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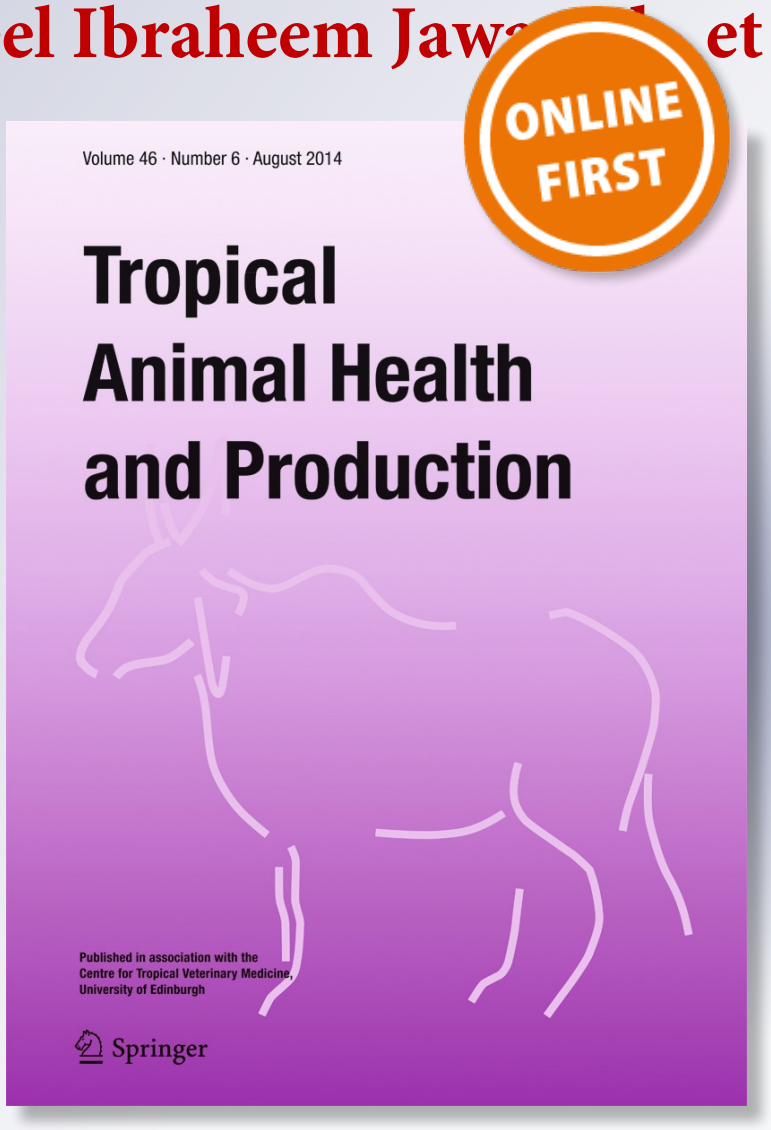

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REGULAR ARTICLES

Molecular identification of chlamydial cause of abortion in small ruminants in Jordan

Huthaifa Salah Ababneh · Mustafa Mohammed Kheir Ababneh · Wael Mahmoud Hananeh · Fawzi Mohammad Alsheyab · Khaleel Ibraheem Jawasreh · Moath Ahmad Al-Gharaibeh · Mohammed Mahmoud Ababneh

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Abstract *Chlamydophila abortus* (*Ch. abortus*) is the etiological agent of ovine enzootic abortion (OEA) and one of the most common infectious agents of abortion in small ruminants worldwide. RFLP-PCR analysis of the outer membrane protein gene (*OMP2* gene) was used for diagnosis and characterization of chlamydial causes of abortion in small ruminants in Jordan. Sixty-six placental tissues and 15 vaginal swabs were collected from aborted ewes and does to identify cause of abortion in Jordan. Thirty-eight placental samples (58 %) and 13 vaginal swabs (87 %) were positive for chlamydial DNA. Shedding of bacteria in vaginal swabs was detected within 7 days after abortion. The results of this study showed that chlamydiosis is one of the important causes of abortion in small ruminants in Jordan. In addition, vaginal swab is an excellent sample for molecular diagnosis of chlamydiosis. DNA sequencing and RFLP analysis of the *OMP2* reveal that all chlamydial cause of abortion in small ruminants in Jordan are due to *Ch. abortus*. While, *Ch. pecorum* was not detected in any sample. *OMP2* gene of the isolated Jordanian strain was identical (100 %) to *Ch. abortus* FAS strain. In conclusion, *Ch. abortus* is an important cause of abortion in Jordan; vaginal swab within 7 days of abortion can be used for molecular diagnosis of chlamydiosis in small ruminants.

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Introduction

Chlamydophila is an obligate, intracellular gram-negative bacterium that causes a broad range of diseases in both human and animals. RFLP-PCR test based on 16S–23S ribosomal intergenic region was used to identify *Chlamydiaceae* species; according to this phylogenic analysis, the family *Chlamydiaceae* consists of two genera: *Chlamydia* (*C.*) and *Chlamydophila* (*Ch.*), and nine species (Longbottom et al. 2002). The most economically important chlamydial agent in small ruminants is *Chlamydophila abortus* (*Ch. abortus*; previously classified as *Chlamydia psittaci* immunotype 1) (Aitken and Longbottom 2007; Stuen and Longbottom 2011). *Ch. abortus* is the etiological agent of ovine enzootic abortion (OEA) and one of the most common infectious agents of abortion in small ruminants worldwide. In sheep, the disease is usually manifested as abortion in the last 2 to 3 weeks of gestation. While goats can abort at any stage of pregnancy, most abortions occur during the last 2 to 3 weeks of gestation (Matthews 1999; Nietfeld 2001). Another member of the genus *Chlamydophila* is *Chlamydophila pecorum*. *Ch. pecorum* is commonly isolated from the digestive tract of clinically inconspicuous ruminants (Mohamad and Rodolakis 2010). Although *Ch. pecorum* had been isolated from placenta and vaginal fluid of aborted small ruminants, involvement of *Ch. pecorum* in small ruminant's abortion is still unclear (Berri et al. 2009). In addition, *Ch. pecorum* has been implicated as a cause for infertility, conjunctivitis, arthritis, mastitis, and pulmonary inflammation in sheep, goats, and cattle (Berri et al. 2009). Human infection of *Ch. abortus* can be acquired when dealing with contaminated sources such as

placentas and fetuses. The symptoms in human range from influenza-like symptoms to life-threatening illness and abortion in pregnant women (Pospischil et al. 2002; Stone et al. 2012). Vaccination is the best option in controlling OEA (O.I.E 1996). Currently, two types of chlamydial vaccines are commercially available; inactivated and live attenuated. Both vaccines have been documented to prevent abortion and to reduce transmission (O.I.E 1996; Longbottom and Livingstone 2006).

Direct examination, bacterial isolation, serological and molecular tests have been described for diagnosis of chlamydiosis. Serological testing is the most commonly used technique for diagnosis and epidemiological studies. In fact, complement fixation test is recommended by the World Organization for Animal Health (OIE). However, vaccinations against *Ch. abortus* may interfere with serological investigations. Molecular techniques such as conventional and real-time PCR have been used to identify *Ch. abortus* in abortion cases. Amplification of the outer membrane protein 2 gene (*OMP2*) was found to be a reliable procedure to diagnose *Ch. abortus* infection. Furthermore, enzymatic digestion of the PCR product can further differentiate *Ch. abortus* from *Ch. pecorum* (Hartley et al. 2001; Marsilio et al. 2005).

Majority of abortion cases in small ruminants are reported in the last month of pregnancy. Infectious diseases such as brucellosis, toxoplasmosis, and chlamydiosis are the usual suspects in Jordan. These diseases can be of considerable economic importance to the sheep and goat industries as well as public health due to their zoonotic nature (Aldomy et al. 2009). Chlamydiosis in small ruminants in Jordan has been documented serologically (Al-Qudah et al. 2004). But, their contribution in abortion has not been investigated. Therefore, the aims of this study were to identify chlamydial cause of abortion in small ruminants in Jordan using RFLP-PCR analysis and DNA sequencing of the *OMP2* gene.

Materials and methods

Animals

Flocks of sheep (n=17) and goats (n=5) with recent cases of abortion in the northern parts of Jordan were used in the current investigation.

Placental and vaginal sample collection

A total of 66 placental samples (cotyledons and intercotyledonary areas) from aborted ewes (n=46) and does (n=20) were collected within 24 h of abortion in plain tube and stored at −80 °C until DNA extraction and PCR analysis. In addition, vaginal swabs were collected from aborted ewes (n=9 from three flocks) and does (n=6 from two flocks) within 7 days after abortion. Swabs were placed in transport medium (Vircell microbiologists, Spain) and kept on ice. Transport medium containing swabs were centrifuged at 10,000 rpm at 4 °C for 3 min. Thereafter, pellets were stored at −80 °C until used for DNA extraction and PCR analysis.

DNA isolation

Total DNA was isolated from placenta and vaginal pellet using DNeasy Blood and Tissue Kit (Qiagen Inc., Mississauga, Canada) according to the manufacturer's instructions. Genomic DNA was visualized in 1 % agarose gel to ensure its quantity and quality. Extracted DNA was stored at −20 °C until PCR analysis.

PCR analysis

Detection of chlamydial DNA was performed by amplification of the *OMP2* with specific forward and reverse primer, F: 5′-ATG TCC AAA CTC ATC AGA GGA G-3′ R: 5′-CCT TCT TTA AGA GGT TTT ACC CA-3′. The PCR was performed in a total reaction volume of 20 μl containing 3 μl of template, 4 μl of 5× Firepol master mix (Solis Biodyne, Tartu, Estonia) and 10 pmol of each primer. Thermal cycling conditions were initial denaturation 94 °C for 2 min; followed by 40 cycles with 1 cycle consisting of 1 min at 94 °C for denaturation, 1 min at 56 °C for primer annealing and 1 min at 72 °C for extension. The last cycle included 5-min incubation at 72 °C for final extension. Positive control of *Chlamydophila* DNA (Vircell microbiologists, Spain) and negative control of nontemplate control was performed with each PCR run. The amplified DNA product was analyzed by agarose gel electrophoresis, and the product was visualized under UV light.

Purification of amplified DNA

Amplified DNA was purified from agarose gel using PCR purification kit (Jena Bioscience GmbH, Germany) according to the manufacturer's instructions. Then, the purified product was stored at −20 °C until DNA sequencing and RFLP analysis.

DNA sequencing

The purified PCR amplicons were sent for sequencing (Macrogen, Korea) to confirm the PCR results. Sequences were viewed with BioEdit software and aligned using the MegAlign software of the DNASTAR package. Nucleotide identity and phylogenetic analysis were done with the above-mentioned software.

RFLP-PCR analysis

PCR-RFLP was used to differentiate *Ch. abortus* from *Ch. pecorum* using the amplified *OMP2* gene. This method is able to differentiate *Ch. abortus* from *Ch. pecorum* by

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Ch.abortus Jordan strain TCCTCGCGCTAACTAGTATGGCGAGTTCATTTGCCAGCGGGAAGATAGAGGCCGCTGCTG
Ch.abortus FAS strain    TCCTCGCGCTAACTAGTATGGCGAGTTCATTTGCCAGCGGGAAGATAGAGGCCGCTGCTG

Ch.abortus Jordan strain CAGAGTCTCTTGCTACAAGATTCATTGCCAGTACCGAAAACTCAAATGACAATGTTTTGC
Ch.abortus FAS strain    CAGAGTCTCTTGCTACAAGATTCATTGCCAGTACCGAAAACTCAAATGACAATGTTTTGC

Ch.abortus Jordan strain AAGCAACAGCCAAGAAAGTTAGATTTGGTCGTAACAAAAATCAAAGACAAGAACAAAAAC
Ch.abortus FAS strain    AAGCAACAGCCAAGAAAGTTAGATTTGGTCGTAACAAAAATCAAAGACAAGAACAAAAAC

Ch.abortus Jordan strain ATACTGGCGCTTTCTGTGATAAAGAATTTTATCCTTGCGAAGGTGGTCAGTGCCAATCCG
Ch.abortus FAS strain    ATACTGGCGCTTTCTGTGATAAAGAATTTTATCCTTGCGAAGGTGGTCAGTGCCAATCCG

Ch.abortus Jordan strain TCGATACTACACAAGAATCTTGCTACGGCAAAATGTATTGTGTCCGTGTTAACGATGACT
Ch.abortus FAS strain    TCGATACTACACAAGAATCTTGCTACGGCAAAATGTATTGTGTCCGTGTTAACGATGACT

Ch.abortus Jordan strain GTAACGTGGAAATTAGCCAAGCTGTACCTGAATATGCAACAGTAGGATCTCCTTATCCTA
Ch.abortus FAS strain    GTAACGTGGAAATTAGCCAAGCTGTACCTGAATATGCAACAGTAGGATCTCCTTATCCTA

Ch.abortus Jordan strain TTGAAATTCTTGCTGTAGGTAAAAAAGATTGCGTTAATGTTGTGATCACTCAACAACTTC
Ch.abortus FAS strain    TTGAAATTCTTGCTGTAGGTAAAAAAGATTGCGTTAATGTTGTGATCACTCAACAACTTC

Ch.abortus Jordan strain CTTGCGAAGTTGAGTTTGTCAGCAGTGATCCTGCGACAACACCAACCTCAGATAGCAAAT
Ch.abortus FAS strain    CTTGCGAAGTTGAGTTTGTCAGCAGTGATCCTGCGACAACACCAACCTCAGATAGCAAAT

Ch.abortus Jordan strain TAATCTGGACAATTGATTGCTTAGGTCAAGGTGAAAAATGCAAAATTACCGTTTGGGTAA
Ch.abortus FAS strain    TAATCTGGACAATTGATTGCTTAGGTCAAGGTGAAAAATGCAAAATTACCGTTTGGGTAA
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Fig. 1 The alignment of the partial sequence (540 nt) of the *OMP2* gene of the *Ch. abortus* Jordan strain (GenBank number KJ739313) and the reference *Ch. abortus* FAS strain (GenBank number DQ494809). A complete matching is seen in all nucleotide that gives 100 % nucleotide identity between the Jordan strain and the FAS reference strain of *Ch. Abortus*

producing different band patterns using Alu1 restriction enzyme. Alu1 restriction enzyme was used as described by Marsilio et al. (2005). The digested products were visualized on 2 % agarose gel electrophoresis. *Ch. abortus* is expected to yield two bands of 352 and 235 bp while *Ch. pecorum* is expected to yield two bands of 397 and 193 bp.

Results

Detection of *OMP2* gene of *Chlamydophila* in placenta and vaginal swab

Thirty-eight placental samples (58 %; ewes=28, does=10) out of 66 and 15 vaginal swab (87 %; ewes=7, does=6) out of 15 were positive for chlamydial DNA with a PCR product size of 587 bp of the *OMP2* gene. Shedding of bacteria in vaginal swabs was detected within 7 days after abortion. No positive results in the *OMP2* PCR were found after 7 days (data not shown). Placenta and vaginal samples were collected from the same animal in five cases. Of those, placenta and vaginal swab of two animals and vaginal swab but not the placenta in one animal were positive for chlamydiosis. Both the vaginal and placental samples of the last two animals were negative for chlamydiosis.

Sequence of *OMP2* gene of *Ch. abortus* Jordan strain

The PCR amplicon of the partial *OMP2* gene was directly sequenced. A fragment with a size of 540 nt was obtained and aligned with the same fragment of the reference *Ch. abortus* FAS strain (Fig. 1). MegAlign software of the DNA star

Table 1 The nucleotide identity percentages of partial *OMP2* gene of the *Ch. abortus* Jordanian strains (KJ 739313) and other *Ch.* related strains

Ch. strains	*Ch. abortus Jordan strain*	*Ch. abortus strain FAS*	*Ch. felis*	*Ch. caviae*	*Ch. pneumoniae*	*Ch. pecorum*
Ch. abortus Jordan strain (KJ739313)	100	–	–	–	–	–
Ch. abortus strain FAS (DQ494809.1)	100	100	–	–	–	–
Ch. felis (AY286314.1)	90.2	90.6	100	–	–	–
Ch. caviae (AE015925.1)	90.2	90.7	90	100	–	–
Ch. pneumonia (U56925.1)	75	75.7	71.7	76.4	100	–
Ch. pecorum (U56924.1)	80	80.4	75.3	75	72.1	100

The highest nucleotide identified (100 %) is seen between the *Ch. abortus* Jordan strain and the FAS strain while the lowest (75 %) is seen between the *Ch. abortus* Jordan strain and the *Ch. pneumoniae*

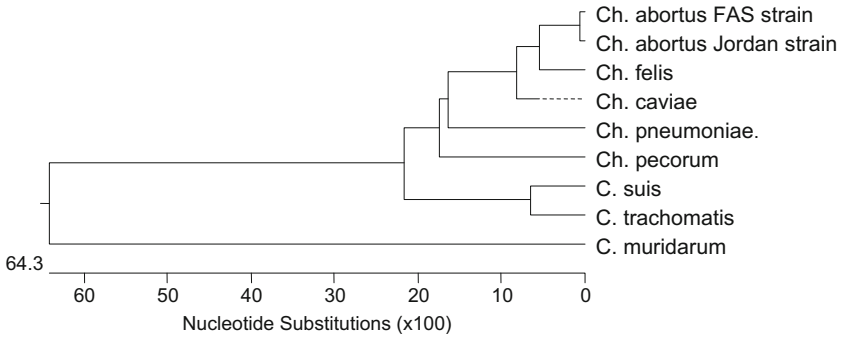

Fig. 2 Phylogenetic tree of the partial *OMP2* gene of the *Chlamydophila abortus* Jordanian strain and other related strains. The *Ch. abortus* Jordan strain lies within the same cluster as the *Ch. abortus* FAS strain

package was used to design the phylogenitic tree. The nucleotide identity percentages of partial *OMP2* gene of the Jordanian strains and other related *Ch.* species are shown in Table 1. *OMP2* gene of the isolated Jordanian strain (GenBank number KJ739313) was identical (100 %) and grouped in the same cluster with *Ch. abortus* FAS strain (GenBank number DQ494809) as shown in Figs. 1 and 2 and Table 1, while the lowest identity (75 %) was between the *Ch. abortus* Jordan strain and the *Ch. pneumoniae*.

Digestion pattern of the PCR amplicon of the *OMP2* gene

PCR-RFLP assay was used to differentiate between *Ch. abortus* and *Ch. pecorum*. Digestion of the PCR amplicon of the 587 bp *OMP2* gene with Alu1 yielded two bands of 235 and 352 bp as presented in 2 % agarose gel electrophoresis image (Fig. 3). The restriction digestion pattern found in all *OMP2* PCR positive (both placental and vaginal) samples indicated that all these samples are infected with *Ch. abortus* and not *Ch. pecorum*.

Discussion

Diagnosis of chlamydial abortion in small ruminants depends on the detection of bacteria in impression smears of the placenta, serological analysis, isolation of *Chlamydophila* or its nucleic acid from aborted animals or vaginal secretions. The result of this study showed that the placenta and vaginal swab within 7 days of abortion were equally suitable samples for PCR detection of *Ch. abortus* in small ruminant. PCR is considered to be the most sensitive diagnostic method for diagnosis of *Chlamydiaceae* in human medicine. *Ch. abortus* can be isolated from the aborting sheep and goat before treatment with antibiotics; however, culture of the pathogen is rarely performed because of technical difficulty and safety. Chlamydiosis is a zoonotic disease, and the causative agent can impair human health during the isolation and identification of *Ch. abortus*. Therefore, molecular assays such as PCR-RFLP offer a safer, fast, and accurate method for detection of chlamydial DNA in cases of abortion. *OMP2* gene shows a high degree of conservation between *Chlamydiaceae* species for most of its sequence. Therefore, amplification of *OMP2* gene alone does not allow the discrimination between chlamydial species. However, analysis of PCR products by RFLP and digestion with *Alu1* is sufficient for species identification. The use of PCR-RFLP in the current study identified the isolates as *Ch. abortus*. Only two bands were generated in PCR-RFLP with *Alu1* digestion. The limited number of fragments expected with the use of Alu1 digestion allow easy discrimination among different chlamydial species (for example, *Ch. abortus* vs *Ch. pecorum*). Several investigators reported that high sensitivity and specificity of RFLP using the Alu1 enzyme (Hartley et al. 2001; Marsilio et al. 2005). Moreover, shedding of *Ch. abortus* through vagina was detected by PCR until 7 days after abortion. Therefore, when the aborted fetus and placenta are lost, vaginal swab is considered to be the optimal sample for diagnosis of chlamydial abortion. Vaginal chlamydial shedding decreases rapidly after abortion, and few chlamydial DNA were detected in the vaginal swabs at the subsequent estrus after abortion (Livingstone et al. 2009). In one animal, *Ch. abortus* was detected in the vaginal swab but not in the placenta. In accordance with the current study, Marsilio et al. (2005) showed that the high collagen content in the placenta could impair DNA quality making vaginal swab a better sample than placenta for PCR (Marsilio et al. 2005).

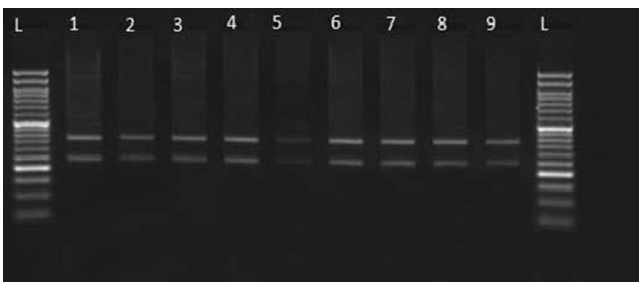

Fig. 3 RFLP-agarose gel electrophoresis image, 1–9: Alu1 digested *OMP2* gene yielding two bands with 235 and 352 bp. This restriction pattern is compatible with *Chlamydophila abortus*. *L*, 50 bp DNA marker. The size of the intense bands in the 50 bp DNA ladder are 200 and 500 bp, respectively

The most important studied causes of infectious abortion in small ruminant in Jordan are Brucellosis (Samadi et al. 2010) and Toxoplasmosis (Abu-Dalbouh et al. 2012). The cause of abortions in the current study was not fully investigated. However, the results of this study showed, for the first time, that *Ch. abortus* is considered to be a major cause of abortion in small ruminants in Jordan. This is supported by the result of an old published serological survey in Jordan (Al-Qudah et al. 2004). In the latter study, all the tested flocks were found to be infected with *Ch. abortus* and the prevalence ranged from 4 to 57 % and 4 to 20 % in sheep and goat flocks, respectively (Al-Qudah et al. 2004). In addition, more than 60 % infection rate was determined serologically in sheep and goat flocks used in the current study (unpublished data). Lack of control strategy of chlamydiosis through vaccination may be the cause behind the high infection rate of chlamydiosis especially in areas where brucellosis is controlled leaving *Ch. abortus* as the major infectious cause of abortion in Jordan.

PCR-RFLP analysis reveals that all chlamydial cause of abortion in Jordan was due to *Ch. abortus*. Restriction with Alu1 gives species-specific band lengths. RFLP yielded two bands of 352 and 235 bp which is consistent with *Ch. abortus*. On the other hand, RFLP of *Ch. pecorum* is expected to yield two bands of 397 and 193 bp. Berri et al. (2009) in Tunisia identified *Ch. pecorum* from the placenta of aborted small ruminants. Rodolakis and Souriau (1989) showed that intradermal or intravenous inoculation of pregnant ewes with *Ch. pecorum* strains isolated from ovine abortion cases did not induce the same abortion rate as *Ch. abortus* (Rodolakis and Souriau 1989). However, the role of *Ch. pecorum* in small ruminants abortion is still unclear; therefore, further studies are required.

The results of this study and other related studies showed that *OMP2* gene is considered as conserved gene (Vladimir and Andrey 2005). Sequencing of the *OMP2* gene in the Jordanian strain was similar with 100 % homology to the reference strain. This is consistent with the findings of Vretou et al. (1996) who reported identical pattern after Alu1 digestion of *OMP2* gene from four different diverse *Ch. abortus* strains. In addition, complete genome sequence of *Ch. abortus* showed high level of conservation in gene sequence in comparison to other members of Chlamydiaceae (Thomson et al. 2005). *OMP2* is responsible for immunization of *Chlamydia* infection (Vladimir and Andrey 2005). Therefore, global *Ch. abortus* vaccine targeting this protein may protect ewes and does against chlamydiosis.

In conclusion, this study showed that *Ch. abortus* plays a substantial role in sheep and goat abortion in Jordan. *OMP2* gene in the Jordanian strain is similar with 100 % homology to the reference strain. Authorities in the ministry of agriculture in Jordan are required to set up appropriate vaccination and epidemiological surveillance to control and eradicate this disease.

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Conflict of interest The authors declare that they have no conflict of interest.

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