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Korrespondenzadresse:
sarah.stalb@cvuas.bwl.de

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Chemisches und Veterinäruntersuchungsamt Stuttgart, Stuttgart, Germany[1]
Chemisches und Veterinäruntersuchungsamt Freiburg, Freiburg, Germany[2]
Landestollwut- und Epidemiologiezentrum, Chemisches und Veterinäruntersuchungsamt Freiburg, Freiburg, Germany[3]
Friedrich-Loeffler-Institut, Institut für Bakterielle Infektionen und Zoonosen, Jena, Germany[4]
Landesgesundheitsamt Baden-Württemberg, Stuttgart, Germany [5]

Detection of tularemia in European brown hares (*Lepus europaeus*) and humans reveals endemic and seasonal occurrence in Baden-Wuerttemberg, Germany

Nachweis von Tularämie in Feldhasen (Lepus europaeus) und in Menschen belegen das endemische und saisonale Vorkommen in Baden-Württemberg, Deutschland

Sarah Stalb[1], Birgitta Polley[1], Klaus-Jürgen Danner[2], Markus Reule[3], Herbert Tomaso[4], Anja Hackbart[4], Christiane Wagner-Wiening[5], Reinhard Sting[1]

Summary

Tularemia is a highly contagious infectious disease which has re-emerged in Germany with varying spatial and temporal distribution. European brown hares (*Lepus europaeus*) and wild rabbits (*Oryctolagus cuniculus*) typically serve as hosts for the zoonotic pathogen *Francisella tularensis* subsp. *holarctica*, but many animals including insects and ticks can represent relevant vectors for tularemia. Since the beginning of 2016, cases of tularemia have seemed to accumulate within the brown hare population in Baden-Wuerttemberg (BW). Simultaneously, the number of human infections has also risen compared to previous years. Natural emergence or re-emergence of tularemia can appear in susceptible populations at any time. Knowledge about the actual occurrence and spreading of tularemia is essential to inform health professionals and risk groups, such as hunters, forest workers and farmers in endemic areas. Therefore, we assessed the spatial and temporal distribution of tularemia in brown hares and wild rabbits in BW, retrospectively based on results obtained from cultural and molecular investigations during routine diagnosis, including 179 brown hares (55 positive animals) and ten wild rabbits (0 positive animals), which were randomly found moribund or dead between January 2010 and June 2016. In addition we assessed surveillance data from notified human cases during the same time period. Our data suggest that tularemia is endemic in BW due to an annual and widespread occurrence. Furthermore, tularemia occurs with seasonal peaks in spring and autumn, in particular in October. There are counties where tularemia was observed in both humans and brown hares; however, there are regions with no coincidence of cases in humans and brown hares, suggesting that other reservoirs such as insects or ticks may play a relevant role as vectors. The results of the present study suggest that tularemia is an endemic zoonosis in BW which poses a permanent infection risk to humans.

Keywords: *Francisella tularensis* subsp. *holarctica*, zoonosis, European brown hare, epidemiology

Zusammenfassung

Die Tularämie ist eine hochkontagiöse Infektionskrankheit, die in Deutschland seit einigen Jahren wieder verstärkt mit unterschiedlicher regionaler und zeitlicher Verbreitung auftritt. Klassische Wirte für den Zoonoseerreger *Francisella tularensis* subsp. *holartica* sind der Europäische Feldhase (*Lepus europaeus*) und das Wildkaninchen (*Oryctolagus cuniculus*), aber auch viele andere Tiere einschließlich Insekten und Zecken können relevante Vektoren sein. Seit Beginn 2016 scheint eine steigende Fallzahl an Tularämie verendeter Feldhasen in Baden-Württemberg (BW) aufzutreten. Gleichzeitig stieg auch die Anzahl humaner Infektionen in BW im Vergleich zu den Vorjahren an. Das natürliche und erneute Auftreten von

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Tularämiefällen in empfänglichen Populationen ist demnach jederzeit möglich. Kenntnisse über das aktuelle Vorkommen und die Verbreitung der Tularämie sind wichtig, um Gesundheitsämter und Risikogruppen wie Jäger, Waldarbeiter und Landwirte in Endemiegebieten informieren zu können. Um die regionale und saisonale Verbreitung der Tularämie bei Feldhasen und Wildkaninchen einschätzen zu können, werteten wir in einer retrospektiven Studie die bakteriologischen und molekularbiologischen Untersuchungsergebnisse von 179 Feldhasen (55 positive Tiere) und zehn Wildkaninchen (0 positive Tiere) aus, die zufällig moribund oder tot in BW im Zeitraum von Januar 2010 bis einschließlich Juni 2016 gefunden wurden. Zudem werteten wir die humanen Meldedaten in diesem Untersuchungszeitraum aus. Unsere Ergebnisse weisen darauf hin, dass die Tularämie in BW aufgrund des jährlichen und weitverbreiteten Vorkommens endemisch ist. Außerdem tritt die Tularämie mit saisonaler Häufung im Frühjahr und im Herbst auf, insbesondere im Oktober. Es gibt Regionen, in denen Tularämiefälle sowohl bei Menschen als auch bei Tieren auftreten, wobei dieser Zusammenhang nicht zwingend ist. Dies lässt die Schlussfolgerung zu, dass auch andere Vektoren, wie Insekten und Zecken, relevant sein können. Die vorliegenden Ergebnisse legen nahe, dass die Tularämie in BW eine endemische Zoonose mit Infektionsgefahr für den Menschen ist.

Schlüsselwörter: *Francisella tularensis* subsp. *holarctica*, Zoonose, Feldhase, Epidemiologie

Introduction

Tularemia is a zoonotic disease which exists endemically in most European countries (Tarnvik et al., 2004). Sporadic outbreaks occur in both humans and animals at different times of the year (Morner, 1992). Even in endemic foci, the occurrence of tularemia varies widely between regions and tularemia may emerge annually within a 5-year period or may be inactive for more than a decade, for yet unknown reasons (WHO, 2007). The re-emergence of tularemia has been reported in several European countries, including Germany (Hauri et al., 2010; Kaysser et al., 2008; Splettstoesser et al., 2007; Splettstoesser et al., 2009; Tarnvik et al., 2004). In Europe, this notifiable infection is caused by *Francisella (F.) tularensis* subsp. *holarctica* (type B), in contrast to the highly pathogenic *F. tularensis* subsp. *tularensis* (type A), which is mostly found in the USA (Olsufjev and Meshcheryakova, 1983; WHO, 2007). Both subspecies affect a broad range of hosts (Ellis et al., 2002). Regional occurrence typically affects only a few mammalian and arthropod species (Ellis et al., 2002). In Europe, tularemia is most frequently seen in hares (*Lepus* spp.) and other small animals such as rabbits, which serve as reservoir hosts and are linked to enzootic transmission (Morner, 1992; Nigrovic and Wingerter, 2008; Runge et al., 2011). Arthropods (ticks, mosquitoes and biting flies) are relevant vectors and may serve as a long-term reservoir (Ellis et al., 2002; Keim et al., 2007; Tarnvik et al., 2004). Tularemia is transmitted to humans by direct contact with infected animals, through contaminated water or food, or by vectors such as mosquitoes or ticks (Gehringer et al., 2013; Morner, 1992). The clinical presentation in humans depends on the respective Francisella subspecies and the route of infection (Nigrovic and Wingerter, 2008). In hares and rabbits, tularemia usually causes an acute and fatal septicaemia, however sometimes shows a subacute to chronic disease course (Gyuranecz et al., 2010; Sting et al., 2013).

Tularemia was first recovered from brown hares in Baden-Wuerttemberg (BW) in 2008 (Sting, 2008). Since then, only sporadic cases of tularemia have been observed in brown hares in BW, which were caused by *F. tularensis* subsp. *holarctica* (biovar 1, erythromycin sensitive) (Mueller et al., 2013; Sting, 2008). In Germany, the number of human cases has risen since 2004; however, tularemia still remains a relatively rare disease (Mueller et al., 2007; Runge et al., 2011; Splettstoesser et al., 2007). In BW in the years 2010–2015 between four and nine human cases and between one and 14 cases in brown hare were notified per year (LGA, 2016). Remarkably, in the first half of 2016 a growing number of tularemia cases in brown hares were observed in BW. Within the same time period, eleven human cases had already been notified in BW (LGA, 2016). Indeed, outbreaks in humans are often correlated with outbreaks in animal populations (Ellis et al., 2002; Sjostedt, 2007). Knowledge about the occurrence and spread of tularemia in animals in endemic areas is essential for public health management. Exposed persons such as hunters, forest workers, and farmers, as well as public health professionals and medical practitioners, should be aware of the risk of tularemia infections, especially in endemic foci. Therefore, we evaluated data on the spatial and temporal distribution of tularemia in the brown hare population of BW from January 2010 until June 2016 retrospectively, including the data obtained during routine diagnostics of randomly found carcasses which cannot be assumed to be statistically representative.

Material and Methods

A total of 179 European brown hares (*Lepus europaeus*) and ten wild rabbits (*Oryctolagus cuniculus*) were examined for *Francisella tularensis* between 01.01.2010 and 30.06.2016. The tested animals were accidentally found dead or moribund by hunters, farmers and hikers within the regional councils of BW and sent in for bacteriological testing. Samples were investigated either by a subspecies specific SYBR-Green based real-time PCR targeting the genomic locus designated Ft-M19 (Bystrom et al., 2005), which obtains a 30-bp deletion unique to identify the

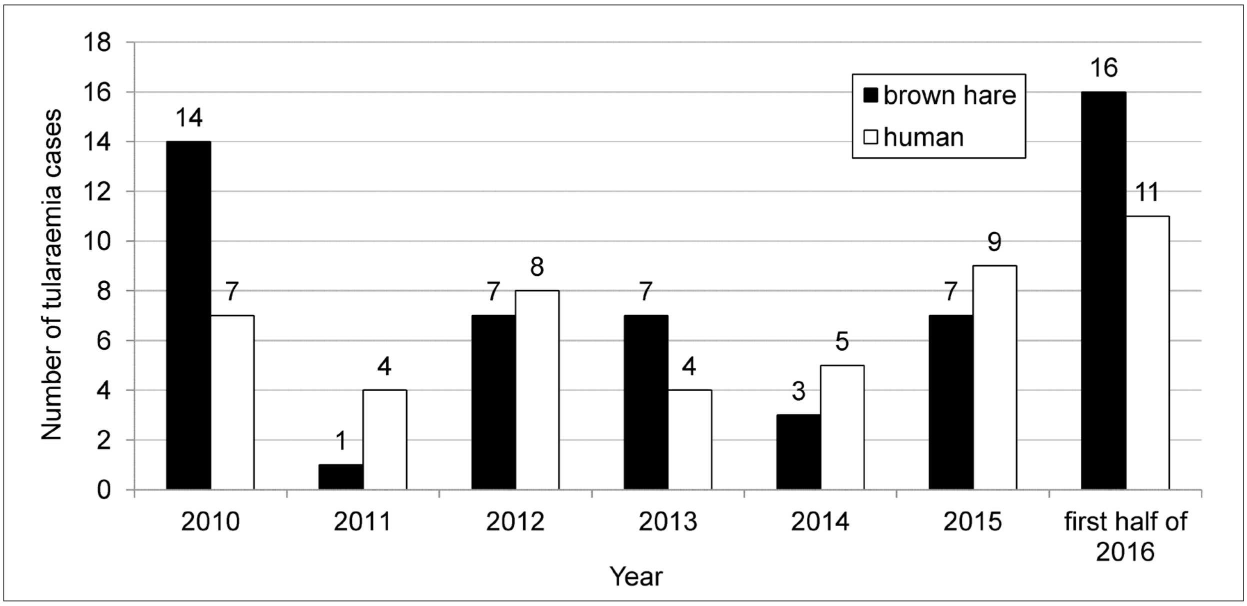

FIGURE 1: *Detection of F. tularensis subsp. holarctica in brown hare and humans in Baden-Wuerttemberg (Germany) between 01.01.2016–30.06.2016..*

subspecies of *F. tularensis* (n = 22), or by culture (n = 33); the latter was followed by MALDI-TOF mass spectrometry identification (MALDI TOF Microflex Biotyper System, Bruker Daltonik, Bremen, Germany), as published before (Johansson et al., 2000; Mueller et al., 2013; Sting et al., 2013). Further identification including phylogenetic characterisation of the isolates using canonical single nucleotide polymorphism assays (canSNPs), was performed at the National Reference Laboratory for Tularemia (Friedrich-Loeffler-Institute, Jena, Germany) as described previously (Mueller et al., 2007).

Antibiotic susceptibility testing was assessed by disk diffusion method towards seven antimicrobial agents (erythromycin, tetracycline, doxycycline, streptomycin, gentamicin, chloramphenicol and ciprofloxacin) as published elsewhere (Tomaso et al., 2005).

In addition, we analysed surveillance data on human tularemia, since acute, laboratory diagnosed tularemia is notifiable in Germany. The surveillance data, provided by IfSG-Meldestelle Baden-Württemberg from 2010 until 30.06.2016 was assessed with special focus on geographical distribution.

Results

Annual occurrence of tularemia

Francisella tularensis subsp. *holarctica* occurs annually and is widespread in BW, resulting in overall 55 positive cases (22 by PCR and 33 by culture) of tularemia detected within the brown hare population and 48 laboratory diagnosed cases in humans between January 2010 and June 2016 (Fig. 1). Positively tested hares originated from 23 of the 34 counties from which hares were investigated. In humans, notified autochthonous cases of tularemia occurred in 20 out of the 44 counties of BW. In brown hare, the number of cases of tularemia varies from one year to another. However, in the first half of 2016 more cases were observed (n = 16) than in the previous years (between one and 14 cases per year).

An average of about 30% of all investigated brown hares perished every year from tularemia. However, particularly low absolute and relative numbers of annual detection rates occurred in 2011 (6%, 1/17) and 2014 (11%, 3/28). Among *F. tularensis* subsp. *holarctica* strains isolated from brown hares (n = 25), genotyping revealed that all strains belonged to the basal genetic clade IV and subclade 18. In addition, all isolates were sensitive to erythromycin, as well as to tetracycline, doxycycline, streptomycin, gentamicin, chloramphenicol, and ciprofloxacin.

In humans, 48 cases (median age 50, range 1–84 years, 31 males) have been notified in BW since 2010. The average annual number of tularemia cases increased from two (2001–2009) up to eight (2010–06/2016). Since 2010, suspected vectors have been hares (13%, 6/48), ticks (21%, 10/48) and mosquitoes (2%, 1/48). Furthermore, five human cases were linked to farming (10%, 5/48), four cases had a travel history to eastern countries such as Kosovo (8%, 4/48) and one case of laboratory infection occurred (2%, 1/48). The source of infection remained unknown in half of the human cases (50%, 24/48).

Seasonality of tularemia

In the brown hare population of BW, tularemia is a seasonal disease, with its highest occurrence during late spring and autumn (Fig. 2). Expressing the number of tularemia cases of brown hare for each month as a percentage of all investigations indicate that tularemia cases accumulate during May (56%, 10/18) and October (73%, 8/11), whereas lowest detection rates of tularemia occur during January (13%, 2/16) and December (17%, 4/24). In humans, excluding five cases with a travel history or without data specification, most cases accumulated in October (21%, 9/43) as well as in summer during June (12%, 5/43) and July (14%, 6/43). Besides that, a high number of cases were notified in January (14%, 6/43).

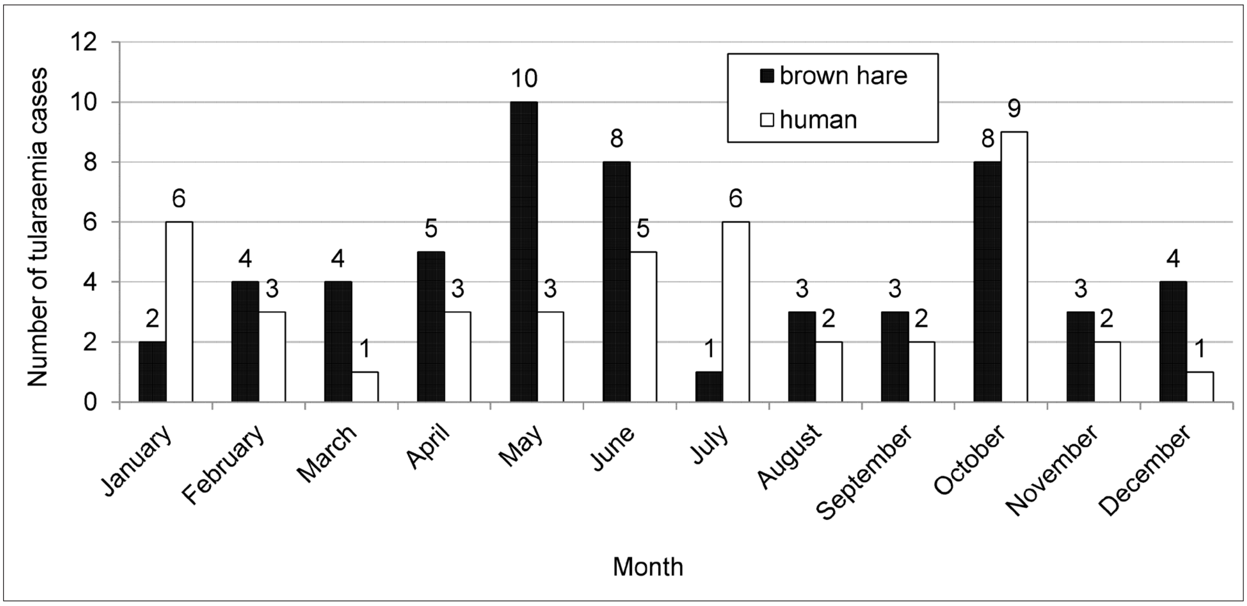

FIGURE 2: *Seasonal occurrence of tularemia in brown hare and humans in Baden-Wuerttemberg (Germany) from 2010 until June 2016. For humans the onset of the disease is presented. Four human cases are excluded due to travel history and no date was available in one case.*

Geographical distribution of tularemia cases

Tularemia has been reported in many regions of BW and some hot-spot regions can be identified. In brown hares, these hot-spots include the counties of Ludwigsburg, Rems-Murr, Schwaebisch Hall, Main-Tauber, Ortenau, Breisgau-Hochschwarzwald, Biberach, and Bodensee (Lake Constance) (Fig. 3). Most diagnosed human cases occurred in the county and city of Karlsruhe (n = 7 and n = 4), Ortenau (n = 5), Biberach, Ludwigsburg (each, n = 3), Goeppingen, Neckar-Odenwald, Rhein-Neckar, Rottweil and Tuebingen (each, n = 2). The detection of *F. tularensis* in the brown hare population coincides with sites of exposition of notified human cases in the following twelve counties (number of cases in brown hare/humans): Alb-Donau (1/1), Biberach (2/3), Breisgau-Hochschwarzwald (4/1), Emmendingen (1/1), Enzkreis (1/1), county of Karlsruhe (1/7), city of Karlsruhe (1/4), Ludwigsburg (8/3), Neckar-Odenwald (1/2), Ortenau (6/5), Raststatt (1/1) and Schwaebisch Hall (4/1). It should be noted that in the counties of Karlsruhe (n = 2), Biberach, Ludwigsburg, Ostalbkreis and Ortenaukreis (each, n = 1) human cases could be traced back to direct contact with infected brown hare. However, *F. tularensis* was also detected in brown hares in eleven counties where no autochthonous human cases were reported in the period between January 2010 and June 2016 including Bodensee (Lake Constance) (n = 5), Freudenstadt (n = 1), Heilbronn (n = 1), Hohenlohe (n = 1), Loerrach (n = 1), Main-Tauber (n = 1), Ravensburg (n = 1), Rems-Murr (n = 8), Reutlingen (n = 1), Sigmaringen (n = 1) and Waldshut (n = 1). In a minor portion of counties (n = 8), human cases were diagnosed even if no cases were observed in brown hares, including the cities of Baden-Baden (n = 1), Esslingen (n = 1), Goeppingen (n = 2), Ostalbkreis (n = 1), Rhein-Neckar (n = 2), Rottweil (n = 2), Tuebingen (n = 2) and Zollernalb (n = 1). In some counties human cases were linked to farming including Biberach, Goeppingen, Ortenau, Raststatt and Zollernalb (each, n = 1). Tick bites were the suspected source of infection in the counties of Karlsruhe, Ludwigsburg (each, n = 2), Ortenau, Breisgau-Hochschwarzwald, Neckar-Odenwald, Tuebingen, Esslingen and the city of Baden-Baden (each, n = 1). In the county of Ortenau, one case was even linked to a mosquitoe bite. Among brown hare, in ten counties no carcasses were submitted for investigation and in another ten counties no tularemia was detected.

Discussion

Annual occurrence of tularemia

The annual and widespread occurrence of tularemia in brown hares and humans point out that tularemia is endemic in BW. In BW, tularemia co-occurs in brown hares and humans with comparable temporal patterns over several years. It is worth noting that, low detection rates occurred, for example in 2011. Since 2014 an increasing number of cases have been recognized, exacerbating since the beginning of 2016 in both brown hares and humans. Annual variation in the occurrence of tularemia is well known from the literature and is still not well understood, but may be related to climatic factors such as temperature and precipitation (WHO, 2007). However, the simultaneous increase of tularemia cases in man and brown hare suggests that brown hare play an important role in the ecology of tularemia and may serve as a reservoir for human infections, as described previously (Gyuranecz et al., 2010). Indeed, a preceding increase in the density of the rodent population size has previously been associated with outbreaks of tularemia (Efimov et al., 2003). In Germany, tularemia in hares occurs in regions with rather humid soil, corresponding with the natural habitat of hares (Mueller et al., 2013). A dense population of brown hare exists throughout BW, with an average of 16 hares/100 km², which is largely dependent on the structure of the landscape ranging from four to 55 hares/100 km² (LAZBW, 2016). In Germany, 40% of human tularemia cases were linked to contact with or consumption of infected hares (RKI,

FIGURE 3: *Geographical allocation of tularemia cases among European brown hare (rectangles) and humans (triangles) in counties in Baden-Wuerttemberg (Germany) from 2010 until June 2016. The symbols are randomly distributed within each county. For brown hare (BH) the data are presented as number of cases/number of investigated animals in the respective counties. For humans (H), the number of notified autochthonous cases is noted. The names of the counties are given as abbreviations according to the vehicle registration system for the county.*

2008); in contrast, this share only accounts for 13% in BW. However, farming (10%) as suspected source of infection might partly be attributed to infected animals due to inhalation of contaminated dust or hay (Tarnvik et al., 1996). This might also be true for cases of unknown sources of infection (50%). In France, an increase in cases in hares has often been associated with an increased number of human cases during the winter (Decors et al., 2011). However, in Sweden no correlation with tularemia and the size of the rodent populations was found (Tarnvik et al., 1996) and mosquitoes have been strongly implicated as vectors of tularemia (Ellis et al., 2002; Tarnvik et al., 2004). Likewise, in BW an increasing number of tick-transmitted tularemia cases in humans have been noted, supported by the detection of *F. tularensis* in ticks (*Ixodes ricinus*) (Gehringer et al., 2013) and tick-born transmission to humans (Boone et al., 2015). However, the importance of various susceptible animals as reservoirs for *F. tularensis* and the change of transmission patterns and epidemiology of tularemia over time are still poorly understood and deserves closer attention (WHO, 2007).

Endemic appearance of tularemia in BW is further supported by the fact that within the brown hare population *F. tularensis* subsp. *holarctica* remains stable over time. All isolates recovered from brown hares retained the same genotype and biovar during the observation period. The basal genetic clade IV and subclade 18 correspond with the so-called Iberian clone, which is widespread in western and central Europe including western parts of Germany (Gyuranecz et al., 2012; Mueller et al., 2013). As expected, all isolates from brown hare in BW were assigned to biovar I (erythromycin sensitive), corroborating with previously published data (Mueller et al., 2013). In addition, the antimicrobial susceptibility pattern of these isolates is typical for *F. tularensis* subsp. *holarctica*, which is naturally resistant to penicillins and cephalosporins (Tomaso et al., 2005). Overall, all isolates of *F. tularensis* subsp. *holarctica* from brown hare in BW belonged to one defined molecular subpopulation and, until now, no introduction of the phenotypically distinct subpopulation (biovar II [erythromycin resistant], clade B.I) from eastern Germany has occurred. However, underlying mechanisms leading to this segregation of two phylogenetic distinct subpopulations of *F. tularensis* subsp. *holarctica* in Germany remain obscure (Mueller et al., 2013).

Seasonality of tularemia

It is striking that tularemia shows a seasonal pattern in BW, with most cases diagnosed in October in both humans and brown hares. Furthermore, increasing numbers of tularemia cases in humans occurred in June and July. This is in accordance with the situation in most endemic countries, where the incidence is usually highest during late spring, the summer months and early autumn (CDC, 2002; Tarnvik et al., 2004; WHO, 2007). In addition in BW, increasing numbers of cases in humans occurred in January, in contrast to relative and absolute low detection rates in brown hares during this month. For example, in France high mortality in hares coincided with sharp drops in temperature of about 8°C within four days from January to March (Decors et al., 2011), and in Norway an unusually cold November and December in 2010 was accompanied by fatal cases of tularemia in the mountain hare (*Lepus timidus*) (Larssen et al., 2011). In comparison, in the USA the most significant risk factor for humans in late spring and summer are arthropod bites and direct contact with infected tissue, whereas a winter incidence peak is associated with rabbit hunts (Ellis et al., 2002). Typical natural foci of tularemia occur in biotopes with mean annual air temperature between 8°C to 10°C (Pikula et al., 2003), emphasizing the survival strategy of the psychrophilic *F. tularensis* bacteria (RKI, 2016). In general, climatic conditions seem to influence tularemia outbreaks depending on the type of reservoir involved and the modes of transmission (Morner, 1992).

Geographical distribution of tularemia cases

In BW as a whole, tularemia must be considered as a persistent potential risk of infection for both, humans and brown hares, especially in hot-spot areas. However, evaluations on the basis of counties are not unambiguous. In less than two thirds of the counties in BW (27%, 12/44), the detection of *F. tularensis* in the brown hare population coincides with the localisation of exposition of notified human cases. The notified human cases were verified with regard to the actual site of infection, because the localisation of notification is not necessarily the site of exposure. In these areas, the detection of tularemia in wildlife should be perceived as a positive indicator. In contrast to these results, in a quarter of the counties in BW (25%, 11/44), *F. tularensis* was only detected in brown hares without autochthonous human cases and in a minor portion of counties (18%, 8/44) human cases were diagnosed even if no cases were observed in brown hares. Thus, tularemia in humans is most probably considerably underreported and underestimated due to unspecific clinical symptoms (Splettstoesser et al., 2009). This probably also applies to knowledge about the actual distribution and occurrence of tularemia in wildlife in BW. This is due to a lack of knowledge about the true number of infected hares, since the number of investigated carcasses depends mainly on random findings in the field reflecting only the tip of the iceberg. Even if the number and co-occurrence of human cases is not sufficient to establish an epidemiological link between human and animal tularemia cases, there is growing body of evidence that other animal vectors in addition to brown hares carry the pathogen (Lutz et al., 2016; Schulze et al., 2016). However, this data offers valuable evidence regarding where and when tularemia circulates in the field and gives a realistic view on where possible exposure to diseased brown hares exists. Indeed, in BW in five counties infections in humans could be ascribed to direct contact to infected brown hares. This may reflect the fact that the pathogen inhabits natural foci. This is supported by a prevalence study where most of the tularemia positive brown hares were mainly restricted to two hot-spots areas (Runge et al., 2011). Many routes of transmission might co-occur in hot-spot areas, as shown in several counties in BW. This further implicates the importance of assessing epidemiological data on geographical and temporal occurrence of endemic tularemia in order to improve the health risk assessment as a basis for protective measures (Splettstoesser et al., 2009).

We conclude that tularemia is endemic in BW, displaying a seasonal appearance and varying occurrence in different years. Our data also show that the number of cases in brown hare does not always correspond with the number of cases reported in humans, suggesting that contact with infected hares is not the only mode of transmission and that arthropod vectors and contaminated dust and water may play a relevant role. Therefore, *F. tularensis* subsp. *holarctica* always has to be regarded as a potential cause of infection in BW, even in years or counties without reports of tularemia in animals. However, the present sample cannot be assumed to be statistically representative. A definitive statement about the current geographical and temporal occurrence of tularemia in BW may be limited compared with representative sampling and therefore monitoring of wildlife in BW should be enlarged significantly.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Address for correspondence:
Sarah Stalb
Chemisches und Veterinäruntersuchungsamt Stuttgart
Schaflandstr. 3/3
70736 Fellbach
Germany
sarah.stalb@cvuas.bwl.de