Journal of Medical Bacteriology

Diagnosis of *Chlamydia abortus* by Isolation in Cell Culture and Real Time PCR in Aborted Sheep and Goats

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ARTICLE INFO

Article type:
Research Article

Article history:
Received: 11 Sep 2024
Revised: 28 Sep 2024
Accepted: 17 Oct 2024
Published: 04 Nov 2024

Keywords:
Abortion, Cell culture, Chlamydia, Real-time PCR, Small ruminants.

ABSTRACT

Background: Ovine enzootic abortion (OEA) caused by *Chlamyidia abortus* is one of the most important abortive disease in small ruminants. Diagnosis of Ovine enzootic abortion depends on the isolation and detection of the agent or its nucleic acid. The aim of the present study was to detect *Chlamydia abortus* using both isolation method and real-time PCR in *Brucella* free flocks in Iran.

Methods: Twenty-eight vaginal and conjunctival swab samples which were *Chlamydia abortus* seropositive, were selected from ewes and does with recent abortion. Then the samples were tested by real-time PCR and positive molecular samples were inoculated into McCoy cells.

Results: Using real-time PCR, 18 samples (64.3%) were positive and 7 (25%) of them were isolated in cell culture.

Conclusion: The present results indicate that *Chlamydia* can play a relatively significant role in the abortion in does and ewes in Iran. Although the isolation of *Chlamydia abortus* have 100% specificity, because of low sensitivity, time consuming, cost and high probability of contamination, it is not suitable for routine laboratory diagnosis. Therefore, applying real-time PCR which have high sensitivity and specificity is recommended.

- ***Please cite this paper as:*** Esmaeili H, Hamedi M, Madani SA, Barin A. Diagnosis of *Chlamydia abortus* by Isolation in Cell Culture and Real Time PCR in Aborted Sheep and Goats. *J Med Bacteriol.* 2024; **12** (4): pp.9-16.

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Introduction

Ovine enzootic abortion (OEA) caused by *Chlamyidia abortus* is one of the most important abortive disease in small ruminants worldwide. This gram negative bacterium causes certain economic losses in Europe, North America and Africa and 45% of ovine abortion in England is related to this agent (1). According to the previous studies, *C. abortus* has a significant role in small ruminant's abortion in our country and the reports showed the infection range between 9-25% (2, 3, 4, 5). Seroprevalence of *C. abortus* was 25.6% among individual animals and 81.4% in sheep and goat flocks in a study conducted in 2015 in 7 provinces of Iran (6). In a molecular study in 2020 in Iran, *C. abortus* DNA was detected in 23.5% of aborted fetuses (3).

Identification of OEA, is related on the bacterial isolation and identification of the agent or its nucleic acid in aborted fetus or vaginal discharge of freshly aborted ewes (7). However, diagnosis of chlamydial agents is neglected in many laboratories, because *Chlamydia* spp. do not grow in routine bacteriological media and isolation of this bacterium is difficult. Moreover, direct microscopy examination by the use of modified ziehl neelsen is neither sensitive nor specific, and because of the similarity of *Chlamydia* appearance with *Brucella* and *Coxiella burnetii*, there is a possibility of misdiagnosis (8).

Isolation of chlamydial agents using either embryonated chicken eggs or cell culture, is difficult, though it is the golden standard for the detection of *Chlamydia* (7, 9, 10), and according to the World Organization for Animal Health, cell culture is the method of choice (7). Madani et al., in 2011 inoculated the samples collected from birds into McCoy cells and 23% of the inoculates showed inclusions in cell culture (9).

There are various molecular based methods for the detection of *Chlamydia* which are fast, easy and sensitive and among them, real-time PCR is preferred because of its high sensitivity and specificity and quantitative of results. According to the advantages of molecular methods, they are more popular than isolation (11). Esmaeili et al., in 2021, detected *C. abortus* DNA in 24.1% of small ruminant's samples by Real-time PCR (4). Livingstone et al., in 2009 in the UK, identified *C. abortus* at oestrus period of sheep with the previous abortion using real-time PCR (12).

Regarding high rate of infectious abortion in small ruminants in Iran (13), similarity of *C. abortus*, *C. burnetii* and *Brucella melitensis* in direct smears and low specificity of serological tests (8, 11), it is important to investigate different diagnostic methods to use them for the detection of chlamydial agents in clinical specimens. The aim of the current study was to detect *C. abortus* using real-time PCR and isolation in cell culture from samples belonged to the aborted sheep and goats in the free *Brucella* flocks.

Materials and Methods

Sample collection

Twenty-eight vaginal and conjunctival swab samples were collected from ewes and does with the history of recent abortion. The animals were selected from the ones identified *C. abortus* seropositive using an indirect ELISA kit, CHEKIT®-*CHLAMYDIA* (IDEXX Laboratories B.V., 1119 NE Schiphol-Rijk, Nederland). In addition, the animals had been tested for *Brucella* spp. infection using Rose Bengal, Wright and 2-mercaptoethanol tests according to the protocol of Iran Veterinary Organization.

Samples were belonged to fourteen sheep and fourteen goats from nomadic and rural flocks from five provinces. Sucrose-phosphate-glutamate (SPG) buffer was used for transporting the collected samples (23). It consisted of 74.6 g/L sucrose, 0.512 g/L KH_2PO_4, 1.237 g/L K_2HPO_4, 0.721 g/L glutamic acid (all chemical agents of SPG buffer were provided from Merck KGaA, Germany), 10% fetal calf serum (Jahad

Daneshgahi, Iran), 100 mg/L from each of vancomycin and streptomycin (Jaber Ebne Hayyan, Iran), 50 mg/L gentamicin (Alborz Darou, Iran), and 50mg/L amphotericin B (Bristol-Myers Squibb, France). Each swab was immersed in 2 ml SPG buffer and was kept in -20 °C.

Real-time PCR

DNA was extracted from 200µ of SPG solution contained swab sample using High Pure PCR Template Preparation kit (Roche, Germany). Real-time PCR was conducted according to the method of Pantchev et al., 2009 (14). The target fragment in this study was a part of *C. abortus OmpA* gene. Each reaction included an internal amplification control.

Forward and reverse primers and TaqMan probe included GCAACTGACACTAAGTCGGCTAC A, ACAAGCATGTTCAATCGAT and FAM-TA AATACCACGAATGGCAAGTTGGTTTAGCG -BHQ respectively.

Nuclease free water was used as negative control in all the stages and the standards of commercial kit were used as positive control. Real-time PCR was performed using 2X TaqMan universal PCR Master Mix, 1µ concentration of each primer/probe mix, 1µconcentration of internal extraction control primer/probe mix and 3µ RNAse/DNAse free water. The final volume of the reaction mixture amounted to 20µ including 10µ master mix and 5µ of sample DNA.

Amplification was carried out by a Rotor-Gene Q Series (QIAGEN): using the following cycling parameters: heating at 95 °C for 10 min (single denaturation step), subsequently 50 cycles of 95 °C for 10s and 60 °C for 1 min (annealing and extension).

Cell culture inoculation

Cell culture procedure was conducted based on the animal cell culture guide retrieved from American Type Cell Culture (ATCC) and McCoy cell line (National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) was used for the sample inoculation and *Chlamydia* isolation. Freshly trypsinized McCoy cells were passaged into the 1-dram shell vial containing one 2cm diameter circular coverslip. The cell culture vials were incubated for 24-48 hours at 37 °C.

The prepared samples were inoculated into four shell vials containing McCoy cells rich coverslip at the bottom. The vials were then centrifuged at 1000 x g for 30 min in order to enhance the attachment of chlamydial elementary bodies to the host cells. The remaining fluid on inoculated cell culture was then discarded and replaced by cell culture media containing 1 mg/lit cyclohexamide (Sigma Chemical Co., St. Louis, MO) and 10% fetal calf serum.

Staining of the cover slip and inclusion body identification

The cultures were examined at two-day intervals. The first monolayer culture medium was removed at day 4 or 5 after inoculation and washed with sterile PBS once. The inoculated cell on the coverslips was fixed with aceton-methanol (Merck KGaA) (50:50, v:v) for 5 min and stained with Giemsa.

The coverslips were then removed from the vials and mounted on a slide using Entellan® (Merck KGaA), and examined microscopically under high power field for chlamydial inclusion bodies. Cultures that appeared to be negative at 7 days post-inoculation, were repassaged with the same method as described above.

Statistical Analysis

The agreement between the two tests was calculated by kappa and Macnemar methods.

Results

Real-time PCR

All the tested samples were *Brucella* negative. Real-time PCR detected the DNA of *C. abortus* in 18 samples (64.3%) (Table 1). Eight positive samples were belonged to ewes and ten of them were from does (Table 2). All the positive samples in real-time PCR, were belonged to ewes and does which had at least one abortion in their history.

Isolation in cell culture

Chlamydia were isolated in cell culture from 7 samples (25%) (Figure 1). Four positive samples were belonged to does and three of them were from ewes. *Chlamydia* was isolated from four conjunctival and three vaginal samples.

Comparative results of the two methods

Real-time PCR identified the highest amount of *Chlamydia* genome related to a goat from a herd in Fars province which had 460654 genes in its vaginal swab sample and Chlamydial inclusion of the sample was detected in cell culture, too. Eight and ten positive swab samples either by real-time PCR or isolation in cell culture, were from ewes and does respectively (Table 1). Seven conjunctival and 11 vaginal samples were positive by using either real-time PCR or isolation in cell culture (Table 2).

Statistical analysis

The calculation of Kappa statistic showed the agreement between the two tests was 32%, so according to the interpretation of Altman (1999), they had fair agreement (15) and by the use of McNemar's test, there was no agreement between the two methods ($P<0.01$).

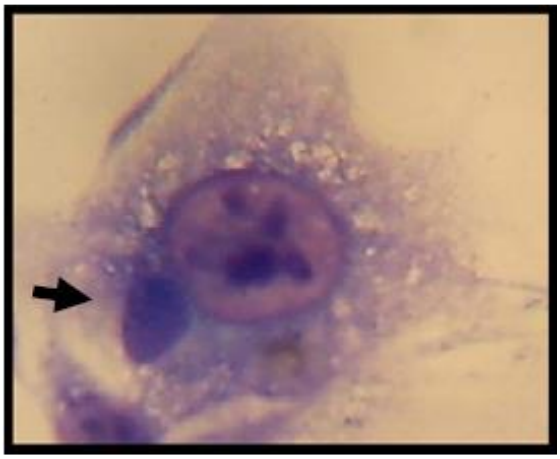

Fig 1. Perinuclear cytoplasmic inclusion body (arrow) of Chlamydial agent isolated from a vaginal swab sample of a doe. × 1000, Giemsa.

Discussion

In the present study, the clinical samples of sheep and goats were evaluated for the detection of chlamydial organisms using real-time PCR and cell culture isolation. Real-time PCR has recently become a common technique in diagnostic laboratories and because of its high specificity and sensitivity, the results are highly reliable (16).

As it was shown in table 1, positive results in 18 out of 28 samples, indicated trustworthy of real-time PCR for diagnosis of OEA in small ruminant's flocks. Another advantage of the molecular method, is its ability in differentiation species of *Chlamydia* while isolation methods merely show chlamydial inclusion. However, as real-time PCR can also detect the DNA of an inactive agent and there may be dead *Chlamydia* in studied samples from recovered animals, false positive results might occur (17). In addition, false negative results may occur due to the inhibitory substances in the collected samples (18). As the present results showed, 10 seropositive animals were not identified by real-time PCR.

Table 1. Absolute and relative frequency distribution of *Chlamydia* positive samples, based on results of real-time PCR and cell culture in ewes and does with the history of abortion.

Real-time PCR	**Positive**		**Negative**		**Total**	
Cell culture	Frequency	%	Frequency	%	Frequency	%
Positive	7	25	-	-	7	25
Negative	11	39.2	10	35.7	21	75
Total	18	64.3	10	35.7	28	100

Table 2. Absolute frequency distribution of the samples based on the type of animal, the sampling site and the incorporated results of the tests.

Animal	Sampling site	Results of real-time PCR or Cell culture		Total
		Positive	Negative	
Ewe	Vagina	4	2	6
	Conjunctiva	4	4	8
	Total	8	6	14
Doe	Vagina	7	2	9
	Conjunctiva	3	2	5
	Total	10	4	14
Total	Vagina	11	4	15
	6	7	6	13
	10	18	10	28

Along with the probability of the presence of inhibitory substances, chlamydial shedding is intermittent (12). In addition, infection with other *Chlamydia* species such as *Chlamydia pecorum* or even infection with some of the gram negative bacteria like *Acinetobacter* spp. may lead to a cross-reactive antibody response (19). Moreover, serological methods may show a previous chlamydial infection which the animal has recovered and become immune and seropositive. Seropositivity may also be due to vaccination (9, 20). However, since there is no vaccination against OEA in Iran, this is not an issue. This can explain how ELISA positive results in the present study, missed by real-time PCR.

As mentioned before, the molecular method is usually preferred for the detection of *Chlamydia*, though isolation of the agent is necessary for confirmation. In the present study, 28 samples were inoculated into the cell culture and chlamydial infection was confirmed in seven of them. In agreement to the current results, in a study

concerning the ruminant chlamydiosis in France in 2009, *Chlamydia* spp. were isolated from only two out of 20 clinical samples. Similar to the present study, the authors used McCoy cells culture to isolate *Chlamydia* (8). According to another study in 2011 in Egypt, 5% of fecal swab samples from sheep and goats were positive for *Chlamydia* spp. using Vero monolayer cells (21).

Despite low sensitivity, isolation is still the golden standard for the diagnosis of chlamydiosis and cell culture has been shown to be the best method for isolation of *Chlamydia* spp (7). However, live and active agents are needed for isolation, but the bacteria may not survive during the sampling, transportation and isolation process in the laboratory, which may lead to the false negative results (22).

Comparing the two methods applied in the present study, there were significant differences in the number of positive samples detected by isolation in cell culture and real-time PCR. In this research, isolation in cell culture was considered as the golden standard, however, real-time PCR was more reliable in the detecting of *Chlamydia* positive ewes and does.

In the current study, only seven positive samples in real-time PCR were isolated in cell culture (Table 1) and these findings were supported by other studies which showed low sensitivity of isolation compared with the molecular methods (8, 23). A study in 2003, compared PCR and isolation method in cell culture for the detection of *Chlamydia*. The results of the survey showed that all isolated samples, were positive using PCR, but in contrast, 15 PCR positive samples, were not isolated in cell culture (24).

In a survey in 1999 in Australia, McElena and Cross, evaluated sensitivity and specificity of PCR and isolation in cell culture for the detection of *Chlamydia psittaci* in poultry. The researchers determined that in the best situation, cell culture method can only detect 17 out of 25 positive PCR samples (25).

The first isolation of *C. psittaci* from birds in Iran reported in 2011 by Madani et al. The researchers inoculated PCR positive samples into McCoy cells and Giemsa was used to stain the inclusion bodies. In this study, only four out of eleven positive PCR samples were positive for chlamydial inclusions in the cell culture and no *Chlamydia* could be isolated from the PCR negative samples (9). The current research was conducted in a similar way of Madani et al. in 2011 but for the detection of *C. abortus*. Nevertheless, the results of the both studies were the same and all the isolated *Chlamydia* in the cell culture were belonged to the real-time PCR positive samples as well.

Infections caused by members of *Chlamydiaceae* family, have been usually underestimated due to the lack of availability of suitable tests and special laboratory facilities for the detection of these intracellular pathogens. Serological tests and detection of the bacterium using direct smears from clinical samples are usually the only available methods of chlamydial diagnosis in most diagnostic laboratories in Iran and their application might lead to misdiagnosis and wrong decisions (26). Moreover, isolation of the agent is not practical in the routine diagnosis procedures, since it is difficult, expensive, time consuming, labor intensive and hazardous. As a result, using the method is limited to the research domain or very specialized laboratories (27). Based on our findings, real-time PCR is a suitable method for rapid and accurate diagnosis of *C. abortus*, so it can be advised instead of isolation and other molecular methods.

Conclusion

In the present study, 64.2% of samples were positive using real-time PCR in the *Brucella* free flocks which highlighted the importance of being aware of the infectious abortifacient pathogens other than *Brucella*. Comparison of the three methods used in the current study: serology, molecular and cell culture, suggested real-time

PCR as a recommendable tool for laboratory routine diagnosis procedures.

Acknowledgements

This project was supported by the grant from the faculty of veterinary medicine, University of Tehran, Iran.

Funding Information

This study was supported by a grant from the University of Tehran (Grant Number:28786).

Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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