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Serological and molecular evidence of coxiellosis and risk factors in sheep flocks in central-eastern Tunisia

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ABSTRACT

In this study, we conducted an investigation to determine the true prevalence of coxiellosis in sheep in central-eastern Tunisia. A total of 492 veterinary samples taken from 110 flocks were screened for coxiellosis using IS1111-based real-time PCR assay. Sheep sera were tested using an indirect enzyme-linked immunosorbent assay. Based on molecular and serological results, the true adjusted animal and herd-level prevalence of coxiellosis were 11.8% and 20.21%, respectively. Bacterial excretion was observed in 17 flocks, and 19 females showed evidence of *Coxiella burnetii* shedding (100%). In addition, a statistically significant association was found between vaginal and milk shedding for sheep. Multivariable logistic regression analysis at the animal-population level indicated that strata and vaccination variables were found to be associated with coxiellosis. Besides, it was shown that this infection increased when the intensive farm was exposed to carnivores and when the cleaning practices were not respected, while it decreased when a suitable quarantine was introduced for any introduction of a new animal. Good hygiene and sanitation practices on-farm should be handled as strategies to deal with this zoonotic pathogen in herds.

1. Introduction

Like in all North African countries, ruminant breeding activity plays a major role in the national economy of Tunisia [1]. It contributes to 35% of agricultural gross domestic product (GDP) and to 5% of total GDP [1]. Tunisia has an estimated the livestock population of 5410 million female units (UF) allowing the production of 1054 million tons of dairy products and 120 000 tons of meat. Despite its economic significance, livestock sector suffers from particular fragility in terms of balance between the supply of food and the increase of ruminant population [1]. In addition, ruminants around the world are exposed to several health problems, such as gastrointestinal parasitic and respiratory infections and abortions [2,3]. Numerous pathogenic agents, including viruses, protozoa, fungi and bacteria, are known to directly affect animal health and reproductive function [4]. Among these infectious agents, we note *Coxiella burnetii* (*C. burnetii*) which is one of the most important bacterial agents, causing coxiellosis in domestic ruminants.

Coxiellosis is a zoonotic disease with a worldwide occurrence [5]. Ruminants represent the main reservoir of this microorganism [5]. When it causes illness in sheep, it usually manifests as abortions during the third trimester of gestation, as well as stillbirths and delivery of weak neonates [6]. After abortion, different factors cause the transmission of coxiellosis like the contact of susceptible animals with contaminated fetuses, placental membranes or fluids, faeces, urine and milk [7,8]. About one third of *C. burnetii* infections of pregnant ewes lead to infection of the udder and excretion of the pathogen in milk during the ulterior lactation [9]. Berri et al. [10] showed that the shedding of *C. burnetii* with the milk secretion of lactating ewes can become chronic and remain for two months.

Some previous studies were carried out in different Tunisian geographic regions and demonstrated the presence of *C. burnetii* antibodies among small ruminants [1,11]. However, an information gap still remains on the existence of this infection in many governorates of Tunisia. Indeed, in the governorate of Sfax, annual investigations made by state veterinarians during vaccination campaigns have not been very

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effective. The present study aimed at (i) determining the seroprevalence of *C. burnetii* and the bacteria shedding in sheep flocks (ii) identifying potential risk factors associated with coxiellosis positivity at animal and herd levels.

2. Materials and methods

2.1. Ethical considerations

The study protocol was assessed and approved by the Regional Office of Agricultural Development of Sfax in collaboration with the Veterinary Research Center of Sfax. All livestock owners declared their verbal consent before the study to animal sampling as well to the related survey questions. Samples were collected by authorized veterinarians of the Veterinary Research Center of Sfax during annual vaccination campaigns following standard procedures of the usual screening scheme on farms and Tunisian ethical guidelines. The animal welfare regulations were strictly respected.

2.2. Study area

The present study was conducted in the town of Sfax (average altitude of 13 m), which is located in the East centre of Tunisia, at 270 km from the capital Tunis. This governorate has a semi-arid climate and a mean annual rainfall of 250 mm. The mean minimal temperature is 12 °C in January, whilst the mean maximum temperature is 33 °C in August (National Institute of Meteorology, Tunisia). The breeding of small ruminants in these regions is usually dominated by an extensive system. This system is characterized by the mobility of animals during the year in search of natural pastures, whose diets and resources depend on climatic conditions. It is also characterized by the use of family labor, low productivity and almost non-existent sanitation coverage. The most important native breed of sheep is the Barbarine which mainly contributes to the production of red meat.

2.3. Epidemiological data collection

Two pretested questionnaires were used separately to collect information on potential management and environmental risk factors related to coxiellosis at the animal and herd levels. The sheep owner was questioned by the principal researcher of this study.

2.4. Animals and samples

In each region, households owning sheep were identified through discussion with the veterinarians of this study during the annual vaccination campaigns. The number of flocks to be studied was calculated using the Win Episcope program, and it was based on the formula $n = [t^2 \text{ Pexp } (1\text{-Pesp})]/d^2$ [12]. For this purpose, an expected herd prevalence of 1.78% [1], a desired absolute precision of 10% and a 95% confidence interval were considered. Hence, one hundred and ten flocks were visited. A total of 164 animals (159 unvaccinated and 5 vaccinated animals against *C. burnetii*) were sampled: 164 blood, 164 vaginal swabs and 164 milk samples were collected by veterinarians and sent to the laboratory. Blood samples were collected in 5 ml EDTA tubes from the jugular vein of each animal. The EDTA tubes were immediately cooled to 4 °C. In a laboratory, blood samples were mixed with an equal volume of 6 M guanidine HCl 0.2 M EDTA (pH 8.0) and homogenized, stored overnight at room temperature (22–24 °C) and, the following day, boiled for 15 min [13], cooled, incubated at room temperature overnight and then stored at 4 °C. Vaginal swab samples were collected from each ewe, using sterile swabs, following vulva disinfection with chlorhexidine solution. All swabs were put into tubes containing 1 ml of 2-sucrose phosphate medium (2-SP). They were kept at 4 °C during transportation in ice-pack containers, and stored at − 20 °C until use. All milk samples were stored at − 20 °C until tested.

2.5. Serological assays

All sera were tested for the presence of IgG antibodies against *C. burnetii* using ID Screen Q Fever Serum Indirect Multi-Species ELISA Kit (i-ELISA) (ID VET diagnostics, Montpellier, France).

Optical densities were read using an ELx808 absorbance microplate reader (BIO-TEK INSTRUMENTS®, INC, Vermont, USA).

The antibody titers were calculated using the formula suggested by the kit manufacturers: Per cent positivity (% S/P) = ((OD sample − OD negative control)/OD positive control − OD negative control) × 100)

As recommended by the manufacturer, all sera were considered to be negative when% S/P < 40%, suspicious when 40% ≤% S/P < 50% and positive when% S/P ≥ 50%. The diagnostic sensitivity and specificity of i-ELISA were estimated to be 100% and 99.6%, respectively, as described.

2.6. DNA extraction

The DNA of all blood, milk and vaginal swab samples collected from ewes was extracted by ZR Fungal/Bacterial DNA MiniPrep™ D6005 Kit (Zymo Research) as described by Barkallah et al. [13].

2.7. Rt-PCR assay

A specific primer pair IS1111-F (5′-GCGTCATAATGCGCCAACATA-3′)/IS1111-R (5′-CGCAGCCCACCTTAAGACTG-3′) was designed to amplify a 201 bp fragment of the IS*1111* gene (insert sequences) of *C. burnetii*. The optimization of the conditions of the PCR reaction was carried out with a calibrated standard range of three dilutions (30, 15 and 7.5 ng/μl) of *C. burnetii* genomic DNA. The PCR efficiency was estimated for 5 primer concentrations (0.05, 0.15, 0.2, 0.3 and 0.5 mM) in order to select the one that gave the highest recorded fluorescence and the lowest threshold cycle (Ct). Thermocycling conditions were evaluated by testing different Ta (54–60 °C) in different periods (5, 10, 20, 30 and 60 s). The optimal quantitative PCR efficacy was obtained using a cycling profile consisting of an initial denaturation step at 95 °C for 3 min, then 40 cycles of 10 s at 95 °C and 10 s at 60 °C. A melt-curve analysis was performed immediately after the amplification protocol (60–95 °C). DEPC treated H_2O was used as a negative PCR control. All these optimization reactions were carried out using the CFX96™ thermocycler (Bio-Rad). Ninety positive cultures for *C. burnetii* were used to test the specificity and sensitivity of the optimized real-time PCR. Culture samples with two positive reactions were scored as PCR test-positive. The diagnostic specificity and sensitivity of the developed real-time PCR were calculated according to a previous publication [15]. The amplified products were sent to GATC Biotech SARL (Germany) for sequencing.

2.8. Statistical analysis

The true prevalence of coxiellosis at the animal level was estimated using Epitools epidemiological calculators and the following formula:

TP = (AP + Sp-1)/(Se + Sp-1) [16]. Herd level prevalence was calculated as the number of herds with at least one positive animal divided by the total number of herds tested. The true herd prevalence (THP) was estimated from the distributions of herd sensitivity (HSe) and specificity (HSp) using the following formula: THP = (AHP + HSp-1)/(HSe + HSp-1), which $HSe = 1 - (1 - AP)^n$ and $HSp = Sp^n$ [17].

Analyzing the risk factor was separately performed at the animal and herd levels. At first, all categorical independent variables were coded. The univariate analysis was performed to relate the outcome variable to each explanatory one at animal and herd levels, using the two-tailed Pearson's X2 test. Only independent variables associated with coxiellosis infection ($p < 0.2$) were selected for the multivariate analyses. All selected variables were tested for colinearity using the Chi-square test [18]. The second step in the analysis consisted in building a