

Molecular Detection and Surveillance of Zoonotic Abortive Agents in Small Ruminants in Tunisia: Epidemiology and Risk Factors

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Abstract

Background

Abortions in ruminants are a major constraint affecting livestock productivity and pose a significant threat to public health due to their zoonotic nature. In low- and middle-income countries, particularly Tunisia, these diseases remain underdiagnosed due to the lack of systematic molecular syndromic surveillance. We assumed that multiple zoonotic abortive pathogens are actively circulating at high levels in Tunisian small ruminant populations, with frequent co-infections, and that molecular diagnostics would significantly improve their detection and epidemiological characterization. This study aims to establish the first nationwide molecular epidemiological survey of the main pathogens responsible for abortions in small ruminants, to identify associated risk factors, and to assess the relevance of molecular diagnostics for national surveillance.

Methods

A cross-sectional study was conducted between July 2020 and February 2023 in 24 governorates. A total of 159 samples from abortion cases from 83 farms (sheep and goats) were analyzed by qPCR for the detection of *Brucella* spp., *Coxiella burnetii*, and *Chlamydia* spp. and by PCR to detect *Toxoplasma gondii* and *Salmonella* spp. Multivariate logistic regression analysis was conducted to assess the risk factors associated with abortion.

Results

At the herd level, at least one pathogen was detected in 63.8% of herds. The most common detected pathogens were *Brucella* spp. and *Chlamydia* spp. (22.6% each), followed by *Toxoplasma gondii* (18.8%), *Coxiella burnetii* (15.7%) and *Salmonella* spp. (1.3%). Co-infections involving two or more agents were detected in 24.5% of positive herds, which highlights the complex etiology of abortions. Multivariable analysis revealed that the absence of brucellosis vaccination was strongly associated with an increased risk of infectious abortion (OR = 9.11; 95% CI: 3.40–24.39; $p < 0.001$), while the presence of abortion on neighboring farms showed a negative association (OR = 0.35; 95% CI: 0.13–0.90; $p = 0.031$). Sampling delay significantly influenced the distribution of PCR-positive samples (Spearman's $\rho = -0.86$; $p < 0.001$), with vaginal swabs maintaining high detection rates beyond 10 days post-abortion. The heterogeneous geographical distribution and seasonality of cases were also characterized: Abortion cases showed a clear seasonal trend, peaking during autumn and winter and decreasing during summer. Most confirmed cases were geographically clustered in the northern and central regions.

Conclusion

This study revealed a high endemic circulation of zoonotic abortifacient pathogens and highlighted the added value of molecular diagnostics for effective surveillance. These findings provide an essential scientific basis for revising national control strategies to reduce economic losses and to limit zoonotic transmission to humans in a context of limited resources.

1. Introduction

Abortions in domestic ruminants, regardless of the cause, remain a major constraint on livestock productivity. Abortions are classified into two main categories: infectious and non-infectious. Non-infectious causes represent 10% of abortion causes [1]. Beyond their direct economic losses (loss of offspring, reduced fertility, and

decreased milk production), abortive agents impose an indirect economic burden through the cost of veterinary interventions, biosecurity measures and herd replacement [2–4]. Importantly, a substantial proportion of these abortive diseases in small ruminants are zoonotic and represent a dual threat to both animal production systems and public health.

The etiology of abortion in ruminants is multifactorial, involving environmental causes and management practices [5]. The contribution of infectious abortion remains unclear, and in 90% of cases where an etiological diagnosis is determined, the cause of abortion is infectious [6, 7]. These infectious causes are of particular concern due to their rapid spread [8]. *Brucella* spp., *Coxiella burnetii* (*C. burnetii*), *Chlamydia abortus* (*C. abortus*), *Salmonella* spp., and *Toxoplasma gondii* (*T. gondii*) are key bacterial and parasitic zoonotic pathogens.

Among these pathogens, *Coxiella burnetii* has emerged as a major cause of abortion in domestic ruminants worldwide. Although Q fever affects multiple domestic species, abortion is most frequently associated with infection in small ruminants [9].

The distribution and relative importance of these abortive agents vary considerably between regions and production systems [10, 11]. Therefore, rapid laboratory diagnosis is essential for timely identification and targeted control measures. Currently, sensitive molecular techniques, such as qPCR, play a key role in the early detection and identification of the specific causative agent, and in implementing the appropriate control measures [12].

Co-infection with multiple abortive agents could increase disease severity and losses and impact public health [13]. Understanding the factors contributing to the co-circulation of these abortive infections is crucial for effective control strategies.

In Tunisia, small ruminant production plays an important socioeconomic role, particularly in rural areas, where sheep and goat farming contribute significantly to household income and national meat production [14].

Several abortive diseases affecting small ruminants are classified as notifiable, given their zoonotic potential and economic impact. Indeed, the surveillance of abortion in livestock is largely based on passive reporting by farmers and veterinarians, supplemented by limited active surveillance activities [15]. In Tunisia, it was reported that sheep and goat owners perceive abortion as a normal event that does not require investigation [16]. One of the primary reasons for this perception stems from traditional husbandry practices and, potentially, a lack of awareness about the true pathological nature and consequences of abortion events [16]. This underestimation of a highly significant syndrome has led to abortion being a neglected sheep health issue, resulting in substantial economic losses due to both direct and indirect costs. For example, outbreak-based studies of toxoplasmosis in Spanish sheep flocks have estimated direct losses ranging from €63 to €172 per abortion depending on the production system and flock type [17]. In addition to these direct losses, abortion outbreaks generate substantial indirect economic costs. These include increased veterinary expenses for diagnosis and treatment, the implementation of control measures such as vaccination and biosecurity improvements, and the costs associated with laboratory investigations.

Previous studies on abortion conducted in Tunisia were based on serological diagnosis. The epidemiological profile of abortion in small ruminants in Tunisia is characterized by the endemic presence of several key pathogens, including *Brucella* spp., *Toxoplasma gondii*, and *Coxiella burnetii*, which collectively account for a substantial proportion of reproductive losses [18]. Brucellosis represents a major abortifacient agent in Tunisia,

and it is endemic in both human and ruminant populations in the country [19]. Seroprevalence studies on *Toxoplasma gondii* have confirmed the widespread presence of anti-*T. gondii* antibodies in small ruminants, indicating frequent exposure and infection [20]. Although *Chlamydia abortus* is a major cause of enzootic abortion globally, its specific prevalence in Tunisia is widely and extensively documented compared to *Brucella* and *Toxoplasma*, but it remains a potential threat for small ruminants [18]. The occurrence and spread of abortive diseases in Tunisian sheep are profoundly influenced by management practices, environmental conditions, and owner knowledge. A critical risk factor is the lack of adequate knowledge, attitudes, and practices (KAP) among sheep owners regarding the causes and consequences of abortion. Poor biosecurity and hygiene practices further exacerbate the risk of infection. The improper disposal of aborted fetuses, placentas, and other contaminated materials is a common practice that facilitates environmental contamination with pathogens like *Brucella* and *Coxiella* [21].

Using serological methods to quantify abortion provides an estimate of the prevalence of abortive diseases but does not indicate whether the infection is current or has occurred in the past. To date, comprehensive molecular investigations addressing multiple abortive agents simultaneously in Tunisian small ruminant flocks remain scarce. Moreover, data on herd-level and management-related risk factors associated with infectious abortion are limited. Addressing the problem of abortion in Tunisian sheep requires a comprehensive approach that includes improved diagnostic capabilities, enhanced owner education, and the implementation of effective biosecurity measures. Increasing the availability and accessibility of diagnostic services is essential for identifying specific causative agents and guiding targeted interventions and for the improvement of the surveillance system of abortion. Therefore, the objectives of this study were to (i) determine the frequency of the main causative agents responsible for abortions in small ruminants in Tunisia using molecular diagnostics techniques, (ii) assess the occurrence of mixed infections in abortion cases and (iii) identify key herd-level and management-related risk factors associated with infectious abortion. This study provides the first nationwide syndromic molecular assessment of abortion in small ruminants and offers evidence-based insights to support abortion surveillance and control.

2. Materials and Methods

2.1. Abortion Surveillance System

An active abortion surveillance system was implemented in 24 governorates and a specific case definition and declaration criteria were established to standardize reporting and to guide livestock owners and private and public veterinarians. An abortion event is defined as either the expulsion of a fetus between the completion of organogenesis and the end of gestation (from 30 days of gestation onward) or the delivery of a full-term newborn that dies within 48 h. Mandatory declaration of an abortion event is required under two specific circumstances: (i) the recurrence of closely timed abortions within a herd (defined as three or more abortions occurring within a seven-day period) and (ii) when the abortion rate exceeds 5% across a breeding season.

Prior to this study's commencement, veterinarians from the participating districts were informed of this study's aim. They received training on the sampling protocol, including the type of samples required and the critical time for collection post-abortion, as well as on biosafety procedures. Regional veterinary services and private veterinarians were tasked with disseminating this information to their respective communities to facilitate the timely reporting of abortion events. This strategy aimed to ensure rapid sampling and investigation of abortion

events. Sheep and goat owners were instructed to report abortion events when the abortion rate in their herds reached 5% [22–23]. Upon the report of an abortion event, the national coordinator was notified to verify the legitimacy of the suspicion and to direct subsequent investigation steps. Biological samples were collected in strict accordance with guidelines elaborated by the national coordinator. Concurrently, epidemiological data were gathered using structured questionnaire comprising a combination of both open- and closed-ended questions. This questionnaire was designed to collect information on herd demographics, the farm's history of abortion, the characteristics of the aborting female (age and breed), and the general livestock management practices within the household.

2.2. Study Design

Abortion surveillance was initially implemented across the 24 governorates of Tunisia. Between July 2020 and February 2023, a molecular investigation was conducted on biological samples collected as part of this surveillance effort. While the surveillance system was established in all 24, biological samples were successfully obtained and submitted for analysis (vaginal swabs, fetal tissues and blood). During this period, a total of 159 samples were received for analysis from the 15 participating governorates (Fig. 1).

A structured questionnaire was elaborated and pre-tested to verify clarity and comprehensiveness. The questionnaire consists of two parts: the first part is specific to the animal (age, species, breed, the status of pregnancy, vaccination status, history and stage of abortion and infertility), and the second part is specific to the farm, such as the history of reproductive problems (abortions, stillbirths, stage of abortion, history of abortion), herd size, risk factors related to herd management, grazing system, presence of companion animals (dogs and cats), and exchange of rams with other farms. Data were collected through individual face-to-face interviews with 83 small ruminant owners.

2.3. Sample Collection and Processing

A total of 159 samples were obtained by veterinary practitioners from 83 small ruminant farms (sheep and goats) with reported abortive cases. Sampling was carried out in compliance with biosafety regulations and followed standardized procedures mentioned in the guidelines elaborated by the national coordinator according to the national abortion surveillance protocol implemented by veterinary services in Tunisia and the World Organisation for Animal Health (WOAH) terrestrial animal health code standards.

Biological samples consisted of vaginal swabs, whole blood and fetal tissues collected from aborting females. A total of 103 vaginal swabs and 33 blood samples, collected in tubes with EDTA (Ethylene Diamine Tetra acetic Acid), and 23 samples of fetal tissues including liver, lung and stomach were obtained from recently aborted females. All samples were collected under aseptic conditions, and strict biosecurity measures were implemented to prevent cross-contamination and ensure personnel protection, including thorough disinfection, the use of personal protective equipment (PPE), and appropriate waste disposal procedures. Immediately after collection, samples were stored at 4–8°C and transported to the laboratory while maintaining the cold chain.

2.4. Molecular Screening DNA Extraction

All samples suspected of containing highly pathogenic zoonotic agents were processed under high biosafety measures to prevent occupational exposure of personnel and environmental contamination.

Vaginal swabs were resuspended in 900 µL of sterile 1× phosphate-buffered saline (PBS), and 500 µL of the resulting suspension was centrifuged for 10 min at 10,000× *g*. The supernatant was discarded, and the pellet was diluted in 100 µL of sterile 1× PBS prior to bacterial DNA extraction.

Regarding organ samples, tissues were aseptically processed by fine mincing using a sterile scalpel, and approximately 30 mg of material was used for DNA extraction. For blood samples, 100 µL was directly processed, and a 1:10 dilution in sterile double-distilled water was also extracted to reduce PCR inhibition.

For *Salmonella* molecular detection, a pre-enrichment step was performed to enhance assay sensitivity prior to DNA extraction: 100 µL of liquid specimen or 30 mg of tissue was inoculated into 900 µL of buffered peptone water and incubated at 37°C for 24 h. DNA was subsequently extracted from the resulting pre-enrichment culture using the thermal lysis method.

For all other bacterial targets, DNA extraction was performed according to the manufacturer's instructions using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). A 100 µL volume of pathogen-free fetal bovine serum (FBS) (Gibco-BRL, Paisley, UK) was included as a negative control to monitor potential contamination. In addition, an internal positive control (IPC), consisting of a chemically synthesized sequence (KoMa) cloned into the pPCR-Script vector, was added to each sample prior to extraction to assess the efficiency of the DNA extraction process.

Conventional PCRs were performed to detect *T. gondii* and *Salmonella* spp. DNA by targeting specific genes, namely, the B1 gene and *invA*, respectively [24–25–26]. The primers and probes used are listed in Table 1.

Table 1

Primers and probes used for conventional and real-time PCR for the detection of *Brucella* spp., *C. burnetii*, *Chlamydia* spp., *Salmonella* spp., and *T. gondii*.

PCR Type	Target	Primer/Probe	Sequence (5'-3')
PCR	<i>Salmonella</i> detection		
	<i>invA</i>	InvA-F	AGACGACTGGTACTGATTGATAAT
		InvA-R	ACAGTGCTCGTTTACGACCTGAAT
	<i>T. gondii</i> detection		
	<i>B1</i>	B22-F	AACGGGCGAGTAGCACCTGAGGAGA
		B23-R	TGGGTCTACGTCGATGGCATGACAAC
qPCR	<i>Brucella</i> spp. detection		
	<i>IS711</i>	Bru IS-F	GCCATCAGATTGAATGCTTTTTTTAAC
		Bru IS-R	AACCAGATCATAGCGCATGCG
		Bru IS-TM	Cy5-CGCTGCGATGCGAGAAAACATTGACC-BHQ-2
	<i>KoMa</i>	KoMa-F	GGTGATGCCGCATTATTACTAGG
		KoMa-R	GGTATTAGCAGTCGCAGGCTT
		KoMa-TM	HEX-TTCTTGCTTGAGGATCTGTCGTGGATCG-BHQ-2
	<i>C. burnetii</i> detection		
	<i>IS1111a</i>	Cox-F	GTCTTAAGGTGGGCTGCGTG
		Cox-R	CCCCGAATCTCATTGATCAGC
		Cox-TM	FAM-AGCGAACCATTGGTATCGGACGTT-TAMRA-TATGG
	<i>Chlamydia</i> spp. detection		
	<i>16S</i>	ChI-F	CCGCCAACACTGGGACT
		ChI-R	GGAGTTAGCCGGTGCTTCTTTAC
		ChI-TM	FAM-CTACGGGAGGCTGCAGTCGAGAATC-BHQ1

PCR reactions were carried out in a 25 μ L mixture consisting of 2.5 μ L of 10 $\times$ DreamTaq buffer (including MgCl₂), 0.5 μ L of each primer, 0.5 μ L of dNTPs, 0.2 μ L of Taq DNA polymerase (5 U/ μ L), 15.8 μ L of nuclease-free water, and 5 μ L of extracted DNA.

For *T. gondii*, PCR amplification was carried out with an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 45 s, with a final extension at 72°C for 5 min.

For *Salmonella* spp., amplification consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 min, with a final extension

at 72°C for 5 min.

Positive controls (*T. gondii* and *S. enterica* DNA) and a negative control (nuclease-free water) were included in each PCR run.

PCR amplicons were analyzed by electrophoresis on 2.5% (w/v) agarose gel for the *T. gondii* (B1 gene) assay and 2% (w/v) agarose gel for the *Salmonella* (*invA* gene) assay and visualized under ultraviolet light.

Brucella spp. DNA was detected using the TaqMan real-time PCR Bru-Multi assay targeting the IS711 gene, a genus-specific insertion sequence of *Brucella* spp., as previously described [27, 19]. Simultaneous detection of *C. burnetii* and *Chlamydia* spp. DNA was performed using a multiplex real-time PCR assay targeting the IS1111a gene for *C. burnetii* and the 16S rRNA gene for *Chlamydia* spp. The primers and probes used are listed in Table 1. Each 25 µL reaction contained 6.25 µL of TaqMan Environmental Master Mix (Life Technologies, Brant, France), 0.75 µL of each primer (10 µM), 0.25 µL of probe (10 µM), 5 µL of extracted DNA, and 10.25 µL of nuclease-free water. All samples were tested in duplicate.

Each run included a positive control for the *Brucella* assay (*B. abortus* DNA, 1 ng/µL) and positive controls for the multiplex qPCR detecting *C. burnetii* (Nine Mile strain DNA, 1 ng/µL) and *Chlamydia* spp. (*Chlamydia abortus* DNA, 1 ng/µL), as well as two negative controls (one extraction control using fetal bovine serum and one no-template control using nuclease-free water) and an internal amplification control using KoMa plasmid DNA [28].

Both qPCR assays were performed on a Bio-Rad CFX96 real-time PCR system. For the *Brucella* assay, amplification conditions consisted of an initial step at 95°C for 600 s, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. For the *C. burnetii* and *Chlamydia* spp. multiplex assay, the thermal cycling protocol consisted of an initial step at 50°C for 2 min, followed by 95°C for 10 min, and then 44 cycles of 15 s at 95°C and 60 s at 60°C. Data analysis was performed using Bio-Rad CFX Maestro Software (1.1). Samples were considered positive at cycle threshold (Ct) values ≤ 37 for *Brucella* spp. and ≤ 40 for *Coxiella burnetii* and *Chlamydia* spp. (Table 1).

2.5. Statistical Analysis

Data from the structured questionnaires and laboratory analyses were entered into a Microsoft Excel database and analyzed using R software (4.5.1). Descriptive statistics were performed to summarize data. Frequencies and percentages for herd-level characteristics, management practices, and the proportion of positive samples for various pathogens were calculated to characterize herd and aborted females.

To explore the patterns of pathogen co-occurrence, co-infections were analyzed at both herd and individual levels. A co-infection network was constructed to visualize the frequency and combinations of pathogen associations, where nodes represented identified abortive agents and edge thickness reflected the number of the detected co-infections.

Risk factors associated with abortions were identified using a two-step analytical approach. First, a univariate analysis using generalized estimating equations (GEE) with an exchangeable correlation structure was conducted to account for the clustering of samples within herds (cluster variable: herd identifier) [29]. Variables with a p -value ≤ 0.20 in the univariate analysis, after the absence of multicollinearity was verified ($VIF < 5$) and their predictive importance was assessed, were included in a multivariate GEE model, with the number of predictors limited to one variable per 10 positive events to prevent overfitting. Results are expressed as odds ratios (ORs) with their 95% confidence intervals (95% CIs), and a p -value < 0.05 was considered statistically

significant. Model performance was assessed using the quasi-likelihood under the independence model criterion (QIC) and the area under the ROC curve (AUC).

To assess the correlation between sampling delay and the proportion of PCR-positive samples, Spearman's rank correlation coefficient (ρ) was calculated due to the non-normal distribution of the data. Maps were generated using Arcgis 10.8.

3. Results

3.1. Characteristics of the Studied Herds with Problem of Abortion and Aborting Females

A total of 159 abortion events belonging to 83 herds in 15 governorates were reported by regional veterinary services during the period of study.

Table 2 summarizes the characteristics of the studied flocks and abortion cases. Regarding flock type, most of the affected herds were mixed sheep and goat farms (57.8%), followed by sheep-only flocks (37.34%), while goat-only flocks were the least represented (4.81%). In terms of flock size, the distribution was relatively balanced, with small flocks (< 75 animals) representing 37.34%, medium flocks (76–200) 27.71%, and large flocks (> 200) 34.93%. Concerning sample types, vaginal swabs were the most frequently collected specimens (64.77%), followed by blood samples (20.7%) and fetal tissues (14.4%), which indicates a predominance of easily accessible diagnostic materials. Finally, regarding parity, most aborted females were multiparous (83%), while nulliparous animals accounted for 17%.

Table 2 Characteristics of the studied herds with abortion problems and aborting females.		
Variable	Category	Number (%)
Flock type	Sheep	31 (37.34%)
	Goat	4 (4.81%)
	Mixed	48 (57.8%)
Flock size	Small (< 75)	31 (37.34%)
	Medium (76–200)	23 (27.71%)
	Large > 200	29 (34.93%)
Type of samples	Vaginal swabs	103 (64.77%)
	Fetal tissues	23 (14.4%)
	Blood	33 (20.7%)
Parity	Nulliparous	27 (17%)
	Multiparous	132 (83%)

3.2. Prevalence of Abortive Pathogens in Sheep and Goat Herds

Between July 2020 and February 2023, a total of 159 samples from abortion cases from 83 farms were investigated. At the herd level, 53 out of the 83 farms (63.8%) tested positive for at least one abortive pathogen. At the individual level, 98 out of the 159 samples (61.6%) were positive for at least one pathogen. As presented in Fig. 2, *Chlamydia* spp. and *Brucella* spp. were the most prevalent agents, with each detected in 22.6% of cases, followed by *Toxoplasma gondii* (18.9%) and *Coxiella burnetii* (15.7%), whereas *Salmonella* spp. were rarely detected, representing only 1.3% of cases.

3.3. Spatiotemporal Distribution of Abortion Pathogens in Small Ruminants

A heterogeneous geographical distribution of the investigated pathogens has been observed across the studied governorates. Most of the abortion cases investigated were concentrated in the northern and central regions, whereas several western governorates had no samples received during the study. Among the detected pathogens, *Brucella* spp. were widely distributed and identified in multiple locations across the country. *Chlamydia* spp. were mainly detected in northern and central governorates, often co-circulating with other pathogens. *Toxoplasma gondii* showed a more localized pattern but accounted for a high proportion of confirmed cases in some southern and central governorates, while *Coxiella burnetii* was detected sporadically across several regions, particularly in the north and the center. All five investigated abortive agents were found to co-circulate in the governorate of Sousse (center east). Four agents (*Brucella* spp., *Chlamydia* spp., *Coxiella burnetii* and *Toxoplasma gondii*) were detected in the governorates of Ben Arous (northeast) and Kébili (southwest) (Fig. 3).

3.4. Seasonal Patterns of Abortive Pathogens Detected in Small Ruminant Abortion Cases in Tunisia (July 2020–February 2023)

Figure 4 represents the seasonal distribution of abortion cases during the study period. A clear seasonal pattern was observed, showing an increased frequency of abortion cases during the autumn (September–November) and winter (December–February) months, while a low frequency during summer (July–August) was noted. Among the identified pathogens, *Brucella* spp. were most frequently detected in February (50%) and October (28%), with no cases in March, July, August, or September. *Chlamydia* spp. showed a peak in November (28%), December (42%), and February (12%) and was also detected in September (13%), while its frequency was low in summer. *Toxoplasma gondii* was the most common in January (36%) and December (26%), with moderate levels in September (22%) and October (15%). No cases were recorded in March, July, or August. *Coxiella burnetii* was detected mainly in autumn, with peaks in September (26%), October (21%), November (27%), and December (23%). Its circulation was low in the winter and summer months (Fig. 4).

3.5. Mixed Microbial Infections

Mixed infections involving two or three pathogens were identified in small ruminants in the present study. Among the 53 flocks that were positive for at least one infectious agent, 13 flocks (24.5%) exhibited co-infections with two or three pathogens. At the individual-case level, multiple microorganisms were often detected in the same sample, and at least two different pathogens were identified in 70.8% (17/24) of co-infection abortion cases; however, three pathogens were simultaneously diagnosed in 29.2% (7/24) of cases.

A co-infection network was constructed to visualize pathogen associations (Fig. 5), where node size reflects pathogen frequency and edge thickness represents co-detection frequency. The most common combination was *Brucella* spp. and *Chlamydia* spp. (20.8% of co-infection cases). In the present study, the two flocks that were positive for *Salmonella* spp. were also positive for *Brucella* spp. (2/2).

3.6. Effect of Sample Type and Sampling Delay on Molecular Detection of Etiological Agents

The detection rates of the abortive pathogens varied according to the type of biological sample analyzed (Fig. 6A). Overall, the highest global detection rate was achieved with vaginal swabs (62.1%), followed by fetal tissues (60.9%) and blood (60.6%). However, this difference was not statistically significant ($p = 0.984$).

Among the pathogens, *Brucella* spp. were the most frequently detected in vaginal swabs (27.2%; 28/103) and fetal tissues (21.7%; 5/23), with a lower detection rate in blood samples (9.1%; 3/33). *Chlamydia* spp. showed a significantly higher detection rate in vaginal swabs (27.2%; 28/103) than in fetal tissues (4.3%; 1/23) ($p = 0.04$). Blood samples also exhibited a notable detection rate for this pathogen (21.2%; 7/33). *Coxiella burnetii* was detected at similar rates across sample types: 21.7% (5/23) in fetal tissues, 21.2% (7/33) in blood and 12.6% (13/103) in vaginal swabs. *Toxoplasma gondii* showed comparable detection rates in fetal tissues (21.7%; 5/23), blood (21.2%; 7/33) and vaginal swabs (17.5%; 18/103). Statistical analysis revealed a significant difference in the detection rates for *Chlamydia abortus* ($p = 0.04$) (Fig. 6B).

The time interval between abortion notification and field investigation ranged from zero to sixteen days. Among all investigated cases, 44.6% (71/159) of samples were taken within 48 h of the abortion event. Variations in this interval were associated with the type of biological sample and the delay between abortion occurrence and reporting.

When analyzing the distribution of PCR-positive samples according to the delay between reporting and investigation, we observed a strong negative correlation between the proportion of total PCR-positive samples and sampling delay (Spearman's $\rho = -0.86$, $p < 0.001$), which indicates a higher proportion of total positives originating from samples collected within the first day post-abortion (Fig. 7). However, when analyzing detection rates among samples collected at each delay interval, we observed no significant linear association with time (Spearman's $\rho = -0.06$, $p = 0.8334$).

Of the 98 confirmed abortion cases, the proportion of positive samples collected at each delay interval showed a strong negative correlation with the time elapsed since abortion (Spearman $r = -0.86$; $p < 0.001$). The dashed vertical line indicates the recommended 48-h sampling threshold.

The detection rates across five time intervals were analyzed for the three sample types: blood, fetal tissues, and vaginal samples. For the blood samples, positivity is highest in the earliest interval (0–2: 67%) and in the 5–7 interval (64%), then declines suddenly after 10 days to only 25%. Vaginal samples show a more gradual pattern, with rates fluctuating between 52% and 72% across the intervals, peaking in the 8–10 period (72%) and remaining high (> 10 : 70%). Although they have only been reported in the first two intervals, fetal tissues show a remarkably high rate of 100% in the 3–4 interval, which corresponds to a period of optimal detectability (Fig. 8).

3.7. Risk Factors

In a univariable GEE analysis accounting for clustering within herds, two factors were found to have a significant association with abortion. These factors included the history of Rev-1 vaccination and the presence of abortion on neighboring farms (Fig. 8). The strongest associations were observed for the history of vaccination against brucellosis (OR = 6.92, $p < 0.001$).

The final model identified these two variables as a potential risk factor associated with PCR positivity among aborting animals. Non-vaccinated animals had significantly higher odds of testing positive for at least one abortive agent compared to vaccinated animals (OR = 9.11, 95% CI 3.40–24.39, $p < 0.001$). By contrast, the presence of abortion on neighboring farms showed a protective effect (OR = 0.35, 95% CI 0.13–0.90, $p = 0.031$). Given the descriptive nature of this study, this result should be interpreted as hypothesis-generating rather than as an identification of risk or protective factors. The model achieved acceptable discriminative ability (AUC = 0.73, 95% CI 0.66–0.80) (Fig. 9).

Odds ratios (ORs) with 95% confidence intervals are displayed; the dashed vertical line indicates OR = 1 (no effect). Variables with $p < 0.05$ are shown in orange.

4. Discussion

The present study provides a descriptive analysis of the main causes of zoonotic abortion in small ruminants in Tunisia using molecular detection of five abortive pathogens, with an investigation of their associated risk factors. To the best of our knowledge, this is the first molecular study that investigates five abortive pathogens using PCR techniques.

At the herd level, nearly two-thirds (63.8%) of farms are infected with at least one pathogen. Similarly, at the individual level, 61.6% of abortion cases are associated with at least one infectious agent. This indicates a significant proportion of cases attributed to non-infectious or unidentified causes, such as nutritional, toxic or genetic factors, or to other infectious agents not included in the diagnostic panel [30–31–32]. Our findings were consistent with previous reports from different regions, including Iran, Spain, Portugal, Greece, and Türkiye, which have similarly high prevalence rates ranging from 50% to 75% that have been documented in small ruminant populations [33–34–35–36].

Among the five investigated abortive pathogens, *Brucella* spp. and *Chlamydia* spp. were the most frequently detected pathogens, accounting for 22.6% of cases, followed by *Toxoplasma gondii* (18.9%) and *Coxiella burnetii* (15.7%). Our findings highlighted the potentially dominant role of *Brucella* spp. and *Chlamydia* spp. as significant infectious causes of livestock abortion in Tunisia and emphasized their important potential risk to public health. This result was in accordance with previous studies that proved that *Chlamydia* spp., *Brucella* spp. and *Coxiella burnetii* were the most prevalent causes of abortion in small ruminants, leading to a high number of abortion outbreaks, such as in Iran and Botswana [37–38]. Similar findings were reported in southeast Iran (28%) [39]. In the present study, the detection rate of *Brucella* spp. was lower than what was reported in Iraq, with an overall prevalence of 35.45% and 23.8% in sheep and goats, respectively [40], and lower than what was reported in Greece in 2024, with a prevalence of 29.8% [41]. However, our detection rates are higher than those detected in Pakistan (6.5%) [42], Algeria (0.39% in sheep and 5.31% in goats) [5], and China (7.68% of the samples were identified as positive for *Brucella* DNA using qPCR) [43].

In the current study, *Chlamydia* spp. were identified as predominant abortifacient pathogens in small ruminants in Tunisia. The prevalence observed here is higher than that reported previously in Tunisia by Barkallah et al. [44] in 2017 (8.54%) and higher than estimations from other regions, including Türkiye (3.5–7.7%) [45], Italy (2%) [46] and Iran (12.3%) [37]. However, comparable prevalence rates were reported in Iran (24.1%) in 2021 [10] (24.8%) and in Türkiye (21.4%) in 2021 and in 2024 (23%) [45–47]. Conversely, the prevalence reported in this study is lower than that observed on the Iberian Peninsula (38.3%) [30] and in Algeria (43.3%) [48].

Coxiella burnetii was determined to be the third most frequently detected abortive agent (15.72%). This prevalence is comparable to that reported in North Khorasan, Iran (14.5%) [33], and to findings from central eastern Tunisian sheep flocks ((11.8%) by [44]). According to published data, higher prevalence rates of *Coxiella burnetii* have been reported in other regions, including Algeria (20.62%) [5], Brazil (30.4%) [49], South Korea (26.1%) [50], Poland (22.2% in sheep and 51.16% in goats) [51] and South Africa (61.11%) [52]. Low prevalence rates were recorded in Kermanshah Province, western Iran, where the estimated prevalence in small ruminants was 1.65% [53].

The molecular prevalence of *Toxoplasma gondii* infection in small ruminants in Tunisia was 18.8%, which is consistent with results reported in Algeria (18.68%) [54] and the Republic of Korea (14.1%) [55]. Previous studies conducted in different regions of Tunisia have shown varying prevalence rates. A low molecular prevalence rate of only 5.7% was reported in sheep slaughtered in households in central eastern Tunisia [56], and 20.3% was reported by Gharbi et al. 2013 [20] in two governorates (Sidi Bouzid and Ben Arous). However, high prevalence rates were recorded in northwest Tunisia (32.3–33.3%) [25] and northern and central Tunisia, with 31% in Sidi Bouzid and 32% in Beja [57]. Our findings are lower in comparison to the prevalence rates of *Toxoplasma gondii* reported in northwest Egypt by Khattab et al. (2022), where the prevalence rates were 43.75% in sheep and 27.93% in goats [58].

The low prevalence rate of *Salmonella* spp. (1.3%) observed in this study was not surprising, as *Salmonella* is not considered a major or highly prevalent abortive agent in sheep and goats [30–59]. It may also be partly explained by the possibility of previous exposure in some flocks, as natural immunity typically follows an outbreak [60] and repeated abortion storms are uncommon within the same flock. Indeed, Amagliani et al. reported that in endemically infected flocks, the incidence of abortion decreases to an average of about 10% due to background protective immunity, and abortions are usually limited to recently purchased sheep or ewes lambing for the first time, which may have contributed to the low prevalence of *Salmonella* spp. observed in the present study [61].

The heterogeneous geographic distribution of the abortifacient pathogens in small ruminants observed in this study reflects the complex interaction of climatic conditions, management practices, and surveillance measures influencing pathogen circulation across Tunisia. The observed predominance of abortion cases in the northern and central governorates likely corresponds to the higher density of small ruminant populations and more intensive farming systems in these regions [62]. However, this distribution should be interpreted with caution, as it highlights gaps in the geographic coverage of the abortion surveillance in this study. This limitation underscores the need for more systematic sampling efforts to accurately capture pathogen distribution nationwide. Studies conducted worldwide pointed out that nutritional, climatic or management factors may influence the differential distribution of these pathogens among regions [8–63]. In addition, according to H. Esmaeili et al., the variation between geographic locations may be explained by the diversity of animal breeds and by climate and environmental factors. The detection of a single agent in some regions should be interpreted with caution, as it may result from limited sample sizes rather than the true absence of other pathogens. This

underscores a gap in passive surveillance systems, where undersampling can lead to underestimation of pathogen diversity and prevalence.

Co-infections in clinical cases of abortion occurred in 24.5% of positive herds. The most common combination identified in this study was *Brucella* spp. with *Chlamydia* spp., accounting for 20.8% of co-infection cases. These findings are consistent with the individual detection rates of these pathogens; indeed, their circulation within herds is likely to facilitate co-infection in animals. It also underscores the importance of using broad-spectrum diagnostic panels that detect multiple pathogens simultaneously, rather than focusing on a single agent. This approach reduces the risk of missing a substantial proportion of co-infected cases.

It has been widely reported that the presence of multiple pathogens within the same host or herd can exacerbate disease severity, alter immune responses, and influence pathogen transmission dynamics [64]. Co-infections could lead to immunodominance, where the immune response becomes closely focused on one pathogen at the expense of another, or to antagonistic T-helper cell polarization, which would impair the elimination of co-infecting agents [65]. Furthermore, *Brucella* spp. and *C. burnetii*, both detected in this study, share the ability to subvert host innate immunity by targeting antigen-presenting cells and modulating cytokine responses [66], which could facilitate the establishment or the persistence of co-infections.

Similarly to our finding, Santos et al. [1] have reported co-infections with *Chlamydia* spp. and *Coxiella burnetii* in 16.4% of confirmed cases in Portugal. Nosrati and collaborators [67] demonstrated that co-infections occurred in 45.2% of cases in Iran. Co-infections with abortive agents in small ruminants have been documented elsewhere, with reported rates of 35.5% in livestock in Botswana [38] and a much higher incidence of 66% on the Iberian Peninsula [68].

The seasonal patterns observed in pathogen prevalence suggest that environmental factors play a key role in influencing abortion rates. Increased incidences during the autumn and winter months may be linked to stressors such as nutritional deficiencies or climatic conditions that promote the persistence of pathogens in the environment and, consequently, their transmission.

Moreover, this seasonality reflects the reproductive cycle of small ruminants in Tunisia, with most abortions occurring during the mid-to-late gestation period, which coincides with autumn and winter [16]. Several studies have associated climate changes, including rising temperatures and erratic rainfall patterns, with the occurrence of abortion outbreaks in goats [63–69]. This seasonal variation warrants further investigation to optimize management practices such as implementing vaccination programs before high-risk periods.

Our study confirms that both sample type and the timing of collection significantly influence the detection of abortive pathogens. Vaginal swabs showed the highest overall detection rate (62.1%) and were particularly effective for detecting *Brucella* and *Chlamydia*. This is consistent with previous studies that have highlighted the value of vaginal swabs, indicating that vaginal discharges constitute an important source of infection for other susceptible hosts and represent the most important sample type for the diagnosis of brucellosis in small ruminants [70]. The detection rate of *Chlamydia* spp. in vaginal swabs was significantly higher than that in fetal tissues (27.2% vs. 4.3%; $p = 0.04$). The authors of [71] reported that PCR performed on vaginal swab extracts enabled the diagnosis of chlamydial abortion in five out of 15 flocks that were negative by bacteriological culture of fetal samples, highlighting the high sensitivity of PCR on vaginal swabs for detecting *Chlamydia* spp. The authors of [72] reported that vaginal swabs from aborting animals were found to be more sensitive for detecting

causative agents than samples taken from fetuses or placental tissues [72, 73]. This could be explained by the presence of multiple PCR inhibitors and, furthermore, by the higher microbial load in vaginal discharge [74]. Regarding sampling delays, although the absolute number of positive samples declined over time, the detection rate (proportion of positive samples among those tested at each interval) did not show a significant linear decline. This suggests that pathogen DNA can be detected for up to 10 days or more, depending on the sample type. Fetal tissues showed 100% detection rates in the 3–4-day interval, while vaginal swabs maintained high detection rates ($\geq 70\%$) beyond 10 days. These findings indicate that delays could negatively impact the detection rate of abortive agents and prove that vaginal swabs remain a valuable diagnostic tool in the several days post-abortion, whereas blood samples become unreliable after 10 days [72, 73]. The shedding of abortion-causing bacteria in the vaginal secretions of small ruminants varies depending on the pathogen: *Brucella* spp. are generally excreted for several weeks following abortion, whereas *Coxiella burnetii* and *Chlamydia abortus* may be shed intermittently over several weeks to months. Moreover, the quantity of bacterial shedding influences the sensitivity of molecular detection in clinical samples [30]. Risk factor analysis using multivariate GEE analysis shows that two factors are independently associated with the global positivity of infectious abortion in small ruminants: a history of vaccination against brucellosis and the presence of abortion on neighboring farms. This result indicates that vaccination history against brucellosis was the strongest protective factor against infectious abortion in small ruminants. Non-vaccinated animals had significantly higher odds of testing positive for at least one abortive agent compared to vaccinated animals (OR = 9.11, 95% CI 3.40–24.39, $p < 0.001$). This strong association underscores the critical role of vaccination programs in controlling abortive diseases in small ruminants in Tunisia. Our results align with previous studies conducted worldwide, which have reported significant reductions in abortion rates following the implementation of systematic and effective vaccination campaigns against brucellosis [33–75–76]. Several studied factors were not found to be associated with abortion in our study, although they have been detected as potential risk factors in several studies, such as the presence of dogs [63], type of farming [76], problems with stillbirths and malformations and infertility [77].

One of the most unexpected findings of our study was the identification of abortion presence on neighboring farms as a protective factor (OR = 0.35, 95% CI 0.13–0.90, $p = 0.031$). Given the descriptive nature of our study (absence of a non-aborting control group), the negative association with neighboring farm abortions may reflect reporting bias, reverse causality, or confounding. A plausible hypothesis is that, in response to a perceived disease threat, farmers may have increased their vigilance and awareness, implemented enhanced biosecurity protocols, and adopted improved husbandry practices to reduce exposure to abortive agents. Additionally, the systematic reporting of abortion events may have contributed to more timely detection by veterinary services. This finding is presented as hypothesis-generating rather than a causal risk factor that can be confirmed in analytical studies with appropriate control groups.

In the current study, several limitations should be acknowledged. First, this study is based on active surveillance data, which may be subject to selection bias. Farmers were more likely to submit samples when abortions were perceived as economically significant or unusual. Consequently, our prevalence estimates may overestimate the true prevalence of infectious abortion in the general population of sheep and goats. Second, the relatively small number of samples from certain governorates limits our ability to detect regional differences in pathogen distribution. Third, we did not collect detailed data on management practices (e.g., biosecurity protocols, use of treatment, feeding practices), which could have provided additional insights into the risk factors identified and likely contributed to the moderate discriminative ability of our final model (AUC = 0.73). Despite these limitations, our study provides valuable baseline data on the molecular epidemiology of abortive pathogens in small

ruminants in Tunisia based on four years of surveillance, as it is the first study that includes five abortive agents. These baseline data are essential for informing evidence-based control strategies and monitoring temporal trends in pathogen distribution.

In reference to future perspectives, extended sampling and data collection as part of a broader surveillance study are essential to provide detailed epidemiological data.

Future studies should include samples from all regions and across multiple breeding seasons to minimize the impact of specific regional and climatic factors or seasonal outbreaks. Furthermore, quantifying the microbial load, particularly in cases of mixed infections, will provide important insights and greater depth to the interpretation of contagiousness. Finally, additional examination of fetal tissue and blood samples to compare with the results of vaginal swabs could provide useful information for interpreting data in the future.

5. Conclusions

The present study provides the first nationwide molecular characterization of abortive pathogens in small ruminants in Tunisia. Our findings confirm the active circulation of multiple zoonotic agents, with *Brucella* spp., *Chlamydia* spp., *Toxoplasma gondii*, *Coxiella burnetii*, and *Salmonella* spp. detected in a substantial proportion of abortion cases. The frequent detection of single and mixed infections supports our hypothesis that abortion in Tunisian small ruminants is mainly driven by multiple concurrently circulating zoonotic pathogens. The results also show that molecular diagnostics approaches significantly improve the detection and identification of abortive agents under field conditions, allowing the identification of co-infections, and provide rapid, accurate and sensitive results while minimizing biosafety risks. In addition, the observed risk factors, particularly the absence of brucellosis vaccination, highlight the importance of preventive measures in reducing the occurrence of infectious abortions in Tunisia.

Overall, this study provides robust epidemiological evidence on the main causative agents of abortion and associated risk factors in Tunisia. Considering the scarcity of epidemiological data on abortion occurrence in this country, the findings of this study contribute to the current scientific knowledge on the status of zoonotic abortive agents in small ruminants in Tunisia and support the need to strengthen integrated surveillance and control strategies targeting zoonotic abortive pathogens in small ruminants.

Abbreviations

PCR polymerase chain reaction

qPCR Quantitative polymerase chain reaction

DNA Deoxyribonucleic Acid

WOAH World Organisation for Animal Health

Declarations

Ethics approval and consent to participate

The animal sampling procedures were approved by the Institutional Review Board of the Institut Pasteur de Tunis (protocol code **2021/22/I/V1**; approval date: **27 December 2023**). All samples were collected by licensed veterinarians in accordance with the guidelines of the World Organisation for Animal Health (WOAH) for animal handling and welfare. Besides the sheep owners were informed about the objectives of the study before sample collection. Written informed consent was obtained from all owners, and animals were sampled only with their permission and in their presence.

Consent for publication

Not applicable.

Availability of data and materials

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Competing interests

The authors declare that they have no competing of interests

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Author Contributions

K.G., S.K. and A.B.: Conceptualization, Investigation, and Methodology. K.G. and S.K.: Software and Data analysis. A.B. I.B.A., A.A and A.M.: Laboratory analysis., M.L., R.G., B.B.H.M., A.D., W.K., H.H., I.B.S.: Field investigation K.G. and S.K.: Writing—original draft. M.N.B.: Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Figures

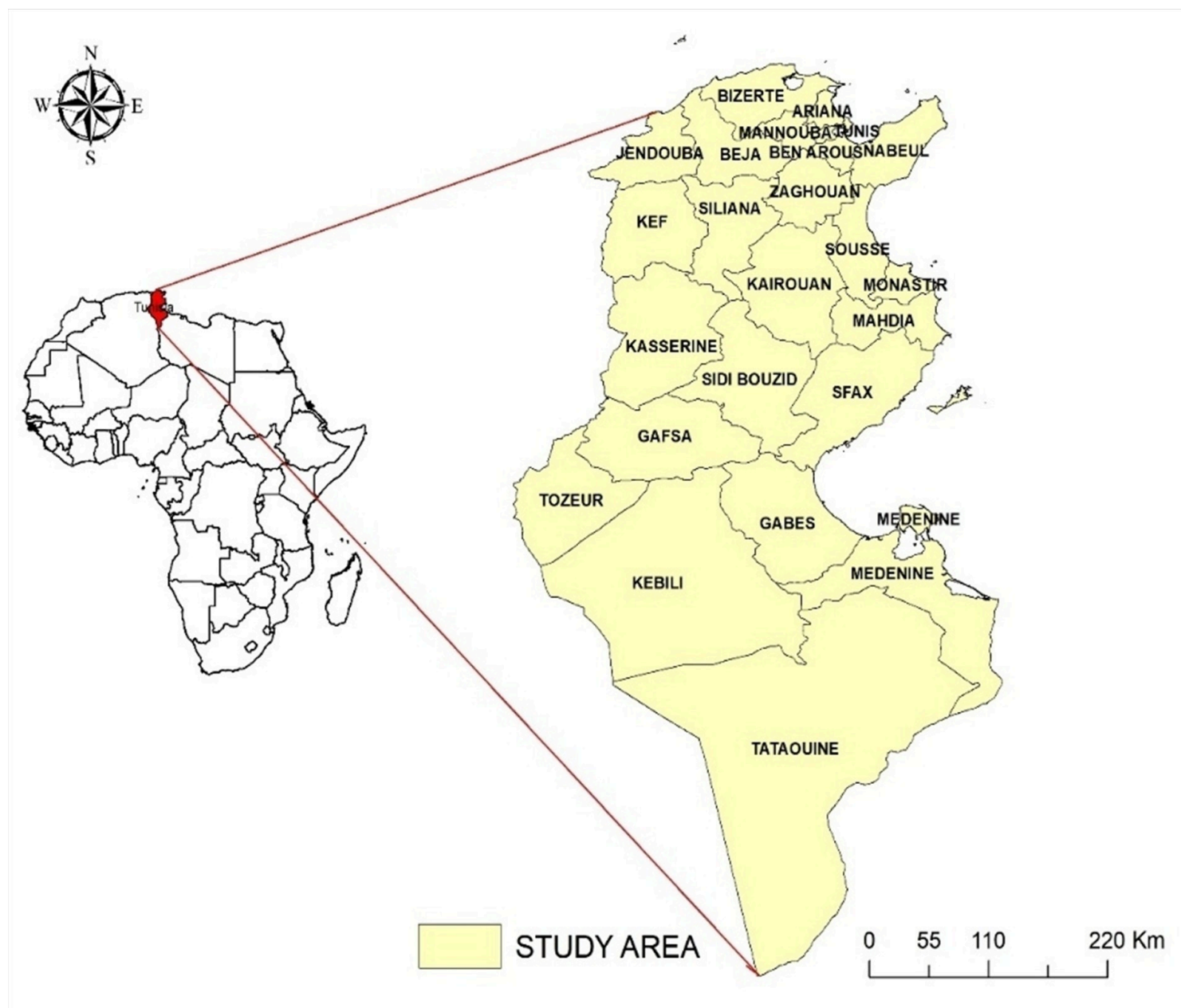


Figure 1

Study area showing the 24 governorates of Tunisia targeted for data collection regarding the surveillance of abortion in small ruminants.

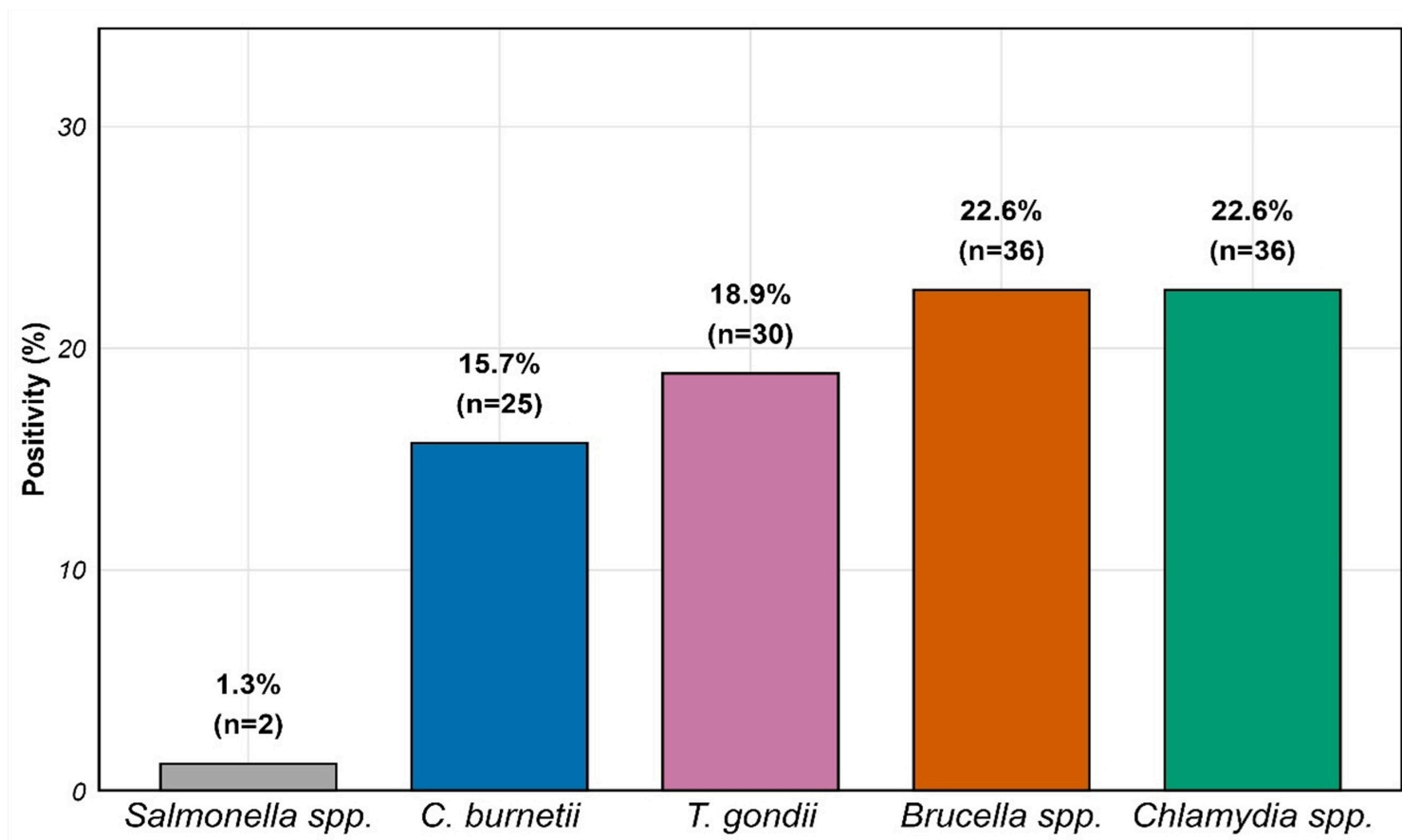


Figure 2

Molecular positivity of abortive agents in small ruminant herds with problems of abortion.

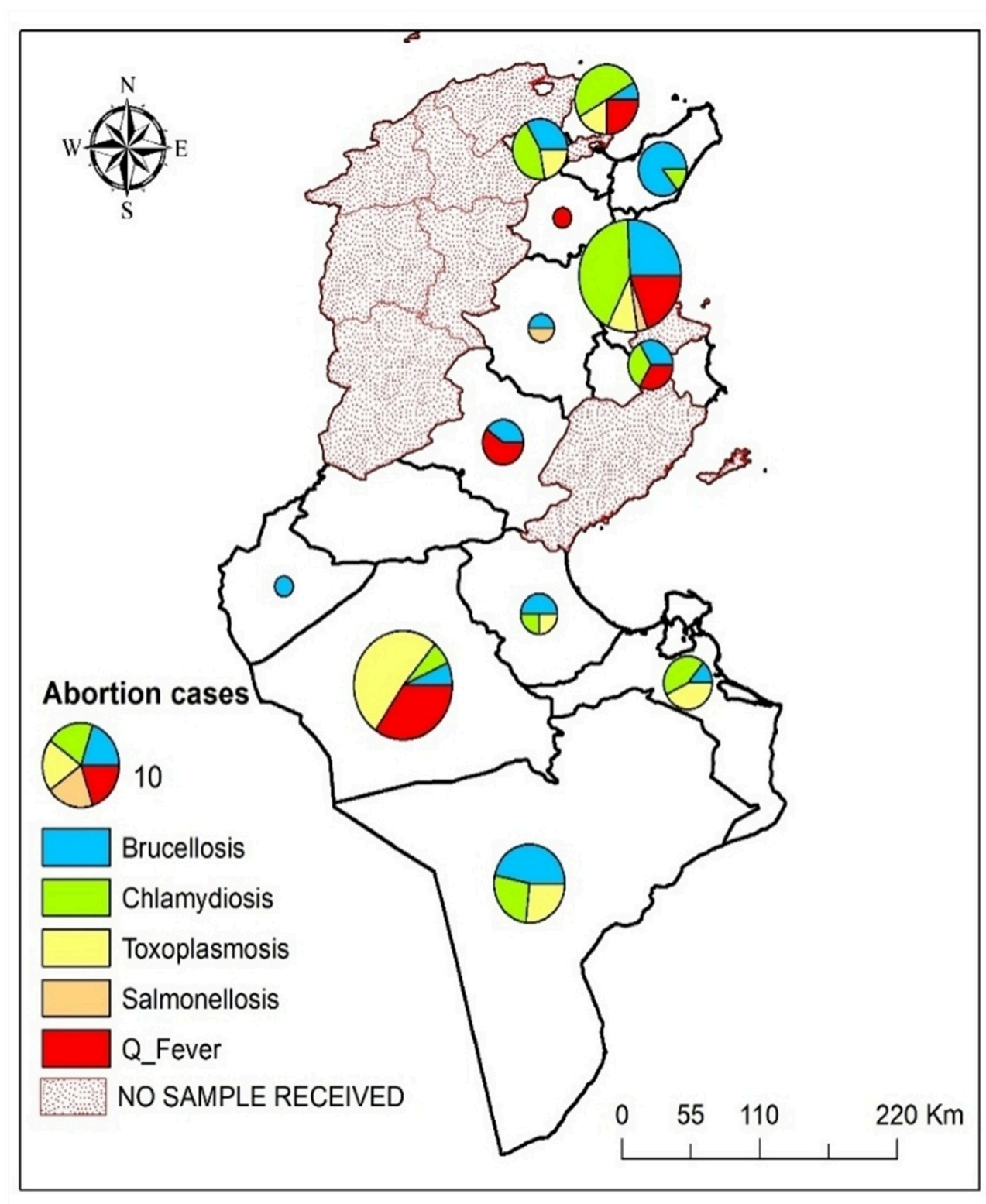


Figure 3

Geographic distribution of abortive pathogens detected in small ruminant abortion cases in Tunisia (July 2020–February 2023).

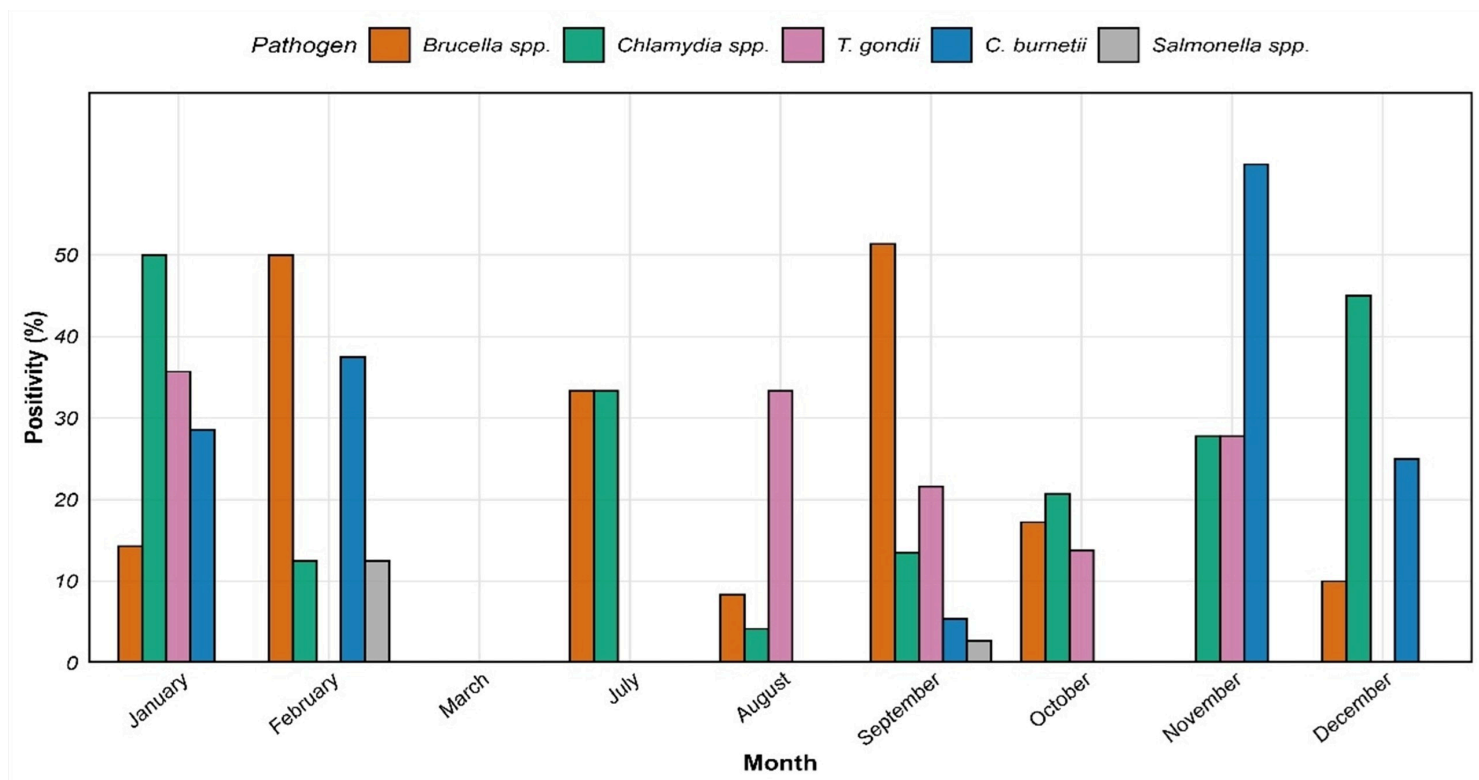


Figure 4

Seasonal distribution of abortive pathogens detected in small ruminant abortion cases in Tunisia (July 2020–February 2023).

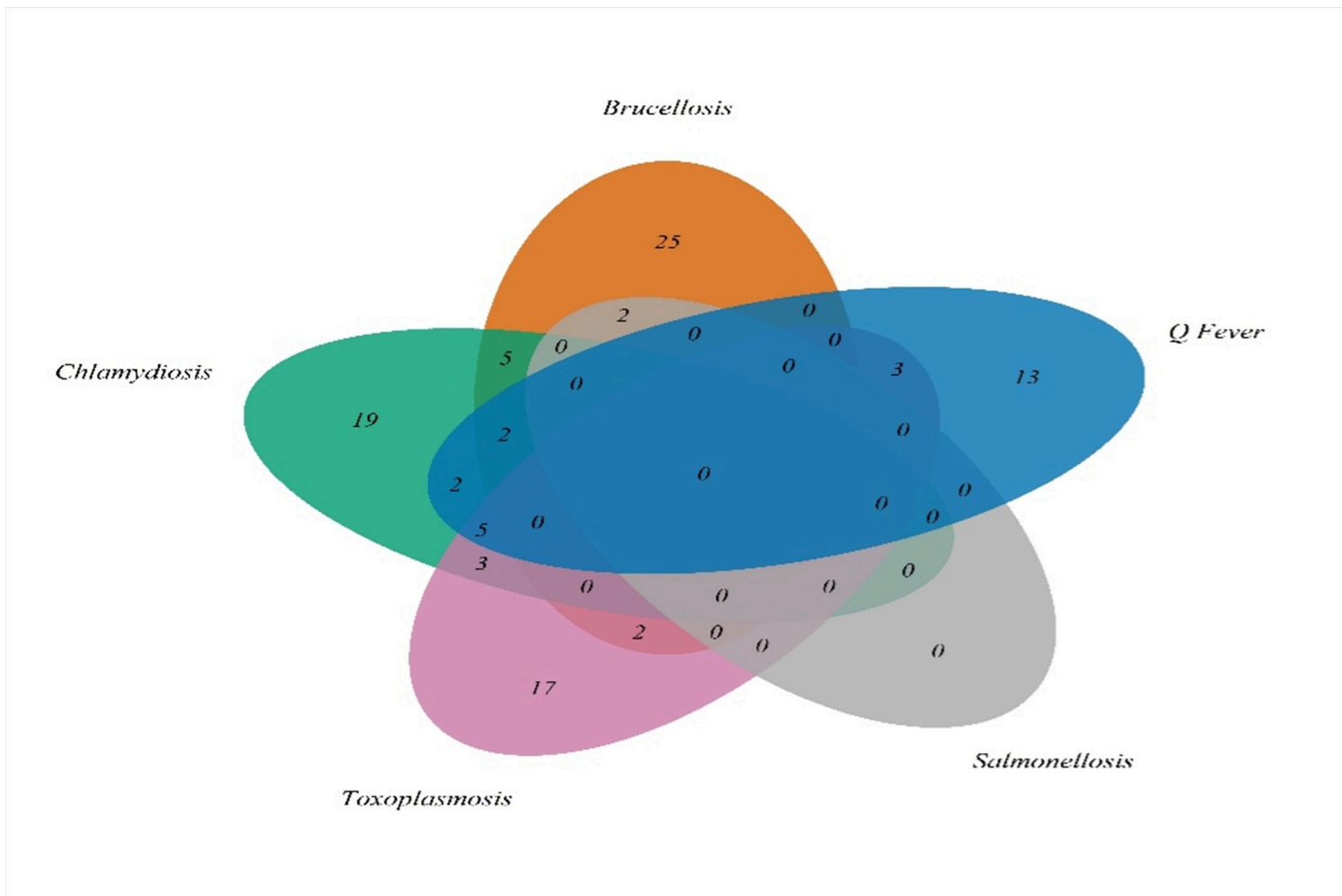


Figure 5

Distribution and co-detection of abortive pathogens in small ruminant abortion cases in Tunisia (2020–2023).

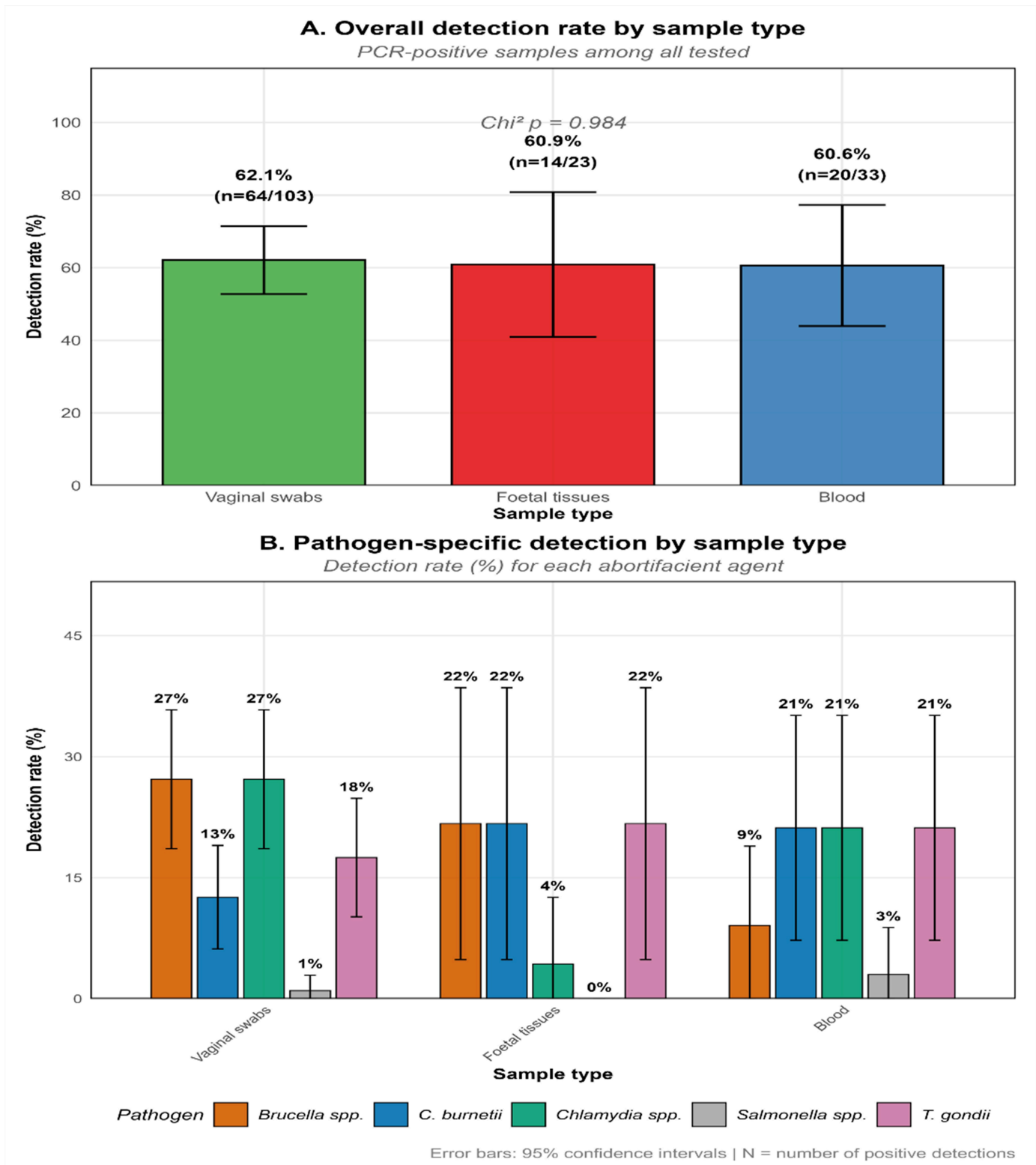


Figure 6

(A) Detection rate of abortifacient pathogens by sample type. (B) Detection percentage of the five zoonotic abortifacient agents in different sample types.

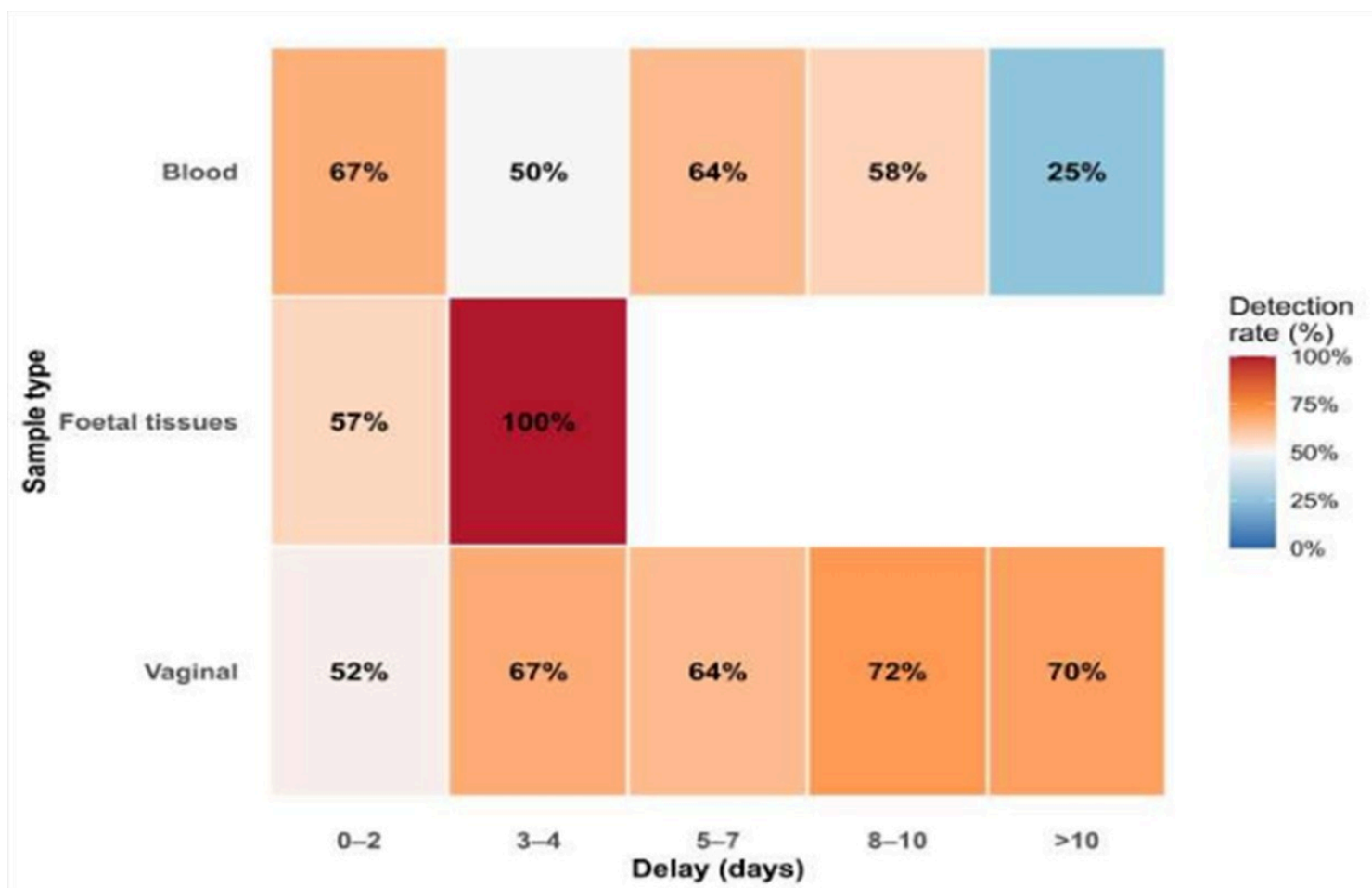


Figure 7

Distribution of PCR-positive samples by sampling delay.

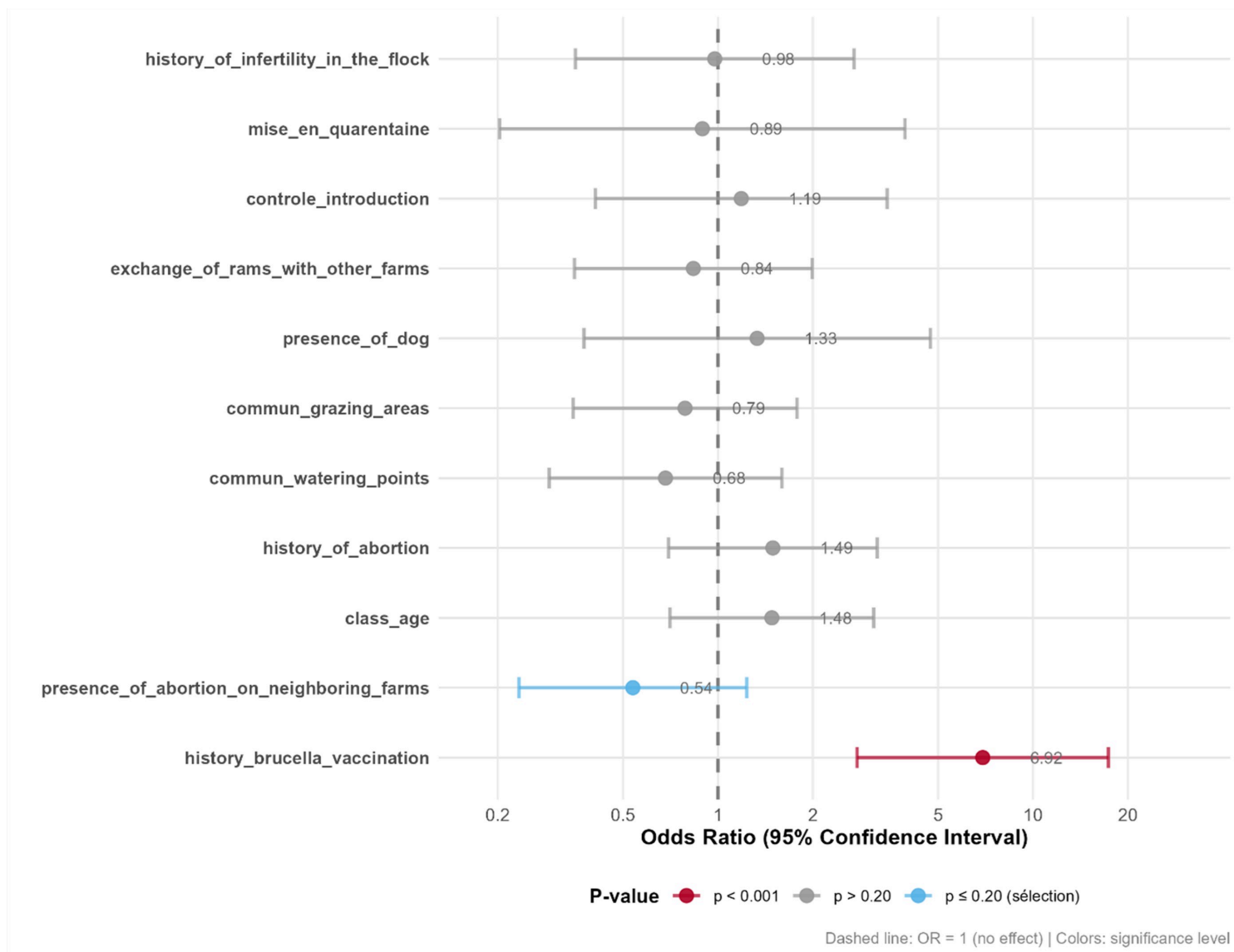


Figure 8

Univariable analysis using generalized estimating equations (GEE) of associated risk factors.

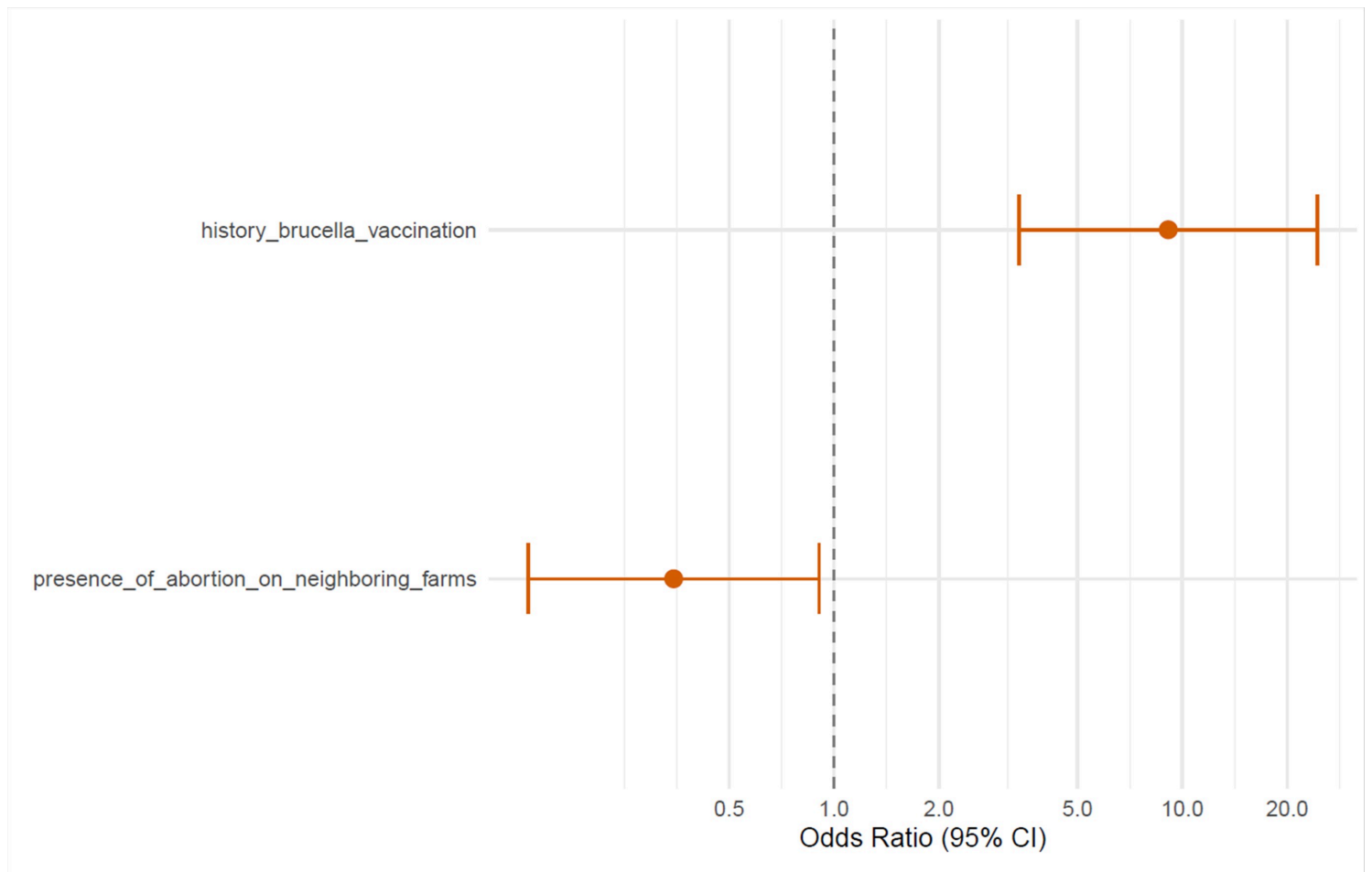


Figure 9

Multivariable GEE analysis showing factors associated with infectious abortion in small ruminants in Tunisia (2020–2023).