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***Chlamydophila abortus* Infection and IgG Antibody are Associated with Abortion During Estrus Period in Cows and Goats**

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ABSTRACT

Chlamydophila abortus (*C. abortus*) usually causes subclinical infections, especially in the pregnancy stage which lead to abortion during the mid-later stage of pregnancy. The aim of this study was to evaluate different methods in detecting *C. abortus* in cows and goats at the subclinical stage and variations in *C. abortus* goat strains. Nested Polymerase Chain Reaction (PCR) was adopted to detect the presence of *C. abortus* DNA from nasal and rectal swabs and vaginal samples [swabs, Control Internal Drug Release (CIDR) and Insemination Catheter (IsC)] and enzyme-linked immunosorbent assay (ELISA) to detect the IgG antibody titers of sera during estrus and follow the trail of gestation and abortion status of the detected cows. Four tests were performed including C1 and C2 of cows and G1 and G2 of goats. C1 results: PCR identified *C. abortus* in 7.4% (10/136) IsC samples and none in all nasal and anal swabs. During pregnancy, the abortion rate of PCR (+) cows (44.4%) was significantly higher than PCR (-) cows (13.5%; $p < 0.05$). About the subclinical infected cows in C2, the antibody titer of the PCR (-) (1.03 ± 0.75) was significantly higher than the PCR (+) group (0.21 ± 0.15 , $p < 0.05$). During cycle induced by CIDR that contains 366 mg progesterone, goats on farms without any abortion history within a year had a significantly lower PCR (+) rate than the goats on farms with an abortion history within a year (9.2%; 10/109 vs. 21.1%, 4/19). This study found that nested PCR could evaluate subclinical infection of *C. abortus* during estrus by use of IsC, CIDR and swab samples. However, subclinical infected animals having high enough antibodies might be PCR-negative. Combination of PCR and antibody detection can apply in early diagnosis of this disease.

Key words: *Chlamydophila abortus*, control internal drug release (CIDR), dairy cow, goat, estrus period

INTRODUCTION

Chlamydophila abortus usually causes limited clinical symptoms at the early stage of infection, although some animals in experiments display signs of short-term mild fever and depression (Buxton *et al.*, 1990; Papp and Shewen, 1996a; Sammin *et al.*, 2006). In goats, *C. abortus* can cause a persistent infection (Thomson *et al.*, 2005; Entrican *et al.*, 2009) and its infection in the uterus during parturition results in significantly changes in temperature, body weight and

biochemical values (Reinhold *et al.*, 2008; Maley *et al.*, 2009). With tetracycline treatment on animals infected without symptoms, an increase in fetus's survival rate is obtained (Quinn *et al.*, 2002); however, such treatment cannot prevent placenta transmission (Andersen and Vanrompay, 2000). *Chlamydia psittaci* can infect ewes reproductive tract during estrus and shed consequently (Wilsmore *et al.*, 1990; Papp *et al.*, 1994; Papp and Shewen, 1996b). The amount of *C. abortus* shed was considered to be lower than the dose which could induce abortion or delivery of a weak lamb for an infected ewe (Livingstone *et al.*, 2009). The risk of transmission during an estrus cycle is considered to be low in goats (Appleyard *et al.*, 1985; Papp and Shewen, 1996b; Livingstone *et al.*, 2009). Additionally, *Chlamydophila* could be transmitted either through artificial insemination or during intercourse (Papp *et al.*, 1994; Papp and Shewen, 1996a).

Previous studies believed that *C. abortus* could only be detected around the 90th day of pregnancy (Buxton *et al.*, 1990) but by using real-time PCR, the pathogen could be found at the 85th day (Maley *et al.*, 2009). Livingstone *et al.* (2009) found that minor quantities of *C. abortus* could be detected from the 14th to the 2nd day prior to ovulation, on the day of ovulation and between the 6th and 13th day after ovulation. However, compared with the normal quantity of 2.7×10^7 particles detected from an aborted fetus, the copies of *C. abortus* detected during an estrus cycle are as low as the lowest limitation of real-time PCR. Our early study proved a high prevalence of *C. abortus* in vaginal swabs determined by PCR (Yu *et al.*, 2012).

Generally, samples collected from pregnant animal are impractical to use because of the difficulty in controlling the timing of collections, insufficient viable samples and a lack of cooperation from farmers (Andersen and Vanrompay, 2000). Because it is difficult to test latent infection and to identify the animals infected through physical examination, an easier sampling and an early diagnostic method is necessary in order to control and prevent the spread of *Chlamydia* spp. Therefore, this study was to establish the *C. abortus* shedding of the subclinically infected cows and goats and to try to find an easier sampling method for early diagnosis of the disease.

MATERIALS AND METHODS

Study areas and period of dairy cattle farms: The study of dairy cattle farms was carried out into two tests, C1 and C2. C1 was conducted between July 2008 and June 2009. 136 cattle from five dairy cattle farms located in southern Taiwan, Yunlin County, were investigated. An insemination catheter (IsC) was collected from each estrus cow after artificial insemination. One hundred and thirty six IsC samples and 58 nasal and rectal swabs were collected. The length of gestation and successful parturition of the 136 cows was fully recorded. C2 was conducted between Oct 2010 and Feb 2011, 194 sets of serum and IsC samples were collected after artificially inseminated from each estrus cow in eight farms in southern Taiwan. Then, after elimination of 66 cows with PCR (-) and ELISA (-) showing subclinical infected with *C. abortus*, the serum collected from 128 cows were analyzed in C2 test.

Study areas and period of dairy goat farms: The study of dairy goat farms was conducted in two tests, G1 and G2. G1 was conducted between Oct 2008 and Aug 2009. One hundred and twenty vaginal swabs were collected from four dairy goat farms with abortive history and without inducing estrus by CIDR in southern Taiwan. *C. abortus* detection was performed for vaginal swabs with or without mucus. Additionally, 104 vaginal swabs were collected from five dairy goat farms without abortion history. G2 was conducted between Oct 2009 and April 2010, six dairy goat

farms (labeled K, L, M, N, O and P) with the use of CIDR to induce ovulation and to initiate estrous cycle were selected. Only the K farm had an abortion history within a year. One hundred and twenty eight dairy goats were selected from these farms ranging from 14-37.

Sample treatment: A vaginal swab was inserted in 8-10 cm and twisted three times. The cotton swab was then flushed and mixed with 2 mL of sterile PBS for 10 min. The 1.5 mL of the mixture were added into an Eppendorf and then centrifuged at 60x g for 1 min. The supernatant was transferred into a new Eppendorf and stored at -80°C. IsC samples containing reproductive excreta were cut into pieces, packed in re-sealable bags and then stored at -20°C. The pieces were flushed and mixed with 3 mL of sterile PBS. The 1.5 mL of mixture were added into an Eppendorf and centrifuged at 60x g for one minute. Then the IsC supernatant was transferred to a new Eppendorf and stored at -80°C. CIDR samples containing reproductive excreta was cut into pieces by sterile scissor and immersed in an Eppendorf with 2-3 mL of PBS. The samples were then vortexed and centrifuged. Due to active progesterone on the surface of inseminators, it was recommended that operators must wear gloves. Blood from tail caudal vein was collected by a specimen tube and then the sample was kept in 5°C storage for three hours before it was sent to a laboratory where the tube was centrifuged at 800x g and 4°C for ten minutes. The CIDR sera were stored at -80°C.

Standard strain: Ovine *C. abortus* B577 (ATCC No. VR-565TM) was imported by Union Biomed Inc from the USA under approval of the Centre of Disease Control, ROC, Taiwan, on 17 December, 2007.

DNA extraction, polymerase chain reaction (PCR), nested-PCR and restriction fragment length polymorphism (RFLP): The primers used to detect the *Chlamydophila* spp. from goat and cattle samples are listed in Table 1. The protocol used to amplify the DNA fragment of *Chlamydophila* spp. from goat samples were followed by the description of Yu *et al.* (2012) and Marsilio *et al.* (2005). After processed by the nested PCR, PCR products were purified by Axyprep™ DNA Gel Extraction Kit (Axygen, USA), following the manual's instructions. Then the amplicon was digested with restriction enzyme *AluI* to produce two DNA fragments with the size of 235 and 352 bp for *C. abortus* (Marsilio *et al.*, 2005). Protocol used to amplify a 330-bp DNA fragment of *C. abortus* from cattle samples were described by Sheehy *et al.* (1996). A mixture of 5 µL of DNA product and 1 µL of 6x loading dye was separated by 2% agarose gel and 0.5% TBE (Tris/Borate/EDTA) buffer at 50 V for 75 min. Later, the gel was stained by ethidium bromide and recorded under UV illumination.

Table 1: Primers used for PCR detection of *Chlamydophila abortus* from goats and cattle

Animal	Gene	Primers	Sequence (5'-3')	Size (bp)	References
Goat	<i>omp2</i>	<i>cmcB-f</i>	CTCGAGATGTCCAAACTCATCAGACGA G	1665	Yu <i>et al.</i> (2012)
		<i>cmcB-r</i>	AAG CTT CTG TAT CAG CAA CGG GTA CC		
	Nested	<i>chla AF</i>	ATG TCC AAA CTC ATC AGA CGA G	587	Marsilio <i>et al.</i> (2005)
	<i>chla AR</i>	CCT TCT TTA AGA GGT TTT ACC CA			
Cattle	<i>omp2</i>	PCR-D ₁	CAA ACT CAT CAG ACG AG	582	Sheehy <i>et al.</i> (1996)
		PCR-D ₂	CCT TCT TTA AGA GGT TTT ACC		
	Nested	PCR-Ab	TCA GTG CCA ATC CGT CGA TA	330	
		PCR-D ₂	CCT TCT TTA AGA GGT TTT ACC		

Serological evaluation: CHEKIT *Chlamydomphila abortus* Antibody ELISA Test Kit (IDEXX, Maine, USA) was used to detect the IgG antibody titer in cattle serum against *C. abortus*, following the protocol described by the company. OD 450 nm was measured using ELISA reader (MERCK, Titertek Multiskan MCC MK II, Germany) and the results were calculated by the following formula: $S/P = (OD_{\text{sample}} - OD_{\text{neg}}) / (OD_{\text{pos}} - OD_{\text{neg}})$. An S/P ratio smaller than 0.3 is considered as negative, while an S/P ratio larger than 0.4 is considered as positive, value between 0.3-0.4 was regarded as doubt positive, according to the ELISA Kit description.

Statistical analysis: Microsoft® Office Excel 2003 was used to analyze the seropositive, antibody titer, average and variance coefficient and standard errors. The relationship between the results of the vaginal samples and the antibody tests from IsC samples in cows were analyzed by Chi-square or Fisher's exact test, according to the sample size. One-proportion t-test described in Minitab® 14 was used to find the difference between the rates of different goat groups. The p-value less than 0.05 was considered as the threshold of significance.

RESULTS

Detection of *C. abortus* DNA collected from IsC, nasal and rectal swabs sample and follow the trail of gestation and abortion status of the detected cows: C1 test of 136 IsC samples and 58 revealed that *C. abortus* was not found in nasal and rectal discharge and was observed only in 7.4% (10/136) of the IsC samples. Investigation of 46 pregnant cows out of 136 cows, abortion rate was 44.4% (4/9) for cows positive for *C. abortus* significantly higher than 13.5% (5/37) for cows negative for *C. abortus* (p = 0.036 by chi-square test and p = 0.05 by Fisher's exact test).

Analysis of serum antibody titer in C2 test: The average antibody titer of the seven *C. abortus* PCR (+) cows was significantly lower than that of 121 PCR (-) cows (0.21±0.15 vs. 1.03±0.75; p<0.05).

Difference in *C. abortus* infection in vaginal swab samples of the goats treated with mucous or CIDR treatment: G1 test was conducted in 71 samples with mucous and 49 samples without mucous from four dairy goat farms with a history of abortion within a year. The positive rate of *C. abortus* from the swabs with mucous was 18.3% (13/71, 95% CI = 0.10-0.29) significantly higher than 0% (0/49, 95% CI = 0.00-0.06) for the swabs without mucous (p<0.05). Examination of five dairy goat farms without a history of abortion and CIDR use showed that *C. abortus* was not detected by PCR (0/104, 95% CI = 0.00-0.03) (Table 2, Fig. 1).

Table 2: Difference in *C. abortus* infection in vaginal swab samples of the goats treated with mucous or CIDR treatment

PCR identification	Mucous ^a			CIDR ^b		
	Positive ^c	Negative ^d	Significance	Positive ^e	Negative ^f	Significance
Positive	13	0	$\chi^2 = 10.06, p < 0.05$	10	0	$\chi^2 = 10.01, p < 0.05$
Negative	58	49		99	104	
Total	71	49		109	104	

^aDetection of *C. abortus* performed by using single PCR from four farms with a history of abortion within a year, ^bDetection of *C. abortus* performed using nested PCR, ^cSwabs with mucous, ^dSwabs without mucous, ^eCIDR samples from five goat farms without abortion history and induced with CIDR, ^fVaginal swabs from other five goat farms without abortion history and CIDR treatment

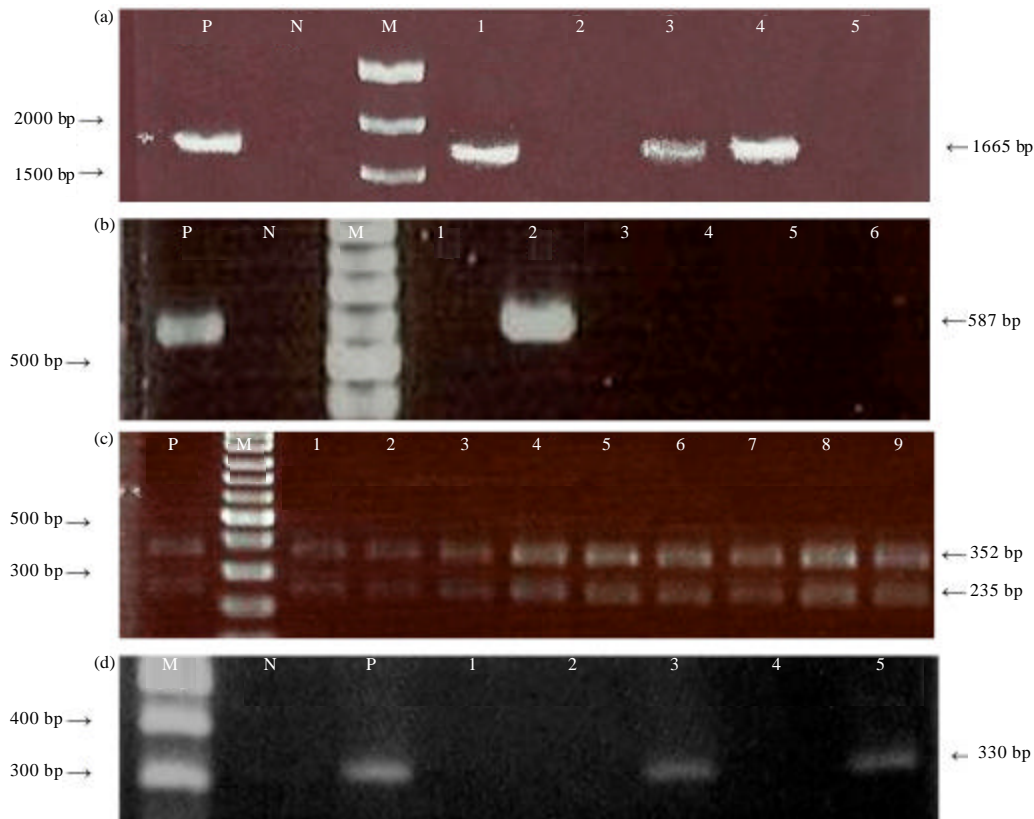


Fig. 1(a-d): (a) Detection of *C. abortus* using *cmcB* primer , Lane 1, 3: Positive sample from vaginal swab, Lane 2, 5: Negative sample from vaginal swab, Lane 4: Positive sample from CIDA (control internal drug release, containing 336 mg progesterone), (b) The nest PCR to detect *C. abortus* from sample in K farm. Lane 1, 3-6: Negative sample from vaginal swab, Lane 2: Positive sample from vaginal swab (c) Results of positive sample of PCR-RFLP by *AluI*. P: Positive control, M: Ladder marker, Lane 1-9 was positive samples, (d) Representative results of *omp2* PCR performed on oestrus cow insemination catheter samples, M: 100 bp size marker, N: Negative control, P: Positive control

The detection rate and the positive numbers of *C. abortus* by single PCR and nested-PCR from vaginal swabs during goat cycles induced by CIDR: In G2 test of CIDR use, the overall prevalence of *Chlamydia* infection was higher in nest-PCR (10.9%, 14/128, 95% CI = 0.06-0.18) than in single-PCR (0%, 0/128, 95% CI = 0.00-0.02) for the goats from these six farms (Table 3). The prevalence of *C. abortus* in farm with abortion history within one year (Farm K) was 21.1% (4/19, 95% CI = 0.03-0.39), while prevalence of the remaining five farms without abortion history within one year was 9.2% (10/109, 95% CI = 0.04-0.16). In compared that the prevalence of *Chlamydia* infection was four (21.1%) out of 19 samples for K farm having an abortion history within a year, *Chlamydia* prevalence was 9.2% (10/109, 95% CI = 0.04-0.16) for the remaining five farms without abortion history within one year (2-3).

Table 3: The detection rate and the positive numbers of *C. abortus* by single PCR and nested-PCR from vaginal swabs during goat cycles induced by CIDR

Farm	K		L		M		N		O		P		Total		95% CI
	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	
Single PCR	0/19	0	0/19	0	0/18	0	0/37	0	0/21	0	0/14	0	0/128	0	0.00-0.02
Nested PCR	4/19	21.1	2/19	10.5	3/18	16.7	2/37	5.4	2/21	9.5	1/14	7.1	14/128	10.9	0.06-0.18

Single PCR: Primers were *cmcB-f/cmcB-r* of *omp2*, Nested PCR: Primers were *cmcB-f/cmcB-r* and *chla AF/chla AR* of *omp2*

Table 4: The genotype distribution of *C. abortus* in six goat farms with the use of CIDR

Farm	Genotype							Total
	IA-a	IA-b	IB-a	IB-b	II	III	IV	
K	2	1			1			4
L	1		1					2
M		1	1				1	3
N				1		1		2
O	2							2
P		1						1
Total	5	3	2	1	1	1	1	14

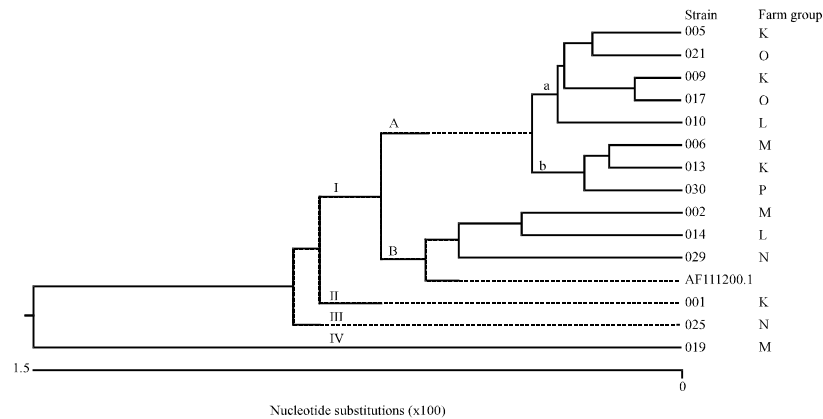


Fig. 2: Phylogenetic tree of 14 *C. abortus* constructed by analyzing the sequences of *omp2* use with DNASTar software (clustal method), The genetic similarities are between 95.2 and 100% among all isolated strains, AF111200.1 is reference strain

Sequence analysis of *omp2* in *C. abortus*: Phylogenetic analysis separated 14 *C. abortus* into 4 clusters and 78.5% (11/14) belonged to cluster I (Fig. 2). The genotype number differed among Farms from single genotype in Farms O (IA-2) and P (IA-b) to three genotypes in Farm K (IA-a, IA-b and II) and M (IA-b, IB-a and IV) (Table 4).

DISCUSSION

Calves with low immuno-protection are more susceptible to infection by *C. abortus* (Jee *et al.*, 2004). The pathogen can reach the digestive tract at early stage of infection, then invades the circulatory system, reaches mainly the reproductive system and then causes abortion

(Entrican, 2002; Papp and Shewen, 1996a; Reinhold *et al.*, 2008; Livingstone *et al.*, 2009). Therefore, the current study confirmed that *Cp. abortus* is frequently present in vaginal swabs and lacks in nasal swabs (Jee *et al.*, 2004) as well as positive association of abortion history and *C. abortus* infection (Reinhold *et al.*, 2008; Yu *et al.*, 2012). However, farms without an abortion history within a year could still have a *Chlamydophila* infection at the low level of *Chlamydophila* shedding (Livingstone *et al.*, 2009). The low *C. abortus* infection in Farm K with subclinical infection cases might be caused by some latent infection or below detectable level of pathogen's DNA, suggesting that *C. abortus* was mainly shed by the animal during her cycle; latent infection exists but difficult in diagnostic.

Normally, the shedding of a pathogen is related to hormone types, mechanism and shedding levels (Yamada *et al.*, 1986; Kaushic *et al.*, 1998). *C. abortus* has been isolated from sheep vaginal swabs between three days before ovulation and four days after ovulation (Papp *et al.*, 1994). The artificial infection of *C. trachomatis* in uterus tends to be successful because of the progesterone priming (Kaushic *et al.*, 1998). In current study, use of the CIDR to induce the cycle increased detection rate of *C. abortus*. Further, application of CIDR, progesterone and both estrogen and progesterone could increase higher titer of bovine viral diarrhea virus was detected during estrous, shorten the diagnostic limitation of *Mycoplasma* spp. and enhance *Pasteurella pneumotropica* detection rate, respectively (Yamada *et al.*, 1986; Taylor-Robinson and Furr, 1990; Fray *et al.*, 2002).

CIDR is helpful in identifying possibly infection in ewes on farms with an abortion history. Livingstone *et al.* (2009) proved that a low-level persistent infection is possible in post-abortion ewes and suggest that a low level of *Chlamydophila*. DNA could be detected during periovulation. CIDR's success rate in inducing the cycles of ewes was between 75% and 90%; however, an animal was in anestrus or already in her cycle it would not respond to CIDR (Cetin *et al.*, 2009). Because consistent application of progesterone could also lead to the animal developing tolerance and drug-dependency (Noakes *et al.*, 2009), the detection rate in this study, especially on those CIDR-using farms, would be a bit underestimated. These results also suggest that even if a farm does not have an abortion history and the PCR result is negative, chronic or latent infection might still exist because Taiwan is the epidemic area of *C. abortus*.

The IgM and IgG level trend was highly associated with the fertility rate in heifers (DeGraves *et al.*, 2004; Biesenkamp-Uhe *et al.*, 2007), implying higher IgG titer would encounter a lower abortion rate. However, IgG antibody titer of seven PCR-positive cows were lower than PCR-negative cows (0.21 ± 0.15 vs. 1.03 ± 0.75 , $p < 0.05$) in this study. In the future study, we will investigate the IgM level to have a better profile of the immune response against *C. abortus* infection.

In this study, the overall detection rate (10.9%) of dairy goat farms at the G2 stage was higher than the rate (5.8%) at the G1 stage. Furthermore, Yu *et al.* (2012) reported that the detection rate from four farms with an abortion history within a year was 10.8%. Apparently, the rate of *C. abortus* infection in Taiwan was stable between 2008 and 2011. The higher rate at the G2 stage could be contributed to a higher shedding of the pathogen because the farms where the study was conducted used CIDR, based on the same PCR primers and diagnostic methods.

As a low level of *Chlamydophila* spp. DNA could be detected in chronic situations, the lower detection rate was expected in dairy goat farms without an abortion history for at least one year. Real-time PCR has been developed to increase the detection rate during the post-abortion period

(Livingstone *et al.*, 2009). Nested-PCR was able to detect *Chlamydophila*. DNA at the beginning of ewe's ovulation and was 100-1000 fold in sensitivity than single PCR did (Livingstone *et al.*, 2009). The phylogenetic analysis indicates diverse of *C. abortus* in goats (Fig. 2, Table 4). Additionally, multiple origins of *C. abortus* in Farms K, L, M and N and mono-origin in Farms O and P were found.

CONCLUSION

In the present study, IsC, CIDR and swabbing samples during the estrus cycle are more acceptable for farmers than other samples when an animal is pregnant. Nested PCR increase the detection rate of *C. abortus* on vaginal samples during periovulation. Since IgG level might associated with abortion rate, when nested PCR combined with ELISA to detect serum antibody against *C. abortus*, the accuracy of predictive ability of abortion will be increased. Diverse genetic origins of *C. abortus* are found in goats.

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