Journal of Advanced Veterinary Research

http://advetresearch.com/index.php/avr/index

Chlamydia abortus in Dairy Farms in Costa Rica

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ARTICLE INFO

Original Research

Accepted:
02 December 2015

Keywords:

Chlamydia abortus
Reproduction
Abortion
ELISA
PCR

ABSTRACT

The aim of the present study was to determine the presence of antibodies against *Chlamydia abortus* in specialized dairy farms. A total of 608 blood samples were collected during 2012 from 24 dairy farms located in the Northern regions of the provinces of Alajuela (15) and Heredia (9), and surveys were carried out to determine management practices in these farms. Serum samples were analyzed by enzyme-linked immunosorbent assay (ELISA) to detect antibodies against *C. abortus* (sensitivity 100.0%, specificity 99.7%). Only one serum yielded positive results (S/P 62 %), two sera weak positive results (S/P 51% and 52%, respectively), while the remaining sera (n=605) were negative in ELISA. Six months later, 22 animals that showed S/P values >22% in ELISA were analyzed again, yielding all negative results. Blood, milk, conjunctival and vulvar swabs from these animals were analyzed by Polymerase Chain Reaction (PCR), and only one vulvar swab tested positive for *Chlamydia* spp. The analysis of the management practices and results obtained with ELISA and PCR lead us to conclude that *C. abortus* is not significantly present (<0.5%) in dairy farms in the Northern regions of the provinces of Heredia and Alajuela in Costa Rica.

Introduction

Chlamydia abortus, formerly known as *Chlamydophila psittaci* serotype 1, causes abortions in cattle, small ruminants, horses, pigs and various wild animals, and may even infect humans, causing influenza-like symptoms and also abortions (DeGraves *et al*., 2004). They are Gram-negative, obligate intracellular bacteria, with a life cycle that alternates between an infectious extracellular form (elementary bodies, EB) and non-infectious but metabolically active intracellular forms (reticulate bodies, RB) (Schachter and Caldwell, 1980). Once released into the environment they may survive for several months, especially in dried fecal material. However, the high lipid content of their cell walls makes them susceptible to the microbicide action of detergents, organic solvents and other disinfectants (Longbottom and Coulter, 2003).

Infections among animals with *C. abortus* occur through direct and indirect contact, since the agent is shed in large amounts in uterine discharges, genital and conjunctival secretions, placentas and fetuses (Longbottom and Coulter, 2003). Generally, transmission occurs by direct contamination of food with conjunctival or genital discharge, which is the most important transmission route. Once ingested, the EB propagate through blood and lymph to other organs (De Graves *et al*., 2004). Experimental studies also found sexual transmission in the epidemiology of the disease. Bulls may excrete chlamydia through semen, which replicate in endometrial cells, causing damage and leading to death of the embryo or infertility of the dam (Bowen *et al*., 1978). *C. abortus* causes multiple

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ISSN: 2090-6277/2090-6269/

non-specific symptoms in cattle, which can be related to other infectious agents, such as repeated breeding at irregular intervals, increased calving interval, increased inseminations, retained placenta, endometritis, vaginitis, metritis, sporadic abortions in the last two or three weeks of pregnancy, premature birth, and stillbirth (Shewen, 1980; Wittenbrink *et al*., 1993; Godin *et al*., 2008). Abortion is probably a result of a systemic infection, but may also occur due to direct inoculation of EB in the reproductive tract during sexual contact. Although cattle may experience clinical disease after inoculation with *C. abortus*, most of the animals present a chronic asymptomatic infection (DeGraves *et al*., 2004).

Diagnosis relies on direct detection of *C. abortus* in placental tissues, fetal organs, excretions from vagina or semen, using cell culture or molecular (Polymerase Chain Reaction, PCR) techniques, while enzymatic immunoassays (ELISA) are mostly used for the detection of antibodies (Gibson da Silva *et al*., 2006; Albarracin, 2011).

Epizootic bovine abortion is considered an important disease in the European and Asian continents (Gibson da Silva *et al*., 2006). In 2001, the first bovine chlamydial abortion was reported in Switzerland; simultaneously, the first zoonotic infection and abortion in a woman was reported in this region, caused by transmission of the agent through caprine abortion material (Borel *et al*., 2006). In North America, bovine abortions caused by *C. psittaci* were diagnosed using isolation and complement fixation assays (Storz, 1971; Schachter *et al*., 1974; Shewen, 1980; Papp and Shewen, 1996).

Investigations in Latin America have been limited to detecting the presence of antibodies against *C. psittaci* in goats and sheep, and to isolating the agent from abortion material (Gibson da Silva *et al*., 2006; Soriano-Vargas *et al*., 2011; Lazcano *et al*., 2012). Only a few studies have been carried out with bovines, probably due to the fact that chlamydial abortion has not been considered a major threat for dairy farms. In the American continent a prevalence of 51.9% was reported in Brazil (Igayara-Souza *et al*., 2004). In Costa Rica, research related to Chlamydiales focused primarily on other species such as birds, horses and sheep (Sheleby-Elias *et al*., 2013; Dolz *et al*., 2013; Jiménez *et al*., 2014; Villagra-Blanco *et al*., 2015), since reproductive failure has been attributed to other infectious agents such as *Neospora caninum* (Romero *et al*., 2005) and Bovine Viral Diarrhea Virus (Chaves *et al*. 2008). The objective of the present study was to determine the presence of antibodies against *C. abortus* in specialized dairy farms in the Northern regions of the provinces of Heredia and Alajuela.

Materials and methods

Sample size

The sample size was calculated with an estimated population of 11000 animals distributed in 70 specialized dairy farms present in the Northern regions of the provinces of Heredia and Alajuela (0.5% overall expected prevalence, 95% confidence level), yielding a total of 582 samples to analyze; however a total of 608 animals were surveyed. Within each farm, the number of animals to be sampled to determine the presence or absence of antibodies against *C. abortus* was calculated with the formula of Cannon and Roe (1982) using Win Episcope 2.0 software, based on a 10% expected prevalence inside each farm, and a 95% confidence level, although some authors have reported intra-herd prevalences of up to 50% (Wang *et al*., 2001; Igayara-Souza *et al*., 2004). Since most of the farms presented similar management conditions and the distribution of the agent was unknown, the same chance of infection on each farm was assumed, thus, all animals within each farm had an equal chance of being infected. A random selection of farms was performed. The study was conducted in 24 dairy farms located in the Northern regions of the provinces of Alajuela (15 farms) and Heredia (9 farms). Since animals with more than one delivery are most likely to be infected with *C. abortus* (DeGraves *et al*., 2004), they were randomly selected in each farm.

Sample collection and surveys

During 2012 (January-September), blood samples were collected and transported in coolers to the laboratory, where they were centrifuged for 10 minutes at 10000 x g and the sera stored at -20°C until processing by ELISA. During 2013 (January-March), a second sampling was performed on selected animals. For these animals, blood samples collected in tubes with and without EDTA, milk samples, and vulva and conjunctiva swabs col-

lected in Eppendorf® tubes containing MEM (Minimum Essential Medium) were taken. Samples were transported in coolers to the laboratory, serum separated by centrifugation and all samples frozen at -20°C until processing by ELISA and PCR.

Immediately after the first blood collection (January-September 2012) a questionnaire was applied to the farmers or managers to get general information (presence of other production animals, entry of new animals), information about housing (quarantine areas, exclusive areas for calving), calf husbandry (type of milk feeding, time of contact with mothers), reproductive management (natural or artificial insemination), and general management practices (disinfection of equipment, delivery approach, abortions, management of mastitis). During the survey, the property was inspected, to verify management practices. During the second sampling (January-March 2013), the manager was interviewed in order to obtain the following information about selected animals: history of mastitis, quarters affected, frequency of these findings, difficulties with becoming pregnant, and other infections.

Enzyme-linked Immunosorbent Assay (ELISA)

The ID Screen® *Chlamydophila abortus* Indirect Multispecies ELISA from IDVet (Montpellier, France) was used. This assay reported a sensitivity of 100.0% and specificity of 99.7% (Pourquier *et al*., 2007). Major Outer Membrane Protein (MOMP) of *C. abortus* was absorbed to the microtiter plates. Serum samples were analyzed in single wells, positive and negative control sera in duplicates. To validate the assay, average of the optical densities (OD) of the positive controls, and difference between averages of OD of positive and negative control sera were verified, to fulfill the limits specified by the manufacturer. With the optical densities obtained from the different sera samples, Serum Positive Percentage (S/P) was calculated, with respect to the average of the positive control sera, using the following formula: S/P = (OD of sample x 100): average OD of positive control. As recommended by the manufacturer, serum samples that yielded S/P percentages less than 50% were considered negative, samples with S/P values between 50-60% were scored as weak positive reactors, and sera with S/P values greater than 60% were considered positive.

Polymerase Chain Reaction (PCR)

Genomic DNA from blood and swabs (200 µl) were extracted using DNeasy Blood & Tissue Kit (Qiagen, Mississauga, Ontario, Canada), following the manufacturers protocol. Milk samples were extracted with the same assay; only one step with Trizol® and chloroform was included, after incubation of samples with proteinase K to remove excess protein and fat that might inhibit PCR. DNA was subjected to endpoint nested PCR to detect species of *Chlamydophila* spp., amplifying part of the gene ompA (outer membrane protein A) as described by Biesenkamp-Uhe *et al*. (2006). In the first PCR forward primer 5'-GCI YTI TGG GAR TGY GGI TGY GCI AC-3' and reverse primer 5'-TTA GAA IC (GT) GAA TTG IGC (AG)TT IA(TC) GTG IGC IGC-3') were used, in the second PCR forward primer 5'-CCR CAA GMT TTT CTR GAY TTC AWY TTG TTR AT-3') and reverse primer 5'-GTA ATT TCI AGC CCA GCA CAA TTY GTG-3', which specifically amplify a 389-404 bp fragment, were used. DNA from *C. psittaci* from a dove from San José, Costa Rica (GenBank KF770962) was used as positive control, while water (Thermo Scientific, Waltham, USA) was used as negative control.

Statistical analysis

Frequencies of the general characteristics and management practices of the farms were calculated. To assess the relationship between *C. abortus* and management practices, the odds ratio (OR) was calculated using a mixed effects logistic regression, with the dairy farm as the random variable. Due the small amount of positive samples, only a univariable analysis was performed for each independent variable. The data was analyzed using SAS/STAT ver. 9.2 (SAS Institute Inc.).

Results

A total of 608 sera were collected from fifteen farms located in the province of Alajuela and from nine farms located in the province of Heredia. The number of animals on these farms ranged between 60 and 535 animals (mean 145), ages of the animals ranged between 33 to 51 months, and mainly

Holstein (37.5%), Jersey (29.2%), Holstein-Jersey (29.2%), and Holstein-Jersey-Pardo (4.1%) breeds were found in the farms.

Characteristics and management practices of these specialized dairy farms are shown in Table 1. All farms used artificial insemination; in three of these farms natural breeding was also used in certain cases. Only nine farms had exclusive quarantine areas, and seven had exclusive calving areas. Four farms reported goats or sheep on their property or in their vicinity, while for eight farms pigs or horses were recorded on the farm or nearby. A total of 21 farms raised their own replacements, and three farms were buying animals from other farms. In 15 farms calves spent less than 12 hours with their mothers, and two farms fed calves with mastitis milk. Only 11 farms buried or disinfected their aborted fetuses, placentas and bio infectious material with calcium oxide, while the remaining farms threw these materials far away from animals or reported that fetuses and placentas were eaten by dogs or never found. Only 13 farms disinfected equipment, in seven farms cows calved in the pasture with the rest of the group, and in most of the farms (18) mastitis was treated immediately with antibiotics, while on four farms these animals were separated. No producer or manager of these farms was aware of *C. abortus*.

Of the total of 608 sera tested by ELISA, only one sample on farm 4 yielded a positive result (S/P 62%) and two samples on farms 12 and 18, respectively, yielded weak positive results (S/P 52% and 53%, respectively), while the remaining sera (n=605) reacted negative. Most negative sera (n=580, 95.4%) showed S/P values lower than 22%, while 25 (4.1%) had S/P values between 22%

Table 1. Results of the survey carried out in 24 specialized dairy farms in the Northern regions of Heredia and Alajuela provinces to determine risk factors for the transmission of *C. abortus* (only positive answers were recorded).

Topics	# Herds (%)
Reproductive management	
Use of artificial insemination	24 (100.0%)
Use of natural mating	3 (12.5%)
Housing information	
Exclusive quarantine areas	9 (37.5%)
Exclusive calving areas	7 (29.1%)
General information	
Goats or sheep on the farm or nearby	4 (16.6%)
Horses or pigs on the farm or nearby	8 (33.3%)
Replaces with own animals	21 (87.5%)
Buys animals from other farms	3 (12.5%)
Calf husbandry	
Calves stay with their mothers less than 12 hours	15 (62.5%)
Use of mastitis milk in calf feeding	2 (8.3%)
Management practices	
Proper disposal of bio infectious material (fetuses, placentas)	11 (45.8%)
Disinfection of equipment	13 (54.1%)
Calving in maternity paddocks	17 (70.8%)
Animals with mastitis were treated immediately with antibiotics	18 (75.0%)
Separation of animals with mastitis	4 (16.6%)

Table 2. The S/P values obtained from 608 bovine samples from 24 specialized dairy farms in the Northern regions of Heredia and Alajuela provinces of Costa Rica, analyzed by ELISA for antibodies against *C. abortus.*

ELISA	S/P values	# Animals (%)
Negative	0 – 21	580 (95.4%)
	≥22 – ≤50	25 (4.1%)
Weak positive	>50 – <60	2 (0.3%)
Positive	≥60	1 (0.1%)
Total		608 (100%)

and 50% (Table 2). The determined overall prevalence in the area was 0.16%.

A total of 22 out of 28 animals that presented S/P values between 22% and 62% were analyzed six months later with ELISA. The only seropositive reacting animal in 2012 reacted seronegative in 2013 (S/P 41%), and additionally, one of the weak positive reacting animal in 2012 (farm 12) reacted seronegative (S/P 14%) in 2013, while the other weak positive reacting animal (farm 18) continued in that condition (S/P 50%). Finally, none of the other 19 samples tested reacted positive (Table 3).

Analysis of whole blood samples (22), milk samples (19), conjunctival swabs (22) and vulvar swabs (22) of these bovines by PCR yielded only one positive vulvar sample, which came from a cow from farm 19 (Table 3, Fig. 1). This animal, however, yielded negative results in both serological analysis (S/P 45% and 26%, respectively). It was not possible to sequence the PCR product due to an insufficient sample, since the animal had been removed from the herd when a third sampling was attempted. The farm on which this animal lived bordered farm 18, where an animal reacted weak positive in the first serological analysis; both farms belonged to the same owner.

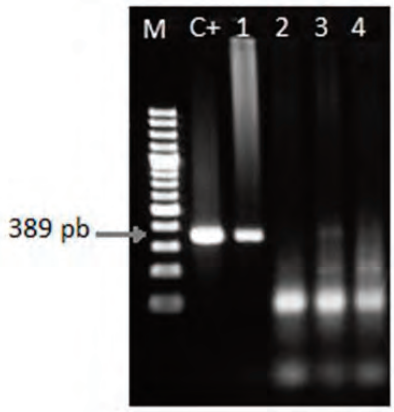

Fig. 1. Agarose gel electrophoresis of the products of the nested PCR for *Chlamydia* spp. (M: molecular marker; C+: positive control; 1: positive vulvar sample from bovine from farm 19; 2-3: negative samples; 4: negative control.

Table 3. Results of ELISA and PCR, history of mastitis and pregnancy of 22 bovines with S/P values ≥22% in the first ELISA analysis

Farm	Animal	ELISA 2012 (S/P)	ELISA 2013 (S/P)	PCR Vulvar swab	PCR Conjunctival swab	PCR Milk sample	PCR Blood sample	Mastitis	Difficulties becoming pregnant
2	37	- (49)	- (17)	–	–	–	–	–	–
4	84	+ (62)	- (41)	–	–	–	–	+	+
5	106	- (23)	- (8)	–	–	–	–	+	–
5	116	- (38)	- (31)	–	–	ND	–	–	+
10	236	- (32)	- (27)	–	–	–	–	–	–
12	255	S (51)	- (14)	–	–	–	–	–	–
12	272	- (37)	- (31)	–	–	–	–	+	–
12	278	- (27)	- (42)	–	–	–	–	–	+
11	285	- (46)	- (41)	–	–	–	–	+	–
11	281	- (26)	- (11)	–	–	–	–	–	–
13	315	- (25)	- (8)	–	–	ND	–	–	+
13	326	- (36)	- (34)	–	–	–	–	–	+
16	388	- (31)	- (22)	–	–	–	–	+	ND
18	447	S (52)	- (50)	–	–	ND	–	–	ND
19	456	- (45)	- (15)	–	–	–	–	+	ND
19	466	- (31)	- (27)	–	–	–	–	–	–
19	468	- (45)	- (26)	+	–	–	–	+	–
21	506	- (23)	- (9)	–	–	–	–	–	+
21	523	- (33)	- (14)	–	–	–	–	+	+
21	525	- (26)	- (25)	–	–	–	–	–	–
22	550	- (24)	- (11)	–	–	–	–	+	–
22	551	- (25)	- (13)	–	–	–	–	–	–

– = Negative result, += Positive result, S= Weak positive result, ND= not determined

The interviews with the managers of the farms during the second sampling made it possible to establish that 47.3% of the animals (9/19) reported problems of chronic or recurrent mastitis. Milk samples and information about udder health were not obtained from three animals, which were not lactating. A total of 7 of 19 animals (36.8%) were reported to have difficulties in becoming pregnant, while in three cases the manager did not know or did not remember the necessary information (Table 3).

Discussion

In this study it was not possible to determine the presence of antibodies against *C. abortus* in specialized dairy farms in the Northern regions of the provinces of Heredia and Alajuela in Costa Rica, since the only animal that tested seropositive yielded negative results in the second sampling, which suggests that the first result might have been a false positive result, consistent with the specificity reported for the ELISA (99.7%). Our results are not consistent with studies in other parts of the world, where high rates of seropositivity of *C. abortus* are reported in cattle herds (Wang *et al*., 2001; Igayara-Souza *et al*., 2004; Niemczuk, 2005; Biesenkamp-Uhe *et al*., 2006; Godin *et al*., 2008) However, recent studies determined low seroprevalences of *C. abortus* in horses (4.8%, 7/146) and sheep (5.3%, 19/359) from different parts of our country (Jiménez *et al*., 2014; Villagra-Blanco *et al*., 2015). There are three possible explanations. First, it is possible that the agent was recently introduced in our country, second, that a low prevalence of the agent in dairy herds are because of the management practices applied and reproductive conditions encountered, or due to false-positive reactions in ELISA, which has to be determined in future studies.

The results obtained by ELISA in both sampling periods with bovines, whose sera yielded S/P values ≥22% in the first sampling period, detected no seroconversion of negative animals; furthermore, positive and weak positive reacting bovines resulted seronegative six months later. Therefore, presence of antibodies against *C. abortus* could not be established in these dairy farms. The occurrence of false positive reactions in ELISA is a plausible explanation for our findings, but if introduction of *C. abortus* occurred in that region more than two years ago, antibody levels may also have declined below the detection limit of the immunosorbent assay (Gibson da Silva *et al*., 2006).

One explanation for the high prevalence of *C. abortus* in cattle reported in the European and Asian continents could be due to the intense production of ovine and caprine, which often graze together with cattle (Shewen, 1980), whereas in Costa Rica the ovine and caprine industry is just emerging. On the other hand, the high seroprevalence of *C. abortus* found mainly in beef cattle in Brazil (Igayara-Souza *et al*., 2004), could be due to the distinct management to which these herds are subjected, which facilitates entry and spread of the agent. The survey established that most farms are managed in ways that prevent entry and transmission of *C. abortus* – for example, the use of artificial insemination, the absence of ovine and caprinein dairy farms,that could represent reservoirs of *C. abortus*, not using mastitis milk when rearing calves, and breeding their own replacements (Bowen *et al*., 1978).

With reference to the results obtained with PCR, confirmation by sequencing the amplified product of the vulvar swab would have unequivocally proven the presence of *C. abortus* in dairy herds of Costa Rica, but a false positive result in PCR could also not be ruled out. In future studies, we recommend taking milk and swab samples on three consecutive days, since excretion of *Chlamydias* are intermittent, and more extensive sampling periods would increase the chances of finding the agent (Jee *et al*., 2004; *et al*., 2005).

In this study, seropositive animals to *C. abortus* were not detected in specialized dairy farms from the Northern regions of Heredia and Alajuela provinces.

Conclusion

It is concluded that if the agent is present in this area, it would be present in very few farms, with a prevalence of lower than 0.5%. We recommend further investigation of the presence of *C. abortus* in bovines of other areas of the country, and the inclusion of ovine and caprine in the investigations, since they are the main host of this agent.

Acknowledgement

We wish to thank Vicerrectoría de Investigación,

Universidad Nacional, for financial support.

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