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Seroprevalence of horses to *Coxiella burnetii* in an Q fever endemic area

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ABSTRACT

Coxiella burnetii can infect many animal species, but its circulation dynamics in and through horses is still unclear. This study evaluated horse exposure in an area known to be endemic for ruminants and humans. We assessed antibody prevalence in horse serum by ELISA, and screened by qPCR horse blood, ticks found on horses and dust from stables. Horse seroprevalence was 4% (n = 335, 37 stables) in 2015 and 12% (n = 294, 39 stables) in 2016. Of 199 horses sampled both years, 13 seroconverted, eight remained seropositive, and one seroreverted. Seropositive horses were located close to reported human cases, yet none displayed Q fever-compatible syndromes. *Coxiella* DNA was detected in almost 40% of collected ticks (n = 59/148 in 2015; n = 103/305 in 2016), occasionally in dust (n = 3/46 in 2015; n = 1/14 in 2016) but never in horse blood. Further studies should be implemented to evaluate if horses may be relevant indicators of zoonotic risk in urban and suburban endemic areas.

1. Introduction

Q fever is a worldwide zoonosis caused by *Coxiella burnetii*, an obligate intracellular Gram-negative bacterium reported in humans and a broad range of animal species, including wild and domestic mammals, birds, and arthropods such as ticks (Maurin and Raoult, 1999). In humans, Q fever ranges from an asymptomatic infection (for around 60% of patients) to severe forms (Eldin et al., 2017; Frankel et al., 2011). Acute Q fever can manifest as a non-specific flu-like syndrome, pneumonia, or hepatitis (Eldin et al., 2017; Frankel et al., 2011). Chronic forms may vary from endocarditis to osteoarticular or vascular infections and chronic hepatitis (Frankel et al., 2011; Raoult et al., 1989). In ruminants, Q fever infections are mainly asymptomatic, but major clinical manifestations are abortions, stillbirths or delivery of weak offspring. Domestic ruminants are considered the main reservoir of the disease. The bacterium is released into the environment during birth or abortion, mostly through parturition products and occasionally in feces or milk as well (EFSA, 2010).

Both animals and humans become infected mainly by inhaling airborne particles contaminated with *C. burnetii*, though tick-borne infection occurs occasionally (Duron et al., 2015). The bacterium may be disseminated by wind (Tissot-Dupont et al., 2004) and withstand unfavorable environmental conditions for periods ranging from several months to years (OIE, 2015).

Human outbreaks are generally associated with the presence of livestock, but infections may also occasionally be associated with other domestic or wild species (EFSA, 2010; Gonzalez-Barrio et al., 2015; Lang, 1990; Marenzoni et al., 2013; Maurin and Raoult, 1999; Stein and Raoult, 1999). Q fever cases have also been reported in horseback riders or people visiting horse facilities, but the source of contamination was likely either small ruminants or ticks present in the horses' environment (Nett et al., 2012; Roest et al., 2013; Runge et al., 2012). A few studies have also mentioned that people working with horses, like veterinarians or breeders, could potentially be at risk for human infections (Karagiannis et al., 2009; Palmela et al., 2012; Sun et al., 2016; Van den Brom et al., 2013). However, the involvement of horses in Q fever

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epidemiology remains unclear (Marenzoni et al., 2013; Roest et al., 2013). Indeed, the few available serological surveys (Joshi et al., 1978; Lang, 1990; Marenzoni et al., 2013; Raseta and Mihajlovic, 1983) suggest that horses may be naturally exposed to *C. burnetii*, but these studies are old and performed with serological tests that are less sensitive than ELISA assays (, 2015).

Additionally, *C. burnetii* DNA has occasionally been reported in samples of aborted equine fetuses or placentas (Leon et al., 2012; Roest et al., 2013; Runge et al., 2012), but the bacterium has never been clearly associated with abortive disorders. Experimental infections have shown that all the challenged horses can develop depression, fever, and/or enteritis, and/or bronchopneumonia (Blinov, 1957; Zotov et al., 1956). These findings support the hypothesis that horses may occasionally be sensitive to *C. burnetii* infection (Marenzoni et al., 2013; Roest et al., 2013), but questions still remain about their potential role as a reservoir or as shedders. Although the bacteria's circulation among ruminants and horses does not always prefigure a human outbreak in the surrounding population, public authorities are interested in implementing reliable monitoring systems able to rapidly identify indicators of zoonotic risk, thereby improving Q fever control. Interestingly, horses are mostly located in urban and peri-urban areas at the interface between domestic ruminants and humans, while human Q fever infections are prevalent worldwide in rural, urban or peri-urban areas (Amitai et al., 2010; Angelakis et al., 2014; Armengaud et al., 1997; Georgiev et al., 2013; Schimmer et al., 2010). A better understanding of *C. burnetii* circulation in and through the horse population is therefore needed to judge whether horses may be a relevant indicator of the bacteria's circulation in various peri-urban areas.

The objective of our work was to concomitantly assess: *(i)* the serological status of horses in a *C. burnetii* endemic area for both humans and ruminants; *(ii)* the presence of *C. burnetii* in the equine environment, including dust and ticks; and *(iii)* the potential expression of clinical disease in horses.

We carried out our study in Southeast France, a region known to be hyperendemic for human Q fever (Fournier, 2014, 2015; Tissot-Dupont et al., 2004, 1999). Indeed, between 2000 and 2009, this region had the highest national incidence of Q fever in humans, with 19 cases per 1 million inhabitants per year, compared to 7 cases in the rest of France (Frankel et al., 2011).

2. Methods

2.1. Field sampling

2.1.1. Study area

The study was carried out in Southeast France, in Camargue (West of the Rhône river mouth) and on the Plain of La Crau (East of the Rhône river mouth) from April 2015 to July 2016. This region was chosen for its high concentration in *(i)* livestock: mostly cattle in Camargue and small ruminants on the Plain of La Crau (Agreste, 2010) *(ii)* human cases historically diagnosed as *C. burnetii* infections and *(iii)* the density of horses living outdoors all year, even in peri-urban areas (Fig. 1).

2.1.2. Stable selection

Equine veterinarians practicing in the area were asked to provide a list of stables to include in the study. In order to maximize the chance of identifying areas at risk for Q fever, we selected preferably stables where horses showed unexplained and chronic fever, chronic weight loss, abortions and/or unexplained respiratory disorders. Additional stables were selected based on their geographic location by targeting areas within a radius of 15 km of human cases reported by laboratories (Fig. 1). Stables were located using a Global Positioning System (GPS) during the survey.

2.1.3. Sampling and data collection

The number of horses selected in each stable was chosen according to the size of the stable's horse population, as previously described (Guidi et al., 2015). A minimum of two and a maximum of 15 horses were sampled on the same location; they were at least one year old and had been housed within the stable for at least one year. We selected preferentially horses that had shown syndromes compatible with Q fever during the previous year, even if clinical signs of Q fever in horses are known to be highly unspecific.

A volume of 18 mL of blood was collected from the jugular vein of each horse into dry and EDTA tubes; serum was recovered after centrifugation (10 min, 3000*g*) and frozen at −20 °C. EDTA tubes were directly frozen at −20 °C. Each horse sampled was examined for the presence of ticks and an average of five (from one to eight) attached but not engorged ticks were collected when present. All the ticks were kept alive during the field sampling period and, after morphological identification, were stored at −80 °C.

A questionnaire was filled in with the owner to collect data on the individual characteristics of each horse (age, gender, activity), its health status (previous and current clinical signs, diagnostic tests and treatments) and stable management (contact with ruminants, deworming practices, tick and flea control).

2.1.4. Dust sampling

Dust was collected from *(i)* one horse shelter or box in each stable during the 2015 field campaign and *(ii)* the outdoor chute for ruminants bred on the farm, in both 2015 and 2016. Dust was sampled with a 16 × 10 cm swab cloth moistened with distilled water (SodiBox, France) and used to wipe horizontal surfaces in five distinct locations, as previously described (Joulie et al., 2015). All the dust samples were stored at 3 °C during the field sampling period and afterwards at −80 °C.

2.1.5. Human data

Data about the occurrence of human Q fever cases diagnosed in the study area were provided by the *Coxiella* National Reference Center (CNR), Marseille. The geographic location of patients corresponds to the address of the diagnostic laboratory where the human serum sample was tested for Q fever.

2.2. Laboratory analyses

2.2.1. Serology

All serum samples were tested using a commercial Q fever ELISA kit (LSIVet Ruminant Serum/Milk, Thermo Fisher Scientific) adapted to screen samples from various mammal species by replacing the ruminant-specific conjugate with peroxidase conjugated protein A/G, which have a strong affinity for both small ruminants and horses according to the producer (ThermoFisher Scientific - Pierce™). The optical density (OD) values obtained were expressed in terms of "mean percentage of sample/positive" (S/P values): $S/P\ value = (OD_{Sample} - OD_{Neg.control})/(OD_{Pos.control} - OD_{Neg.control}) \times 100$. Positive and negative internal controls were included in each plate. A collection of sheep (n = 111) and goat (n = 102) reference sera (144 true negative and 70 true positive sera) was used to determine the optimal seropositivity threshold of the multi-species ELISA test. This threshold was defined from a ROC curve and set to an S/P value of 43% (confidence interval 35–51%) using XLStat, trial version 2017.1.

Accordingly, horses were defined seropositive when S/P > 43% and seronegative when S/P < 43%. We considered that a horse seroconverted when the S/P values varied from below to above the interval confidence between 2015 and 2016. A stable was considered seropositive when at least one of the horses sampled was seropositive.

2.2.2. Preparation of tick, dust and blood samples

Ticks were first identified morphologically using reliable

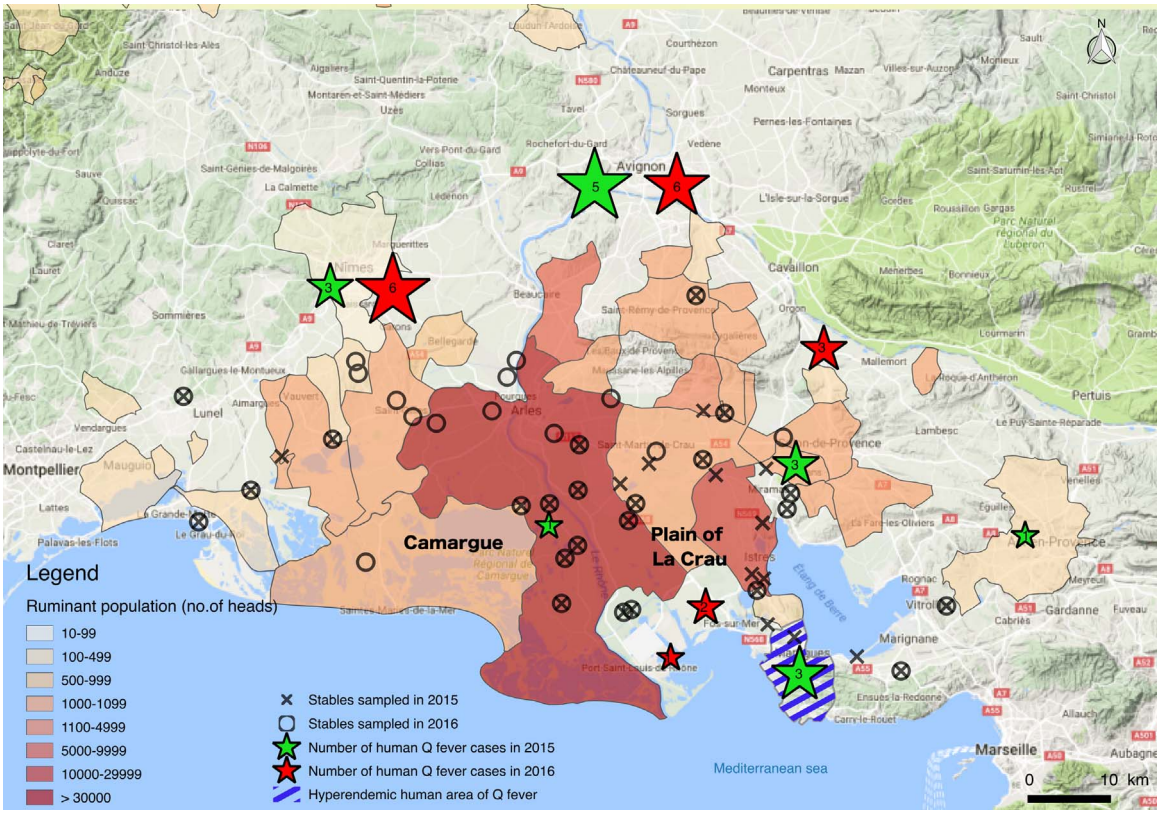

Fig. 1. Map showing the distribution of stables sampled in 2015 and 2016, the location where human cases were diagnosed during both years, and the number of ruminants according to the last agricultural census in France (2010).

identification keys and species descriptions (Estrada-Pena et al., 2004; Pérez-Eid, 2007) and then washed. As previously described (Michelet et al., 2014), the first wash was with 100% ethanol, followed by three consecutive washes with ultra-pure water. Ticks were then crushed using a Precellys homogenizer (Bertin Technologies, France) with two metal beads in 300 µL of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, France) and 30 µL of fetal bovine serum (Sigma-Aldrich, France). DNA was immediately extracted from the tick homogenates.

Dust samples were suspended in 40 mL of PBS. After homogenization, 200 µL of this suspension was mixed with 15 µL of sheep fetal thymus (SFT) cells constituting internal controls for DNA extraction.

Whole blood samples recovered in EDTA tubes were used undiluted for extraction purposes.

2.2.3. DNA extraction and PCR assays

A DNA Purification QIAamp Mini kit (QIAGEN, Courtaboeuf, France) was used to extract DNA from both dust and tick samples. DNA from blood samples was extracted using a NucleoSpin Blood L kit (Macherey Nagel, Hoerdt, France). A real-time PCR method that targets the IS*1111* and GAPDH genes was used to detect *Coxiella* and host cellular DNA respectively (Joulie et al., 2015) using a CFX96 thermocycler (Biorad, France). Briefly, tick and blood DNA extracts were analyzed undiluted and a volume of 0.1 µl of GAPDH DNA concentrated to 30 ng/µl was added to each sample as an inhibition control; the samples were considered positive from the regression mode of the Biorad CFX96 manager software. Dust DNA extracts were diluted to the 10th level and tested with reference to a calibrated standard based on serial dilutions of genomic DNA reference material prepared from the Nine Mile phase II RSA 493 isolate (ANSES Sophia-Antipolis, France). We considered as a limit of quantification per unit volume (LOQ) the lowest concentration of the Nine Mile standard (2.10^3 genome equivalents per milliliter (GE/mL) corresponding to 8.10^5 GE per swab cloth). A similar approach was used to estimate the maximum LOQ per unit volume (LOQ_{max}) using the highest concentration of the Nine Mile standard (5×10^6 GE/mL corresponding to 2.10^9 GE per swab cloth).

2.2.4. Data analyses

All statistical analyses were carried out in R (R version 3.1.0). Our alpha level for statistical significance was set at 0.05. The link between seropositivity of horses and contact with ruminants was studied with Pearson's chi-squared test, or a Fisher's exact test when one of the compared groups contained fewer than six horses. Maps were created with the Qgis software (version 2.8 'Wien').

3. Results

Overall, 37 stables were surveyed in 2015 and 39 in 2016, 24 of which were investigated both years (61.5%). In all, 338 and 294 horses were sampled between late April and mid-May 2015 and between late May and late June 2016 respectively. One hundred and ninety-nine (199) horses were sampled both years (68%). We detailed in Table 1 the proportions of (*i*) horses and (ii) stables in which at least one horse displayed clinical signs compatible with an infection by *C. burnetii* depending on the year of sampling.

Table 1
Distribution of stables and horses displaying clinical signs potentially associated with a *C. burnetii* infection.

	Proportion of stables with at least one horse displaying compatible clinical signs to a *C. burnetii* infection (%)	Proportion of horses displaying compatible clinical signs to a *C. burnetii* infection (%)
2015	54 (n = 20/37)	12 (n = 42/338)
2016	36 (n = 14/39)	6 (n = 17/294)

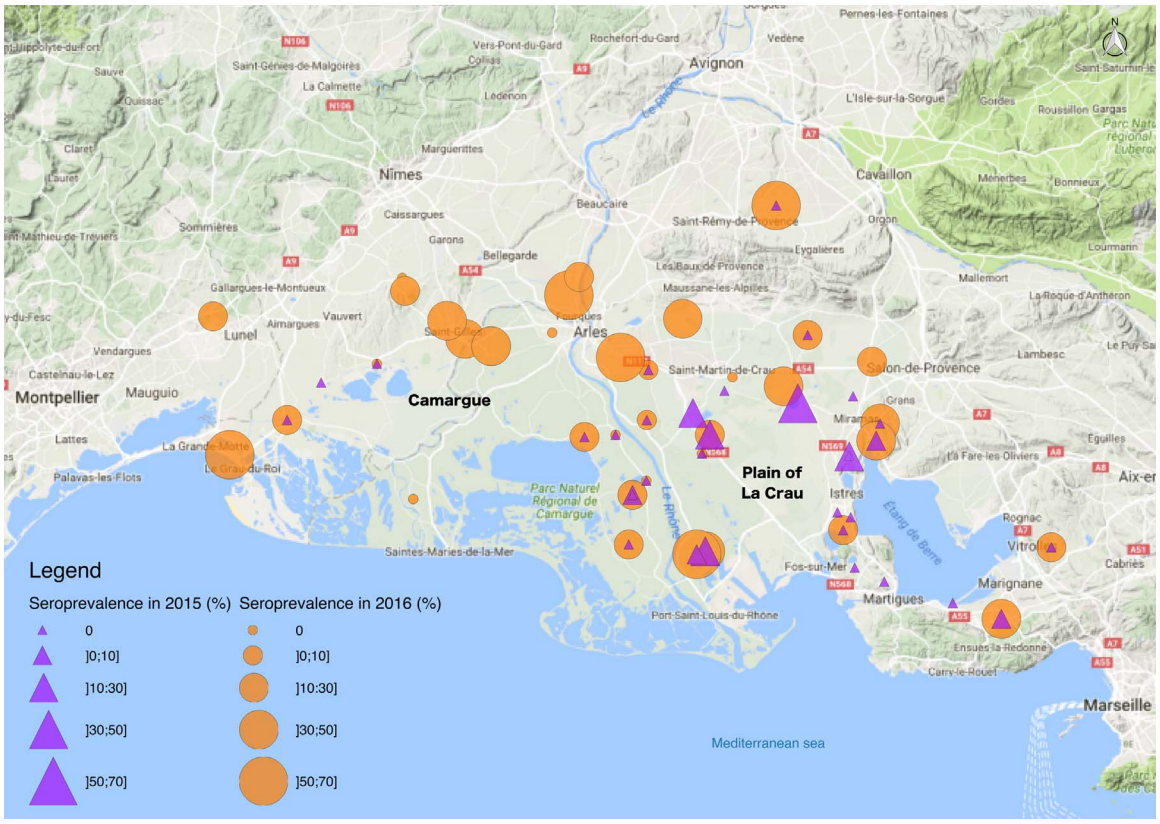

Fig. 2. Map showing intra-stable seroprevalence in 2015 and 2016.

The study populations in both years were homogenous in terms of breed, sex and age. Indeed, six breeds were represented among all the horses sampled. Camargue horses were the most represented (n = 140), but other purebred (n = 59) and crossbred (n = 103) horses were also included, as well as ponies (n = 36). The sex distribution was 30% mares, 4% stallions and 66% geldings. The age varied from 1 to 32 years, with a mean age of 11 years.

3.1. Serological survey

3.1.1. Spatial distribution of seropositive stables

In all, 21 stables were distributed throughout the Camargue region (West of the Rhône river mouth) and 27 on the Plain of La Crau (East of the Rhône river mouth) (Figs. 1 and 2). Of all these stables, 12 in 2015 (32%) and 21 in 2016 (54%) included at least one seropositive horse (Fig. 3). The average proportion of seropositive horses per stable was 5% [3%–7%] in 2015 and 14% [10%–18%] in 2016. Of nine stables with between 30% and 50% of seropositive horses, three were located on the Plain of La Crau. The other six were located around the town of Arles (Fig. 2). We found one stable with more than 50% of seropositive horses (n = 10), located in the southwestern part of the study area near the town of Le Grau-du-Roi. All these stables, except the one in the southwestern part, were close to areas where Q fever human cases had been diagnosed (Figs. 1 and 2).

Twenty-one stables had ruminants in their environment (cattle for 43% and small ruminants for 19%). However, we found no association between horse seropositivity and contact with ruminants, whether direct (i.e. sharing the same pen as ruminants or being used for the working of bulls) or indirect.

Twenty-four stables were investigated both years. In these stables, the average proportion of seropositive horses per stable was 6% [3%–9%] in 2015 and 15% [10%–20%] in 2016.

3.1.2. Seropositivity in horses

Out of 338 horse sera tested in 2015 and 294 in 2016, 13 (4%) and 35 (12%) were seropositive respectively (Fig. 4*a*). Positive S/P values found in 2015 ranged from 47.2 to 98.2 and from 43.3 to 223 in 2016. The seroprevalence observed tended to increase (p = 0.08) in horses

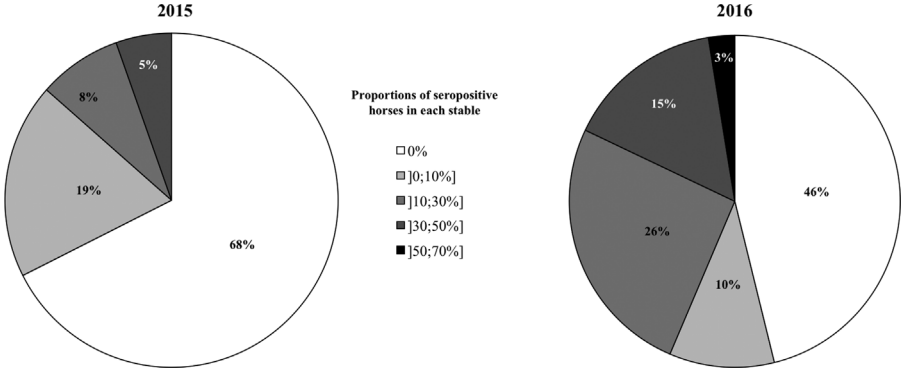

Fig. 3. Circular diagrams showing the distribution of intra-stable seroprevalence depending on the number of seropositive horses.

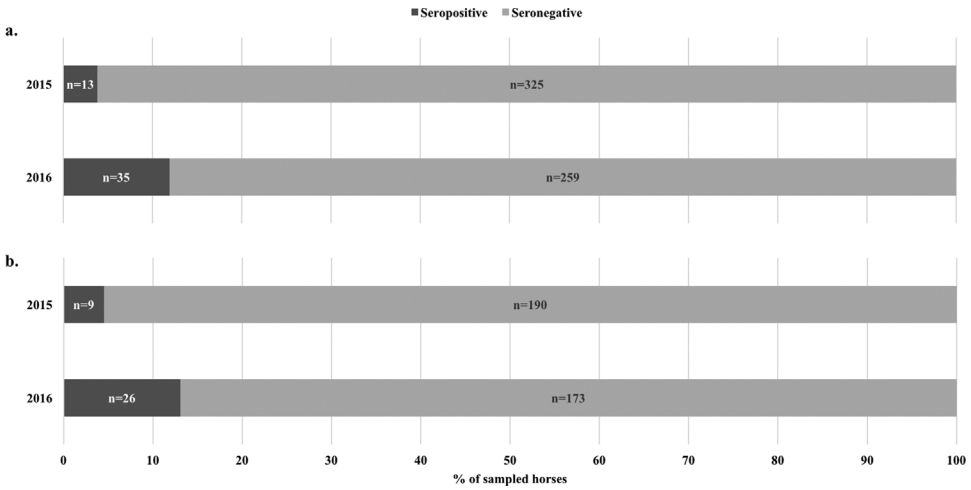

Fig. 4. Histograms showing in 2015 and 2016: (a) overall horse seroprevalence and (b) horse seroprevalence for those sampled in the two consecutive years.

sampled both years (n = 199), ranging from 5% [2%–8%] in 2015 – 13% [8%–18%] in 2016 (Fig. 4*b)*. Of these, 13 seroconverted in 2016 (7%); eight remained seropositive on the second year (14%), and one seroreverted (0.5%). The 13 horses that became positive between the two sampling years were located in 11 stables, seven on the Plain of La Crau and four in Camargue. Five of these 11 stables still included at least one seropositive horse in 2015.

Out of the 199 horses sampled in 2015 and 2016, 25 distributed in 15 different stables were seropositive at least one of the two years. Among them, one (in 2015) showed chronic fever and weight loss, which is compatible with an infection by *C. burnetii*. In these 15 stables, we observed a 30% increase in S/P values for 60% of horses (n = 9), without any suggestive clinical signs.

3.2. *Coxiella burnetii* DNA detection

3.2.1. Blood

All blood samples were negative by PCR.

3.2.2. Dust samples

C. burnetii DNA was detected in 3/46 and 1/14 of the dust samples collected in 2015 and 2016 respectively (Fig. 5). In 2015, two of the three PCR-positive dust samples were harvested from ruminant pens, while the third was from a horse shelter. In 2016, the positive sample was identified in a ruminant pen already positive in 2015. All the positive dust samples were found on the Plain of La Crau (Fig. 5). The highest bacterial burden (about 4.36×10^9 GE per swab cloth) was detected in dust samples collected from the same ruminant pen in 2015 and in 2016.

3.2.3. Ticks

One hundred forty-nine ticks (111 *Rhipicephalus* spp., 24 *Haemaphysalis* spp., nine *Hyalomma* spp. and five *Dermacentor* spp.) collected on horses in 2015, and 305 (269 *Rhipicephalus* spp. and 36 *Hyalomma* spp.) in 2016 were analyzed by qPCR to detect *Coxiella* DNA (Table 2).

Overall, 40% (n = 59/149) and 34% (n = 104/305) of ticks collected in 2015 and 2016 respectively were found positive by qPCR (Table 2). *Coxiella* DNA was detected in *Rhipicephalus* spp., *Haemaphysalis* spp. and *Rhipicephalus* spp.

Positive ticks were mostly found in 14 stables with seropositive horses, including seven on the Plain of La Crau and seven in Camargue (Figs. 2 and 5), but we did not observe any statistical association between seropositive horses and positive ticks ($p > 0.05$).

4. Discussion

4.1. Main findings

Few studies have investigated the horse's potential role as a risk indicator for the transmission of Q fever to humans. This study first showed that horses were exposed to *C. burnetii* in a hyperendemic area for humans. Second, in the sample of stables monitored both years, we observed that the prevalence tended to increase at the stable-level between 2015 and 2016 in some stables but no clinical sign compatible with Q fever infections was observed in any of the horses. Finally, we reported *Coxiella*-positive PCR in the equine environment, especially in dust where ruminants were housed or in ticks feeding on horses.

4.2. Serological studies in horses are scarce and difficult to compare

The use of a single ELISA test for both sampling years allowed us to reliably compare our results between both sampling periods. Here, we used the LSI Vet kit largely used for ruminant serological assays that we adapted for use in a non-ruminant species. However, it remains difficult to compare our results with other serological surveys conducted on horses (Agerholm et al., 2015; George and Marrie, 1987; Martinov, 2007; Pitre, 1960) due to heterogeneity in terms of specificity and sensitivity of the serological methods used (complement fixation, seroagglutination and diverse ELISA tests). Moreover in these published papers, information on the parturition periods in ruminants (main reservoirs), the age of sampled horses as well as on farm density in the investigated areas are generally lacking.

Lastly, the seroprevalence rate described per stable could have been overestimated in that study as we choose to classify stables positive when at least one horse was seropositive. Nevertheless, this choice was made in order to identify as accurately as possible at risk areas for Q fever.

4.3. Clinical signs of Q fever in horses are unknown

Despite our selection criteria for horses, we could not confirm any potential clinical sign that could be observed in association with Q fever disease in horses. Based on experimental studies, parenteral administration of *C. burnetii* triggered a seroconversion along with clinical signs such as fever, depression, conjunctivitis, rhinitis, broncho-pulmonary signs and/or enteritis (Zotov et al., 1956; Blinov, 1957). Clinical signs that we assumed to be compatible with Q fever disease in horses included chronic fever, weight loss of unknown origin, idiopathic lower respiratory disease, abortions and stillbirths, which are non specific (Smith, 2014). Differential diagnosis comprises many other etiologies including piroplasmosis, leptospirosis, anaplasmosis, borreliosis, or

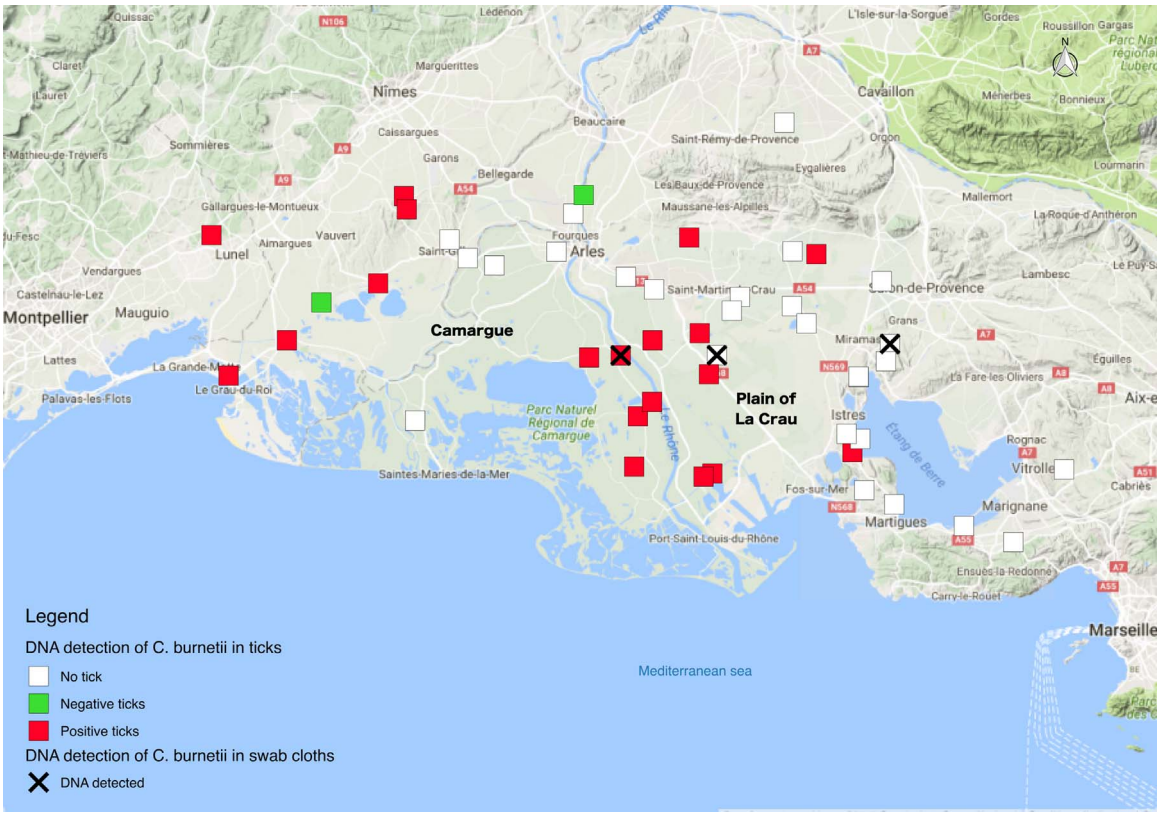

Fig. 5. Map showing the distribution of ticks and dust samples collected in 2015 and 2016.

Table 2
Number of positive and sampled ticks depending on their classification.

Tick classification	Sampling year			
	2015		*2016*	
Genus	*No. of positive ticks*	*No. of ticks sampled*		*No. of ticks sampled*
Rhipicephalus spp.	44	114	96	269
Haemaphysalis spp.	5	21	0	0
Hyalomma spp.	8	9	8	36
Dermacentor spp.	2	5	0	0
Total	**59**	**149**	**104**	**305**

gastro-intestinal parasitism. The final diagnosis of such chronic syndromes in horses represents a significant challenge to veterinary practitioners, but the cost of arriving at a clear diagnosis may be dissuasive to the owner.

The fact that few horses showed compatible clinical signs is consistent with what is usually observed in ruminants or in humans. Indeed, the majority of sheep (Astobiza et al., 2011; Berri et al., 2001; Khaled et al., 2016), goats (de Cremoux et al., 2012a), cattle (Guatteo et al., 2012) or humans (Million et al., 2009) solely seroconvert without reporting any clinical signs. Additionally, horses may also either not display any Q fever clinical signs even though exposed to the bacteria, or the Q fever forms have gone unnoticed due to insufficient surveillance.

4.4. *Coxiella* DNA is present in the equine environment

Two stables on the Plain of La Crau had positive dust samples. Interestingly, they are both in the specific area where most of the seropositive stables were found. The high density of sheep could be a source of environmental contamination and the Mistral wind could contribute to the dispersion of the bacterium (Tissot-Dupont et al., 2004, 1999). The topography of the landscape may also facilitate dissemination of the bacteria, especially along the Rhône River. Therefore, it would be beneficial in the future to investigate the presence of *C. burnetii* in horse litter and in dust from horse shelters through DNA detection.

A significant proportion of ticks found on horses were PCR-positive for *Coxiella*. However, the role of *Coxiella* positive ticks in the transmission of Q fever to horses remains questionable (Duron et al., 2015). Recent findings have shown that ticks may carry endosymbiotic bacteria genetically close to *C. burnetii* and referred to as *Coxiella*-like bacteria (Duron et al., 2015), which are potentially non-pathogenic for ruminants, humans or horses. Interestingly, these newly-discovered bacteria have also recently been described in ticks sampled from horses (Seo et al., 2016). Because many genes used to detect *C. burnetii* may also be amplified from *Coxiella*-like bacteria (Jourdain et al., 2015; Seo et al., 2016), there is a risk of misidentification without subsequent confirmation by sequencing long DNA fragments. Consequently, further investigations to distinguish *C. burnetii* from *Coxiella*-like bacteria are needed to better assess the exposure of horses to vector-borne Q fever.

4.5. *Horses could be sentinels for the circulation of the bacteria in urban or periurban areas*

A previous study reported the detection of *C. burnetii* in equine blood or urine by PCR, but in low burdens and a limited number of horses (Tozer et al., 2014). In our study, antibodies against *C. burnetii* were frequently detected but no bacterial DNA could be amplified from horse blood. Although non-significant, our study suggested a slight increase in seroprevalence of horses sampled both years. Some horses seroconverted and others remained seropositive. This finding reflects the fact that the population re-sampled in 2016 was likely exposed or re-exposed to *C. burnetii* between the two sampling periods in the Camargue and Plain of La Crau regions. However, as in ruminants, antibody dynamics in horses is unknown (de Cremoux et al., 2012b;

Guatteo et al., 2012; Rousset et al., 2009) even though immunity may persist for many years in humans (ECDC, 2010).

Such epidemiological studies are useful to describe the prevalence and distribution of disease in populations. They are generally implemented for diseases which could have a significant economical and/or zoonotic impact. Our study shows that the impact of Q fever disease on health status of horses in the studied area seems to be quite low. Further studies are needed to evaluate if the horse could be used as a relevant indicator of transmission risk for Q fever in this area. Horses are a domestic species typically at the interface between infected domestic ruminants and humans, especially in the studied peri-urban areas.

Ethics statement

Each owner signed an informed consent document which exposed the main results expected and the confidentiality of all the information collected (protected by the French National Commission of Information Technology and Liberties, agreement number 1822460). The sampling protocols were submitted to and validated by the ethics committee of VetagroSup (National Veterinary School of Lyon, France), agreement number 1534.

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