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Original Article

Isolation of *Chlamydia* spp. from Ewes and Does in Iran

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ABSTRACT

Enzootic ovine abortion is caused by *Chlamydia abortus* and may result in abortion among small ruminants during the last 2-3 weeks of pregnancy. Enzootic abortion is diagnosed by isolation of the agent or detection of its nucleic acid in the products of abortion or vaginal excretions of freshly aborted females. Isolation of chlamydial agents in cell culture is the gold standard, so in the present study this method was employed. Twenty-eight vaginal and conjunctival swab samples were selected from ewes and does that had recently aborted. The samples were inoculated to McCoy cells. The inoculated cells were fixed, stained by Giemsa staining, and mounted on slides. Finally, the slides were observed by an optical microscope for the presence chlamydial inclusion bodies. Chlamydia was isolated from four conjunctival and three vaginal samples. All the negative cultures were passaged a further two times. Cell culture was identified as the most convenient method for the isolation of *Chlamydia* and remains essential to document the viability of the organism. Isolation of *Chlamydia* in the present study, highlights the importance of paying more attention to the bacterium as one of the main abortifacient pathogens along with other infectious causes of abortion.

Keywords: Abortion, Cell culture, *Chlamydia*, Isolation, Small ruminants

Isolement des chlamydies contaminant les brebis et les chèvres en Iran

Résumé: *Chlamydia abortus* est responsable d'avortement enzootique chez les petits ruminants dans les 2 ou 3 dernières semaines de gestation. La détection de l'avortement enzootique est possible via l'isolement des protoplastes ou le décèlement de l'acide nucléique dans les sécrétions vaginales d'un animal qui a récemment avorté. L'isolement des chlamydies après culture cellulaire représente la norme de référence utilisée pour déterminer le niveau réel de l'infection par cette bactérie. Dans cette étude, un total de 28 échantillons vaginaux et conjonctivaux a été prélevéà partir des chèvres et brebis ayant subies récemmentun avortement. Ces échantillons ont été inoculés dans des cellules McCoy. Ces dernières ont ensuite été fixées et colorées avec le Giemsa, avant d'être montées entre lame et lamelle et examinées à l'aide d'un microscope optique afin de détecter la présence éventuelle de chlamydies. La chlamydia a été isolée à partir de 4 prélèvements conjonctivaux et 3 échantillons vaginaux. Les cultures négatives ont été inoculées une seconde fois. L'isolement des chlamydies à partir de cultures cellulaire sreprésente la méthode la plus fiable et reste essentielle pour la détection des protoplastes. Dans cette étude, l'isolement de cette bactérie chez les troupeaux étudiés montre la nécessité d'accorder une attention bien plus grande à la chlamydia ainsi qu'à d'autres protoplastes impliquées dans l'avortement.

Mots-clés: avortement, culture cellulaire, Chlamydia, Isolement, petits ruminants

INTRODUCTION

The family Chlamydiaceae formerly had two genera (*Chlamydia* and *Chlamydophila*), but as this type of classification is not used consistently in the scientific literature, it was recently proposed to transfer all the *Chlamydophila* species to the genus *Chlamydia* (Vos et al., 2011). This gram-negative bacterium multiplies in the cytoplasm of eukaryiotic cells in a unique cycle of development (Rodolakis, 1998). Sheep and goat abortion is one of the most important annual economic losses in Iran, which has a significant effect on meat production and herd health status (Esmaeili et al., 2015). Chlamydiosis is one of the most common causes of abortion in small ruminants worldwide, and the few studies performed in Iran indicate the high frequency of chlamydial infection in flocks. Ghorbanpoor et al. (2007) reported *Chlamydia abortus* infection in 8.9% of the aborted sheep in Ahvaz Province, Iran (Ghorbanpoor et al., 2007). Esmaeili et al. (2015) found *Chlamydia abortus* as the cause of 25.6% of abortions in small ruminants across 11 provinces of Iran (Esmaeili et al., 2015). Despite vaccination, abortion caused by *Chlamydia abortus* is a constant problem in those countries with small ruminant industry; for instance, in the UK, 45% of abortions are caused by *Chlamydia abortus* (Longbottom and Coulter, 2003). Infection with this agent is a great concern for public health (Berri et al., 2009). *Chlamydia abortus* may cause abortion and influenza-like illness in humans (OIE, 2012). Chlamydiosis is routinely diagnosed by direct smears stained with modified Ziehl-Neelsen's (MZN) stain, but this method is neither sensitive nor specific, and because of the similarity of chlamydial appearance with *Brucella* and *Coxiella burnetii*, there is a possibility of misdiagnosis (Berri et al., 2009). For laboratory confirmation, the isolation of *Chlamydia* is necessary, which is possible with inoculation onto cell culture monolayers, into the yolk sac of embryonated chicken eggs, or into mice (OIE, 2012). The gold standard method for *Chlamydia* isolation is inoculating the bacterium into embryonated eggs or cell culture, and according to the World Organization for Animal Health, the latter is the method of choice. *Chlamydia* can be isolated by using variable cell lines, but baby hamster kidney (BHK), buffalo green monkey (BGM), and McCoy are most commonly applied for this purpose (OIE, 2012). To our knowledge, there are no chlamydial cell culture isolates from small ruminants in Iran, and in the present study, this method was used for the first time. Although there are several diagnostic methods for chlamydial identification, cell culture isolation is the gold standard, and we attempted to isolate *Chlamydia* in order to elucidate the current state of chlamydial infection in samples from freshly aborted small ruminants.

MATERIALS AND METHODS

Sample collection. Twenty-eight vaginal and conjunctival swabs were taken from the small ruminants that had aborted in the last month of pregnancy. These animals were clinically suspected of enzootic abortion, as well as examined for *Brucella* infection according to the Iran Veterinary Organization (IVO) protocol using Rose Bengal, Wright, and 2-mercaptoethanol tests (IVO). The samples belonged to 14 sheep and 14 goats from nomadic and rural flocks in five provinces (Table 1). Sucrose-phosphate-glutamate (SPG) buffer was used for transporting the collected samples. It consists 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 the 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 50 mg/l amphotericin B (Bristol-Myers Squibb, France). Each swab was immersed in 2 ml SPG buffer and kept in -20 °C.

Cell culture inoculation. McCoy cell line (National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) was used for sample inoculation and *Chlamydia* isolation (OIE, 2012). McCoy cells were grown in the 1 dram shell vial containing a circular coverslip 2 cm in diameter. The cell culture vials were incubated for 24-

Table 1. Clinical specimens taken from ewes and does from different provinces and the characteristics of *Chlamydia*-positive specimens regarding the sample type and their host

Province	Animal	Samples			Positive		
		Conjunctival Samples	Vaginal Samples	Total	Conjunctiva	Vagina	Total
Fars	Doe	4	3	7	2	1	3
	Ewe	2	0	2	0	0	0
Lorestan	Doe	0	4	4	0	1	1
	Ewe	2	3	5	1	1	2
West Azerbaijan	Doe	0	1	1	0	0	0
	Ewe	1	1	2	0	0	0
Bushehr	Doe	0	1	1	0	0	0
	Ewe	0	0	0	0	0	0
Khusestan	Doe	1	0	1	0	0	0
	Ewe	3	2	5	1	0	1
Total		13	15	28	4	3	7

48 h at 37 °C, and then the samples were inoculated into four shell vials containing McCoy cells at the bottom. The vials were centrifuged at 1000 × *g* for 30 min in order to enhance the attachment of chlamydial elementary bodies to the host cells. Thereafter, the supernatants were discarded and replaced with cell culture media containing 1 mg/l cyclohexamide (Sigma Chemical Co., St. Louis, MO) and 10% fetal calf serum.

Staining of coverslip and inclusion body identification. The cultured cells were examined at two-day intervals. The first monolayer culture medium was removed at day 4 or 5 after inoculation and washed with sterile phosphate-buffered saline (PBS) once. The inoculated cells on the coverslips were fixed with aceton-methanol (Merck KGaA, Germany; 50:50, v:v) for 5 min and stained with Giemsa staining as previously described. The coverslips were then removed from the vials and mounted on a slide using Entellan (Merck KGaA, Germany) and examined microscopically under high power field for chlamydial inclusion bodies. The cultured cells that appeared to be negative at 7 days post-inoculation were re-passaged with the same method as described above.

RESULTS

All the ewes and does were negative for brucellosis. After McCoy cell culture inoculation of 28 clinical samples, 7 (25%) specimens were positive for *Chlamydia* infection (Figure 1). Four positive samples belonged to does and three of them belonged to ewes (Table 1). According to Table 1, *Chlamydia* was isolated from four conjunctival and three vaginal samples (Table 1).

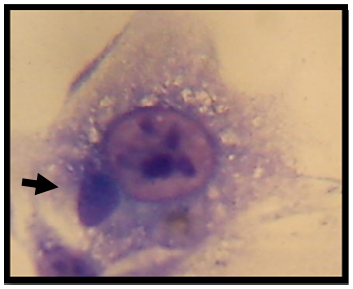

Figure1. Perinuclear cytoplasmic inclusion body (arrow) of Chlamydial agent isolated from a vaginal swab sample of a doe × 1000, Giemsa

DISCUSSION

In the present study, *Chlamydia* spp. were isolated from specimens belonging to ewes and does with the history of recent abortion. While there are numerous reports of *Chlamydia* isolation from small ruminants in the world (Kaltenboeck et al., 2005; Berri et al., 2009) the microbiological and epidemiological studies of chlamydiosis in animals are scarce in Iran. Tabatabayi and Nadalian (1981) reported keratoconjunctivitis due to chlamydial infection in a sheep flock in Iran. They isolated the *Chlamydia* spp. using embryonated chicken eggs and mice (Tabatabayi and Nadalian, 1981). The first isolation of *C. psittaci* from birds in Iran has been

recently reported in 2011. Like our study, the researchers inoculated samples into McCoy cells and used Giemsa staining for identification. In that study, only 4 out of 17 samples were positive for chlamydial inclusions in the cell culture (Madan et al., 2011). *Chlamydia* spp. were isolated from ruminants in different countries (Amin, 2003)Domeika et al., 1994), but since the sensitivity of isolation methods was low, there were a few positive samples in results of most studies. Amin (2003) isolated *Chlamydia* in BHK monolayer cells from ruminant semen samples in Egypt. His study indicated that 10, 4, and 7 semen samples from cows, buffalos, and rams were positive, respectively (Amin, 2003). In a study concerning the ruminant chlamydiosis in France, *Chlamydia* spp. were isolated from only 2 out of 20 clinical samples (Berri et al., 2009). Different immune staining methods such as immunoperoxidase (IP), immunofluorescence (IF), and simple chemical staining like methylene blue or Giemsa staining can be applied to identify the chlamydial agents in the cell culture system (Woodland et al., 1978). In this study, Giemsa staining was employed to visualize the characteristic perinuclear chlamydial inclusions in monolayer cells. This is an easy, quick, and convenient method for identification of chlamydial agents in cell culture (Yoneda et al., 1975). However, regarding the low sensitivity of the Giemsa staining, it is possible to miss some positive samples during the isolation and identification procedures. We tried to lower this possibility by parallel inoculation of each sample into four cell cultures and two blind passages of each negative sample. Isolation is the gold standard for the diagnosis of chlamydiosis, and nowadays, cell culture is the method of choice for this purpose (OIE, 2012). However, as isolation is a difficult, time consuming, labor-intensive, and hazardous method (Berri et al., 2009), its use is limited to the research domain or advanced specialized laboratories (Rodolakis, 1998). Another disadvantage of cell culture isolation is the requirement for live agents, but the bacteria may not survive during the sampling, transportation, and isolation processes in the laboratory (Vanrompay, 2000). In the present study, all the swab samples were transferred immediately into the SPG buffer and then transported to the laboratory on frozen ice packs. However, loss of some of the live organisms during the long distance transportation is possible. Despite the mentioned disadvantages, *Chlamydia* isolation in cell culture is identified as the most convenient method and is essential to document organism viability. Our samples were taken from animals with the history of recent abortion. The clinical signs due to different abortive agents can be very similar (IVO, 2014), and as it has been shown in the present study, *Chlamydia* could play a relatively significant role in the abortion in does and ewes in Iran. To prevent adverse effects of abortifacient pathogens, rapid and definitive diagnosis is required. Nonetheless, in most studies, the main causes of abortion were merely detected in 44% of cases (Leon-Vizcaino et al., 1987). Serologic tests and detection of chlamydial agents using direct smears from clinical samples are usually the only available methods of chlamydial diagnosis in most diagnostic laboratories. Unfortunately, these diagnostic methods are not sensitive and specific enough (Rodolakis, 1998), and their application might lead to misdiagnosis and wrong decisions. Molecular procedures are easy, fast, and sensitive methods for the detection of *Chlamydia*, but because of cross-reaction, detection of dead agents, and contamination of samples, false positive results may occur (De Puysseleyr et al., 2014). Ovine enzootic abortion may cause significant financial losses. It was shown that even in countries that applied *Chlamydia* vaccination in small ruminants, chlamydiosis could cause significant losses due to abortion; for instance, in the UK, the single most common cause of abortion in sheep is *Chlamydia abortus* (Longbottom and Coulter, 2003). In 2015, a serological study in Iran showed that 81.4% of flocks had at least one infected animal (Esmaeili et al., 2015). These results indicated the presence of *Chlamydia abortus* in flocks and the risk of OEA in healthy animals. According to the economic importance of chlamydial abortion and its zoonotic

implications, it is necessary to pay more attention to this bacterial infection in flocks of small ruminants.

In the present study, isolation of *Chlamydia* confirmed the ovine enzootic abortion infection in the studied flocks. Regarding the difficulties in cell culture isolation of *Chlamydia*, we cannot recommend it as a routine diagnostic method, but according to our findings, both clinicians and microbiologists should be aware of the possible role of chlamydial agents in abortion, particularly in brucellosis-free flocks. On the other hand, it should be mentioned that the dual infection with both abortive bacteria is quite possible in ruminants. Finally, the authors highlight the importance of national strategies to monitor and control the chlamydial infections in flocks of small ruminants in Iran.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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