directive 2010/63/EU for animal experiments. Blood sample collection was approved by the federal state governments of Baden-Wuerttemberg (AZ 35-9185.82/0351, AZ 35-9185.82/D-18/01, AZ 35- 9185.82/A-1/18, AZ 35/9185.82/Ganter 18.01.2018) and Bavaria (RUF-55.2.2-2532-2-651-5, ROB-55.2- 2532.Vet_03-18-10). All animals were handled in accordance with high ethical standards and national legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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