Bayesian estimation of sensitivity and specificity of a PCR method to detect *Coxiella burnetii* in milk and vaginal secretions in sheep and goat samples

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ABSTRACT

Coxiella burnetii is a gram-negative and polymorphic rod bacterium that causes Q fever, a common zoonotic disease distributed worldwide. Widespread occurrences of the disease outbreaks indicate the importance of coordinated animal and human health efforts to control these outbreaks. Different tests are available to determine the *C. burnetii* infection status of a flock, but false negative responses may occur, as infectious animals can shed bacteria in milk intermittently, especially during an asymptomatic infection. In this study, a Bayesian latent class model was implemented to estimate the sensitivity (Se) and specificity (Sp) of a PCR method for the detection of *C. burnetii* in milk samples (PCR_M) and vaginal swabs (PCR_V) from Iranian sheep and goats. A nested PCR assay was conducted to detect infected animals among 170 milk samples and 170 vaginal swabs from goat flocks and 170 milk samples and 170 vaginal swabs from sheep flocks. We implemented a Bayesian latent class model to estimate the Se and Sp of a PCR method for the detection of *C. burnetii* in milk samples and vaginal swabs from sheep and goats. Estimations were based on the cross-classified results of PCR_M and PCR_V from the sheep and goat subpopulations. Positivity was 17.6 and 33.5%, respectively, for PCR_M and PCR_V samples among sheep. In goats, the apparent prevalence was 32.9 and 56.4% in PCR_M and PCR_V samples testing positive, respectively. This indicated the lower sensitivity of PCR_M. The Se of PCR_V was significantly higher than Se of PCR_M, which

corresponded to a higher rate of vaginal-positive, milk-negative PCR samples. In contrast, Sp of PCR_V was lower than Sp of PCR_M, representing the higher false-positive rate of vaginal swabs. The PCR_V outperformed PCR_M in terms of identifying latently infected sheep and goats; however, neither method could identify all latently infected sheep and goats, thus the combination is recommended to maximize our ability to identify infected animals. The true prevalence of *C. burnetii* infection was higher in Iranian goats than sheep.

Key words: *Coxiella burnetii*, Bayesian estimation, sensitivity, specificity, PCR

INTRODUCTION

Coxiella burnetii is a polymorphic, gram-negative bacterium that causes Q fever, a common zoonotic disease distributed worldwide that was first detected in Australia in 1937 (Maurin and Raoult, 1999). Domestic livestock is considered the main source of the pathogen and infects the environment by shedding of *C. burnetii* in milk, feces, urine, saliva, vaginal discharge, placenta, and amniotic fluid. Human outbreaks tracing back to sheep and goat exposure have been reported in several locations (Rizzo et al., 2016). In addition, the widespread occurrence of disease indicates the importance of coordinated animal and human health efforts to control these outbreaks.

Coxiella burnetii infection is often asymptomatic but can lead to reproductive disorders (Kampen et al., 2012). Reproductive secretions are more important and have more prominent role in the transmission and spread of bacteria. Various tests are used to determine the *C. burnetii* infection rate in a herd or flock. These tests are based on either the detection of the immune response of the host (i.e., antibody ELISA tests) or

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detection of the microorganism, such as PCR methods that detect bacterial DNA. A PCR can be performed using primers targeting different genes, such as multicopy insertion sequence *IS1111* (Berri et al., 2000); however, false-negative responses may occur, as infectious animals can shed bacteria in milk intermittently, especially during the asymptomatic and latent stage of the infection where bacteria remain localized in the placenta. Antibody-ELISA tests can also give false-positive responses due to the persistence of antibodies from past infections (Worswick and Marmion, 1985). In the latter case, few reactors within a flock or herd may result in falsely classifying a herd or flock that is free from infection as infectious.

To the best of our knowledge, the sensitivity (**Se**) and specificity (**Sp**) of PCR tests have not been evaluated yet in sheep or goats, which can be partly ascribed to the absence of a perfect reference test, often referred to as the gold standard. In the absence of a gold standard, a valid alternative for evaluating the accuracy of diagnostic tests is the use of Bayesian latent class models, which is an affordable, reliable, noninvasive alternative for the evaluation of diagnostic test accuracy and does not require a priori knowledge of the true infection status. Bayesian latent class models were introduced by Hui and Walter (1980) and have been successfully used for over 20 yr (Collins and Huynh, 2014). They are increasingly gaining acceptance (Kostoulas et al., 2017) and the World Organisation for Animal Health has recently adopted their use for Se and Sp estimation (OIE, 2015). We implemented a Bayesian latent class model to estimate the Se and Sp of a PCR method for the detection of *C. burnetii* in milk samples (**PCR_M**) and vaginal swabs (**PCR_V**) from Iranian sheep and goats.

MATERIALS AND METHODS

Target and Source Population

This study was carried out in Kerman Province located in the southeast of Iran, geographically located between 56°55' and 57°15'E and 30°10' and 30°20'N. The climate in the province varies across the regions. The north, northwest, and central areas experience a dry and moderate climate, whereas in the south and southeast the weather is warm and relatively humid (Ghazanfarpour et al., 2013). Kerman province is divided into 23 counties. The samples came from the following counties: Kerman, Mahan, Rayen, Bam, Bardsir, Jiroftand, and Kahnoj. In total, 4,107,068 sheep and goats were reared in Kerman province according to the Iran Veterinary Organization in 2014 (<http://www.ivo.ir>).

The target population included all ewes and goats over 12 mo old (according to the records of the farmers). The sampling unit was the flock and the design was a random sampling. In each flock, 10% of the population older than 1 yr were sampled randomly.

Participants

Sampling was done randomly from January 2014 until June 2014. Samples were collected from 40 flocks. In each flock, 10% of the clinically healthy female population older than 1 yr without disorders in their pregnancy (approximately 10 to 20 livestock per flock) was sampled under simple random sampling. The minimum number of animals to estimate a prevalence of *C. burnetii* in small ruminants with a 95% level of confidence and 20% absolute precision was 340 individual milk and vaginal swab samples: 170 milk samples and 170 vaginal swabs from goat flocks and 170 milk samples and 170 vaginal swabs from sheep flocks. Milk samples were collected directly from udders in sterile tubes. Cotton swabs were moistened with sterile PBS and, after sampling from vaginal mucosa, the swabs were placed in a micro-tube containing 1 mL of sterile PBS.

Definitions

We tested the Se, the probability that PCR indicates *C. burnetii* among those actually infected with *C. burnetii*, and Sp, the fraction of those without *C. burnetii* who will have a negative test result. We also sought the apparent prevalence, the proportion of animals from a representative sample of the population that is positive to the nested PCR method, and the true prevalence, the proportion of livestock that present *C. burnetii* in the population at a time. The term conditional (in) dependence relates to paired test results. An explanation of conditional dependence given disease status can be found in Gardner et al. (2000). Briefly, assume that paired results of 2 tests are available for 100 infected (healthy) individuals. If Se1 (Sp1) = 0.80 (0.90) and Se2 (Sp2) = 0.90 (0.80), the first test would correctly classify 80 (90) and miss 20 (10) infected (healthy) individuals. If we use the second test on the falsely classified individuals, we would expect that, if the tests are conditionally independent given the true infection status, 18 (8) of the infected (healthy) individuals will be correctly classified. Essentially, conditional independence captures the notion that knowing the results of the first test does not modify our expectation about the outcome of the second test.

Test Methods

DNA Extraction. Briefly, 600 μ L of each milk sample was centrifuged at $14,000 \times g$ for 15 min at 4°C and the fat layer was discarded; the pellet of milk cells was then resuspended in the cell lysis buffer (Tris-HCl, EDTA, and SDS) and 3 μ L of proteinase K solution (Roche, Mannheim, Germany) was added to the lysate. Afterward, the digest was incubated at 55°C for at least 3 h and 200 μ L of potassium acetate solution (60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, 28.5 mL of H₂O) was added and protein/SDS complex was precipitated by centrifugation at the maximum speed ($14,000 \times g$) for 3 min at 4°C in a microfuge. The upper layer was then transferred to a new microtube containing 600 μ L of cold isopropanol. The solution was mixed and the precipitate of DNA was recovered by centrifuging the tube at $14,000 \times g$ for 60 s at 25°C. Finally, the supernatant was removed and 600 μ L of 70% ethanol was added to the DNA pellet, the tube was centrifuged at maximum speed ($14,000 \times g$) for 60 s at 25°C, the upper layer was removed, and the DNA pellet was allowed to dry. Finally, the pellet of DNA was dissolved in 100 μ L of distilled water and the extracted DNA was stored at -20°C until being used (Sambrook and Russell, 2001).

For DNA extraction of vaginal secretions, the swabs were first centrifuged at $14,000 \times g$ for 10 min at 4°C and the DNA extraction was done as for the protocol mentioned above.

PCR Analysis. Nested PCR was conducted by using the primers trans1, trans2, 261F, and 463R as previously described (Parisi et al., 2006). The first amplification for the nested PCR was performed in a total volume of 25 μ L, containing 5 μ L of DNA sample, 0.3 μ L of Taq DNA polymerase (Bioneer, Daejon, Korea), 2.5 μ L of PCR buffer (pH 8.3), 1.5 μ L of MgCl₂, 0.5 μ L of dNTPs, and 1.5 μ L of each primer at a concentration of 10 mM. For the second PCR, the reaction mixture was processed as described above apart from DNA template. The DNA amplifications were performed in a MG thermal cycler (Eppendorf, Hamburg, Germany). Positive and negative control (ultrapure water) samples

were included in all amplifications. The sequences of the primers and PCR conditions are presented in Table 1.

Statistical Analysis—Bayesian Latent Class Model

To estimate the Se and Sp of the PCR for *C. burnetii* in milk and vaginal samples, we used a Bayesian latent class model (Branscum et al., 2005). Estimations were based on the cross-classified results of PCR_M and PCR_V from the sheep and goat subpopulations. The 3 main assumptions of this modeling approach are that (1) PCR_M and PCR_V are conditionally independent of each other (Gardner et al., 2000), (2) a distinct difference in prevalence exists between the 2 subpopulations, and (c) the Se and Sp of each test remains constant across the subpopulations. Beta distributions [*Be* (*a*, *b*)] were used as priors for the parameters of interest: Se, Sp, and the prevalence of *C. burnetii* in the sheep and the goat subpopulation. The 2-test, 2-subpopulation model for conditionally independent tests is identifiable; therefore, no additional prior information is required to achieve convergence, and a uniform, noninformative β prior distribution *Be* (1, 1) over the range from 0 to 1 was adopted for all parameters (i.e., Se, Sp, and the prevalence). Assessment of convergence for the Markov chain Monte Carlo was based on a combination of checks because convergence diagnostics of the Markov chain Monte Carlo are not foolproof. Therefore, a combination of the Raftery and Lewis method (Raftery and Lewis, 1992), the Gelman–Rubin diagnostic (Gelman and Rubin, 1992), autocorrelation checks, and visual inspection of the trace plots and summary statistics was used. Parameter estimates were based on analytical summaries of 49,500 iterations of 3 chains after a burn-in phase of 500 iterations. This was adequate because the Raftery and Lewis method (Raftery and Lewis, 1992) method suggested that analytical summaries of 45,000 iterations after a burn-in of 15 iterations were needed. All checks suggested that convergence occurred and autocorrelations dropped off fast. Models were run in the freeware program OpenBUGS (Spiegelhalter et al., 2007).

Table 1. Primer sequences for nested PCR *IS1111* genes

Target	Primer	Sequence, 5'-3'	Amplicon (bp)
<i>IS1111</i>	Trans1-F	TATGTATCCACCGTAGCCAGTC	687
	Trans2-R	CCCAACAACACCTCCTTATTC	
	261F	GAGCGAACCATTGGTATCG	203
	463R	CTTTAACAGCGCTTGAACGT	

Table 2. Cross-classified [positive (+) or negative (–)] results of PCR in milk and vaginal secretions of 340 Iranian sheep and goats

State	Sheep	Goats
Milk+/swab+	18	38
Milk+/swab–	12	19
Milk–/swab+	39	58
Milk–/swab–	101	55

RESULTS

There were 30 and 57 positive PCR_M and PCR_V samples among sheep, corresponding to an apparent prevalence of 17.6% (95% CI = 12.2–24.2%) and 33.5% (95% CI = 26.4–41.1%), respectively. In goats, 56 and 96 PCR_M and PCR_V samples tested positive; the respective apparent prevalence was 32.9% (95% CI = 25.9–40.5%) and 56.4% (95% CI = 48.6–64.0%). The cross-classified results of PCR_M and PCR_V are in Table 2. Posterior medians and 95% probability intervals (in parentheses) for the Se of PCR_M , Sp of PCR_M , Se of PCR_V , and Sp of PCR_V were 42 (32–59), 93 (83–99), 70 (57–89), and 83% (68–98%), respectively. The true prevalence of *C. burnetii* infection was 74% (47–95%) in goats, which was higher than the corresponding estimate in sheep of 31% (10–53%). Results are in Table 3.

DISCUSSION

To the best of our knowledge, this is the first study on the evaluation of the diagnostic sensitivity and specificity of PCR_M and PCR_V for *C. burnetii* in sheep and goats. The apparent prevalence of *C. burnetii* in our study was different between PCR_M and PCR_V in both sheep (17.6 and 33.5%, respectively) and goats (32.9 and 56.4%, respectively), which can be ascribed to the lower sensitivity of PCR_M (Table 3). The true prevalence of *C. burnetii* infection in goats was higher than the corresponding estimate in sheep, which is consistent with the results of other studies (Eibach et al., 2012; Rizzo et al., 2016; Obaidat and Kersh, 2017). This can be partly ascribed to livestock management practices, such as permanence of adult goats on the farm, or the grazing habits of goats in mixed flocks that result in higher exposure, and hence a higher risk of acquiring the infection (Rizzo et al., 2016).

The Se of PCR_V was significantly higher than Se of PCR_M , which corresponded to a higher rate of vaginal-positive, milk-negative PCR samples. Notably, most of these samples came from animals without a history of abortion. This is in line with previous reports in sheep that, following a disease outbreak during the pregnancy season, shedding will continue while no clinical signs

Table 3. Medians (95% probability intervals) for the sensitivity (Se) and specificity (Sp) of the PCR in milk (PCR_M) and vaginal secretions (PCR_V) and the true prevalence (Pr) of *Coxiella burnetii* infection in Iranian sheep and goats, under the 2-test, 2-subpopulation model and assuming that PCR_M and PCR_V are conditionally independent

Parameter	Median (95% probability interval)
Se	
PCR_M	0.42 (0.32–0.59)
PCR_V	0.70 (0.57–0.89)
Sp	
PCR_M	0.93 (0.83–0.99)
PCR_V	0.83 (0.68–0.98)
Pr	
Sheep	0.31 (0.10–0.53)
Goat	0.74 (0.47–0.95)

of the disease are present (de Cremoux et al., 2012). In sheep, shedding of *C. burnetii* in milk occurs during the first month postpartum, but it persists longer in vaginal secretions (Rodolakis et al., 2007; Astobiza et al., 2010). In fact, vaginal secretion is the most important way of shedding *C. burnetii* in sheep (Astobiza et al., 2010). In goats, several studies also indicate that latently infected but apparently healthy goats may also shed the organism in their vaginal secretions (e.g., Agerholm, 2013) while there is absence of the organism in milk samples. The persistence of *C. burnetii* in vaginal swabs in relation to milk samples resulted in a markedly higher Se of PCR_V compared with Se of PCR_M . On the other hand, Sp of PCR_V was lower than Sp of PCR_M , representing the higher false-positive rate of vaginal swabs. Indeed, bacteria have been detected for a long time after abortion in vaginal secretions of sheep, even after subsequent successful pregnancies (Berri et al., 2001).

Even though PCR_V outperforms PCR_M in terms of identifying latently infected individuals, neither milk samples nor vaginal secretion samples can determine all latently infected sheep and goats in a flock (Berri et al., 2000). Hence, a combination of the 2 methods will maximize the ability to identify more infected animals within an infected flock, provided no financial constraints exist. However, the parallel interpretation of both tests will result in a drop in the specificity of the identifying infected animals.

We used a Bayesian latent class model that assumed conditional independence between PCR_M and PCR_V . The assumption of conditional independence is expected to be valid for tests based on a different biological principle (Toft et al., 2005). In our case, though PCR_M and PCR_V are based on the same principle (i.e., detection of *C. burnetii* DNA), the targeted organ differed. We believe that the assumption of conditional inde-

pendence holds because *C. burnetii* shedding occurs via several routes with no concomitancy (Guatteo et al., 2011), as is also evident from the high number of discordant results (Table 2). Sheep and goat infections are associated with shedding considerable amounts of bacteria in placentas, birth fluids, feces, and milk, but none of these routes is interrelated and the duration of excretion varies (O'Neill et al., 2014; Joulié et al., 2015; Freick et al., 2017). Another key assumption in our modeling approach was that of the distinct difference in prevalence between the 2 subpopulations (sheep and goats), which also proved to be valid by the estimated true prevalence of infection in sheep and in goats. Finally, the assumption of constant Se and Sp across subpopulations, in our case between sheep and goats, was expected to be valid because the course of the infection did not differ between sheep and goats and differences in the intrinsic susceptibility to *C. burnetii* between sheep and goats have not been described in the literature.

Ours is the first study highlighting aspects of the *C. burnetii* epidemiology in Iran. Further studies are needed to clarify issues such as the distribution, pathogenesis, and molecular epidemiology to enable the formulation of locally applicable control methods for Q fever, which can be implemented by animal and human healthcare workers (Mohabbati Mobarez et al., 2017). Therefore, it is important to know more about shedding of *C. burnetii* to better control coxiellosis in the herds because of a lack of background knowledge and useful management tools (Joulié et al., 2015). In our study, *C. burnetii* were detected from clinically healthy animals without disorders in their pregnancy. This is in agreement with previous studies that confirmed the presence of carriers without clinical signs. These latently infected animals contribute to infection persistence at the flock level and pose the highest threat for the silent transmission of bacterium (Agerholm, 2013).

The PCR_V outperformed PCR_M in terms of identifying latently infected sheep and goats; however, neither method could identify all latently infected sheep and goats and their combination is recommended to maximize identification of infected animals. The true prevalence of *C. burnetii* infection was higher in Iranian goats than sheep.

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