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# Isolation and Identification of *Chlamydia abortus* from Aborted Ewes in Sulaimani Province, Northern Iraq

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Abstract

Abortion in small ruminants is a significant problem in Iraq and causes severe economic losses in sheep farms. *Chlamydia abortus* causes enzootic abortion in ewes and is associated with reproductive problems in sheep in Sulaimani province – Northern Iraq. During a lambing season in 2017, abortion was widespread among several sheep flocks in different regions of Sulaimani (Kalar, Said Sadiq, and Chamchamal), and *C. abortus* was one of the causes. Accordingly, we carried out this study to isolate and identify *C. abortus* in aborted ewes in these regions. We collected 30 samples of aborted fetuses from five herds in which abortions had been observed. The pathogen isolation was done by inoculation into embryonated chicken eggs and conventional PCR was used to identify *C. abortus* in clinical specimens. *C. abortus* was identified in one of the 30 aborted fetuses (3.33%) from the Kalar district, and all the remaining 29 samples (96.66%) were found positive to *Brucella abortus*. The gene *ompA* encoding the outer membrane protein of *C. abortus* was sequenced and got the accession number MK643153 in NCBI GenBank. The sequence was named *C. abortus* strain Sul/2017. Our isolate showed 99.79% homology with Sul/014 (accession No. KY399850) and differed from the latter by two amino acid substitutions at E115K and K259N. The topology of the phylogenetic tree based on the *ompA* gene showed that the isolate belongs to *C. abortus* and has a common ancestor with isolates of sheep in Iraq and Tunisia with accession numbers KY399850 and HQ62243, respectively.

Key words: enzootic abortion in ewes, *Chlamydia abortus*, embryonated egg, polymerase chain reaction

## Introduction

*Chlamydia abortus* (family: *Chlamydiaceae*) is a non-motile, coccoid, pleomorphic, Gram-negative bacterium. It is an obligate intracellular parasite that causes different diseases in humans and animals (Everett et al. 1999, Madico et al. 2000, Silva et al. 2006).

Ovine chlamydiosis is also called ovine enzootic abortion (OEA) or enzootic abortion of ewes (EAE). It is one of the most significant causal agents of reproductive failure in small ruminants, which is induced by *C. abortus* (DeGraves et al. 2004, Spičic et al. 2015). The pathogen efficiently colonizes the placental trophoblasts (Borel et al. 2006, Campos-Hernández et al. 2014).

Chlamydiosis causes abortion in sheep and goats in the final 2–3 weeks of pregnancy with the appearance of a stillborn and grossly inflamed placenta (Nietfeld 2001, Walder et al. 2003). Also, *C. abortus* constitutes a hazard to pregnant women, as many cases have been reported, which were caused by *Chlamydia* (Pospischil et al. 2002, Walder et al. 2005).

Abortion in the late stages of gestation by *Chlamydia* results in severe losses in many sheep-raising areas globally, especially where herds are intensely crowded during the parturient period (Halbert 2008, Rodolakis and Mohamad 2010). The disease can lead to the delivery of full-term stillborn lambs. Some of the delivered lambs fail to survive longer than two days. It is also common for an infected ewe to deliver one dead lamb and one or more weak or healthy lambs during multiple births. Infection usually spreads into a new flock by the introduction of infected animals. The disease usually causes a small number of abortions in the first year. In the next year, the rate may increase to infect around 30% of the ewes (Longbottom and Coulter 2003).

Etiologic diagnosis of ovine chlamydiosis depends on bacteriological testing on embryonated chicken eggs and chlamydial DNA amplification by polymerase

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chain reaction (PCR) (Sachse et al. 2009, Szymanska-Czerwinska et al. 2013).

Infections of *C. abortus* in sheep have generally been documented serologically in Sulaimani province, in the north of Iraq. However, studies on pathogen isolation and detection by PCR are rare in this region. Therefore, we conducted this study to isolate *C. abortus* in sheep in Sulaimani province, Iraq using egg-inoculation and PCR molecular techniques. We also conducted a phylogenic analysis to compare our isolate with other *Chlamydia* species that were deposited in GenBank using the *ompA* gene as a comparison.

## Experimental

### Materials and Methods

**Study area and sample collection.** During the birth season of 2017, March to May, abortion in sheep flocks was reported by veterinarians in several regions of Sulaimani province in the northeast of Iraq. The farms had a history of abortion in the previous seasons. The management system of sheep herds in these districts is classical; the animal herds belong to different owners. The sheep are fed indoor on grain, hay, and silage and are left to graze on pasture. Different animal species may graze together on the same pasture or share rams for fertility between herds. Farmers in these areas rear local breed or Arabic (Awasi) sheep.

We collected 30 samples of aborted fetuses from five herds of nonvaccinated sheep in different regions around Sulaimani province. Fifteen samples were from Kalar, ten from Said Sadiq, and five from Chamchamal (Fig. 1). The samples composed of tissue collected by using disposable blades and scissors from recently aborted fetuses (liver, spleen, and lung) and their dams (placental caruncle and cotyledon) that had abortions in the previous 2–4 days. Collected samples were put in plastic containers, labeled, and transferred in a cooled box to the Research Center at the College of Veterinary Medicine/University of Sulaimani for isolation and identification of *C. abortus*.

**Isolation of *C. abortus*.** Tissue samples from cotyledons, placentas, and fetal organs were collected and processed for egg inoculation. Homogenization of the tissues was accomplished by grinding using a sterile pestle and mortar. The homogenized tissues were suspended in the sucrose-phosphate-glutamate (SPG) medium containing sucrose (0.22 mM), $K_2HPO_4$ (7.1 mM), L-glutamic acid (4.9 mM), fetal bovine serum (10%), streptomycin (100 µg/ml), gentamicin (50 µg/ml), and nystatin (50 µg/ml) to make a 10% suspension. The suspension was centrifuged at 2000 rpm for 5–10 minutes. After that, the supernatants were collected and aliquoted in small volumes. Part of the supernatant was used directly for the isolation of *C. abortus* in embryonated eggs, and McCoy cell or the extraction of DNA, and the remainder was stored at –70°C.

For isolation of *C. abortus* in the yolk sac membranes of chicken eggs, specific-pathogen-free, 6 to 8-day-old embryonated hen's eggs were inoculated with the tissue suspension of the aborted animals.

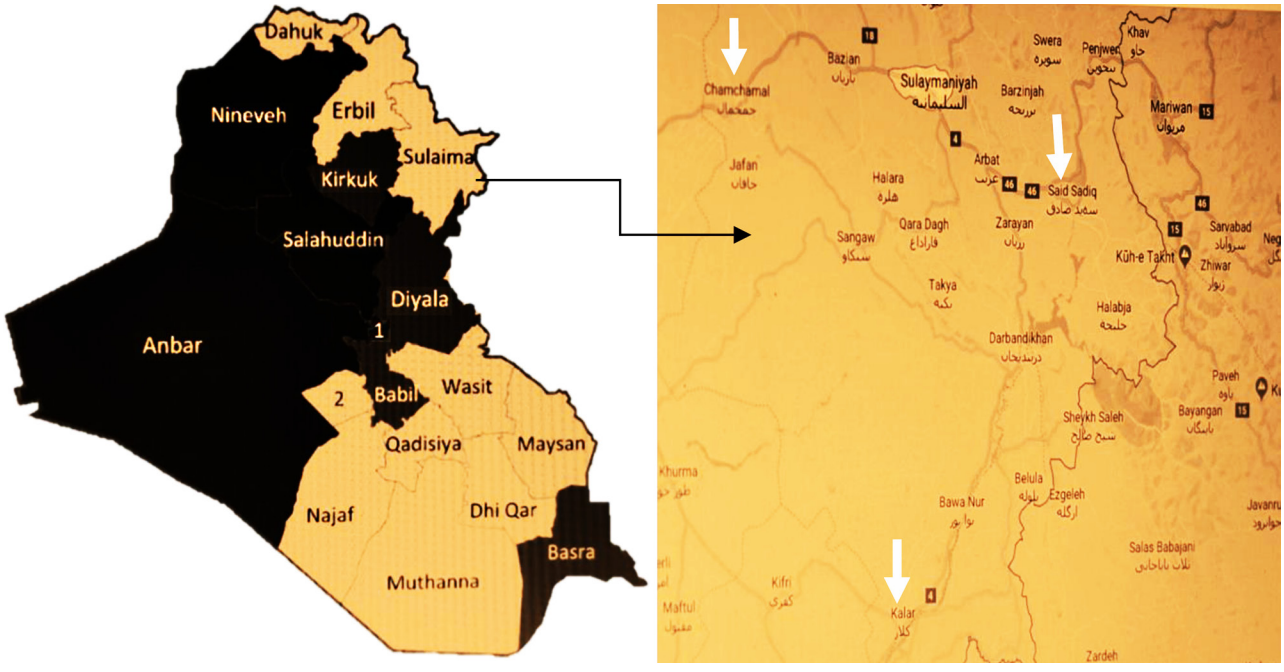



Fig. 1. Map of Iraq with Sulaimani province, showing the districts, Kalar, SaidSadiq, and Chamchamal, where samplings were carried out.

Table I
The targeted genes and PCR primers used for the detection of *Chlamydia abortus* and *Brucella abortus* in aborted ewes.

| Target gene | Primer name | Primer sequence (5'–3') | Amplified fragment length (bp) | Reference |
|---|---|---|---|---|
| *ompA* | omp-F | ATGAAAAAACTCTTGAAATCGG | 1058 | Arshi et al. 2011 |
| | omp-R | CAAGATTTTCTAGACTTCATTTTGTT | | |
| *bcsp31* | B4-F | TGGCTCGGTTGCCAATATCAA | 223 | Baily et al. 1992 |
| | B5-R | CGCGCTTGCCTTTCAGGTCTG | | |

After six days of incubation, the vitality of the egg to be inoculated was checked with a candling lamp. Candling (holding an intense light below the egg to observe the embryo in a darkened room) was used to determine and mark the location of the embryo and egg's air sac. The shell was cleaned with 70% ethanol, and the air sac was marked with a pencil, followed by drilling a small hole into the shell over the top of the air sac. After injecting about 0.2 ml of the inoculums (10% suspension of tissue), the hole was sealed with nail varnish (Soomro et al. 2012). The eggs were incubated at 37°C and candled daily. Eggs that showed embryonic death during 4–10 days after inoculation were kept while the eggs in which the embryo died within 24 hours after inoculation were discarded. Eggs containing dead embryos were kept overnight at 4°C, and the yolk sac membranes were harvested using aseptic techniques in a biosafety cabinet after the rinsing the eggshells with alcohol. The yolk sac was washed two times with pH 7.2 phosphate-buffered saline and cut into small pieces. The pieces were then ground with a pestle and mortar to prepare a cell suspension, as described earlier (Li et al. 2015). The presence of *C. abortus* was confirmed by staining impression smears with Giemsa. The smears were prepared from highly vascularized areas of the yolk sac membranes of chicken eggs. The smears were then fixed by absolute methanol and stained with Giemsa solution. The stained slides were examined with a light microscope using 1000× magnification for the presence of purple inclusion bodies (Dagnall and Wilsmore 1990).

**DNA extraction.** The samples were subjected to DNA extraction using a DNA extraction kit (GeNet Bio, South Korea). The procedure was conducted following instructions provided by the manufacturer.

**Oligonucleotides and PCR amplification.** We used a set of genus-specific primers for discrimination of *C. abortus* and *Brucella abortus* from common infectious agents that cause abortion in sheep. The omp-F and omp-R primers were designed from the *ompA* gene sequence of *C. abortus,* which encode an amplicon size was 1058 bp and were previously used by Arshi et al. (2011), while B4-F and B5-R primers were designed from the *bcsp31* gene sequence of *B. abortus*. Primers encode an amplicon of 223 bp (Baily et al. 1992). The name, sequence, target gene, the predicted amplified fragment, as well as the melting temperature, are listed in Table I The primers were developed by Macrogen® (South Korea) for our study.

The total DNA was subjected to simplex PCR amplification using PCR PreMix (GeNet Bio, South Korea). The reaction was executed in 0.2 ml PCR tubes. The constituents of the PCR tube were 10 µl master mix, 4 µl DNA, and 1 µl (10 pmol) of each of the forward and reverse primers. The final volume of 20 µl was accomplished by adding 4 µl diethylpyrocarbonate (DEPC)-treated water.

The thermal cycler program was started with denaturation at 95°C for five minutes. After that, 40 cycles of denaturation (95°C for 30 seconds), annealing at 58°C for 30 seconds for *C. abortus* and 57°C for *B. abortus*, and extension (72°C for 45 seconds) were run with the final extension at 72°C for five minutes.

The PCR products were examined by loading 7 µl on 1% agarose gel in 1 × Tris/Borate/EDTA (TBE) buffer. The gel was stained with 5 µl Safe dye, and electrophoresis was done using 120 volts for 50 minutes. The Safe-Blue Illuminator/Electrophoresis System was used to visualize the bands of amplicons, which were analyzed based on the pattern of migration by comparing them to a 100 bp DNA ladder.

**Sequencing of the *ompA* gene and phylogenic analysis.** The PCR products of the *C. abortus ompA* gene were sequenced in the Macrogen Sequencing Facility in South Korea. Sequencing was performed several times to verify the identity of each nucleotide, and the gene sequences were presented to GenBank and got the accession number MK643153.

Phylogenic trees were produced using the *ompA* genes of 75 strains of *Chlamydia* species (Fig. 3). The sequences were obtained from GenBank and MLST (http://pubmlst.org/chlamydiales/) websites for *Chlamydiales*. Multiple alignments were done with the Clustal W method (Thompson et al. 1994), and MEGA 7 was implemented to execute a phylogenetic analysis with Neighbor-Joining. The bootstrap measures were determined from 1000 repeats of the original data.

Table II
Detection of *Chlamydia abortus* and *Brucella abortus* in different herds of sheep from three districts in Sulaimani province by PCR.

| Name of district | Number of samples collected | Number positive for *C. abortus* (%) | Number positive for *B. abortus* (%) |
|---|---|---|---|
| Kalar | 15 | 1 (6.66) | 14 (93.33) |
| Said Sadiq | 10 | 0 | 10 (100) |
| Chamchamal | 5 | 0 | 5 (100) |
| Total | 30 | 1 (3.33) | 29 (96.66) |

## Results

**Samples.** In the current study, 30 samples were taken from aborted fetuses from different herds of sheep from three districts in Sulaimani. One sample (3.33%) from aborted ewes was found positive for *C. abortus* both by yolk sac inoculation and PCR (Table II). All the remaining 29 samples were positive to *B. abortus* (96.66%).

**Inoculation of embryonated eggs.** Examination of embryonated eggs revealed the death of chick embryo 4–5 days after inoculation, and the infected yolk sacs were thin-walled, and their blood vessels were severely congested (Fig. 2). Yolk appeared as a bright-colored liquid, and the growth of the embryo was stunted. Embryos were suffering from curled toes, and their bodies were covered with hemorrhage. Only one impression smear of the yolk sac membrane of an egg was found positive for *C. abortus*. Giemsa stain revealed chlamydial elementary bodies as small stained purple cocci occurring individually and in clusters in the cytoplasm of infected cells.

**Identification of *C. abortus*.** In the present study, *C. abortus* was positive for the *ompA* gene, based on agarose gel electrophoresis, which demonstrated the expected amplicon of about 1058 bp. The results were affirmed by determining the sequence of the PCR product, and the sequence received the accession number MK643153 in NCBI GenBank and was named *C. abortus* strain Sul/2017.

**Sequence analysis.** The sequence of the partial *ompA* gene of *C. abortus* strain Sul/2017 showed 99.89% homology with two isolates from the UK with the accession numbers LN554882 and LN554883. However, the isolate in our study showed 99.79% homology with a previous isolate from Sulaimani (Sul/014, accession No. KY399850). Sequence alignment of Sul/2017 with 100 isolates of *C. abortus* showed that the Iraqi strain had one exclusive single nucleotide polymorphism (SNP, A59050) that caused amino acid substitution A92G in the *ompA* gene. However, Sul/2017 differed from Sul/2014 by two amino acid substitutions at E115K and K259N.

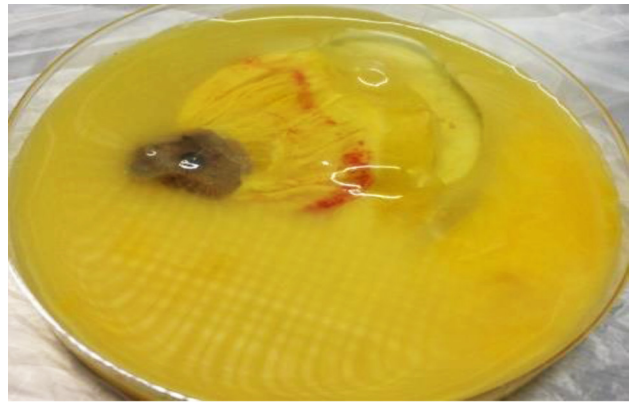
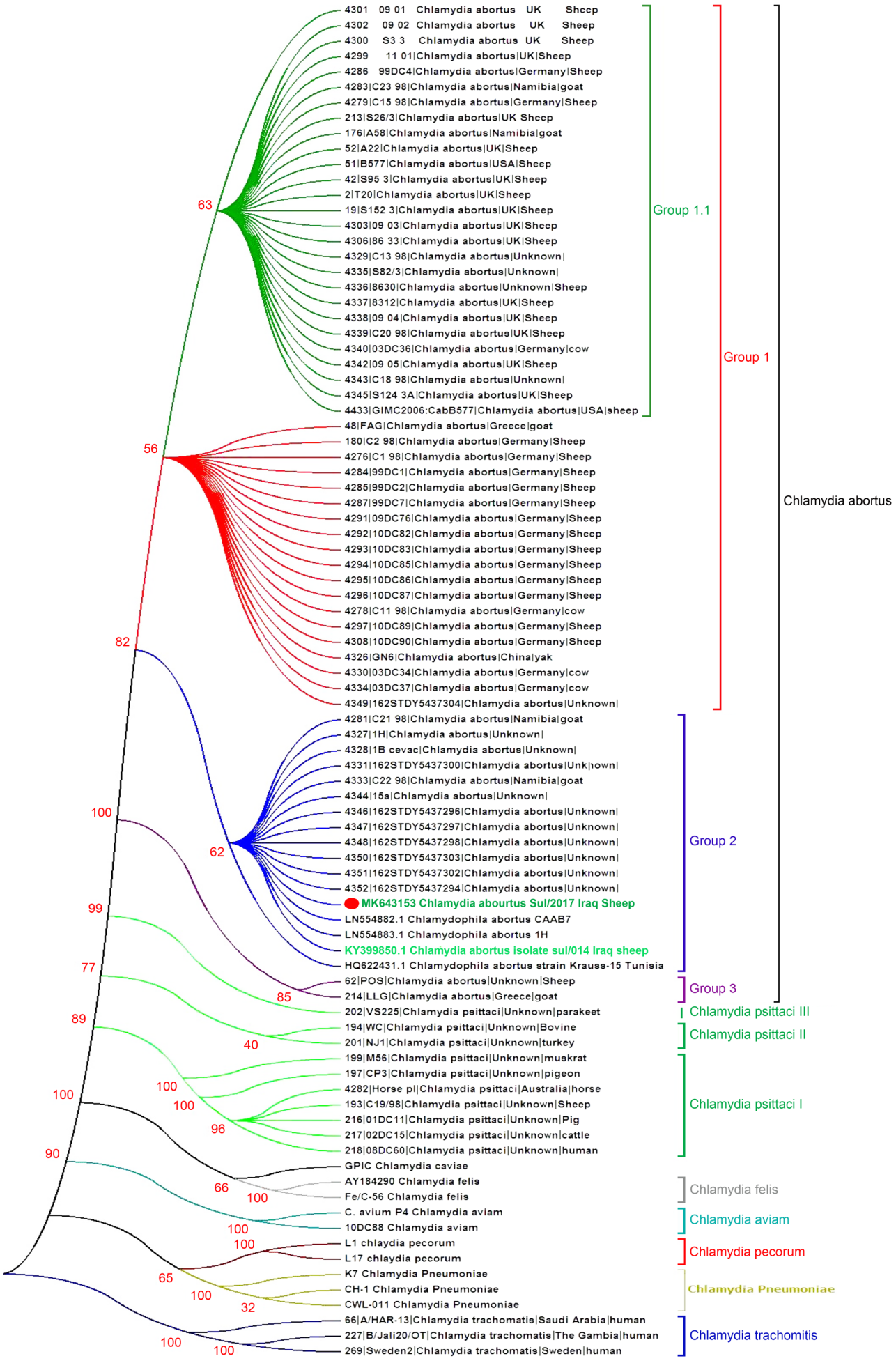


Fig. 2. Embryonated egg showing a dead chick embryo five days after inoculation. The infected yolk sacs were thin-walled, and their blood vessels were severely congested. Yolk appeared as a right-colored liquid, and the growth of the embryo was stunted.

**Phylogenic analysis.** The phylogenetic tree topology, based on the *ompA* gene, showed that *Chlamydia* Sul/2017 from Iraq belonged to *C. abortus* (Fig. 3). The phylogenetic tree revealed that Sul/2017 has a common ancestor with isolates from sheep in Iraq and Tunisia with accession numbers KY399850 and HQ62243, respectively. The partial *omp1* gene of Sul/2017 has been compared with 75 sequences of *Chlamydia* genus that were published in GenBank and MLST websites for *Chlamydiales* (http://pubmlst.org/chlamydiales/). According to the phylogenetic tree, the isolates were distinctly divided into seven clusters; each cluster represented specific species of *Chlamydia* (Fig. 3). The cluster of *C. abortus* was subdivided into three groups. The topology of the phylogenetic tree showed

Fig. 3. Phylogenic trees based on the *ompA* gene showed that the Sul/2017 chlamydia from Iraq belonged to *C. abortus* and revealed that Sul/2017 has a common ancestor, respectively. The partial *ompA* gene of Sul/2017 has been compared with 75 sequences of *Chlamydia* species that were published in GenBank and MLST websites for *Chlamydiales* (http://pubmlst.org/chlamydiales/). The tree shows that Sul/2017 has a common ancestor with isolates of sheep in Iraq and Tunisia with accession numbers KY399850 and HQ62243 and with Sul/2014, CAAB7, H and Krauss-15 isolates that were in a group 2 of *Chlamydia abortus*.


4301 09 01 Chlamydia abortus UK Sheep
4302 09 02 Chlamydia abortus UK Sheep
4300 S3 3 Chlamydia abortus UK Sheep
4299 11 01|Chlamydia abortus|UK|Sheep
4286 99DC4|Chlamydia abortus|Germany|Sheep
4283|C23 98|Chlamydia abortus|Namibia|goat
4279|C15 98|Chlamydia abortus|Germany|Sheep
213|S26/3|Chlamydia abortus|UK Sheep
176|A58|Chlamydia abortus|Namibia|goat
52|A22|Chlamydia abortus|UK|Sheep
51|B577|Chlamydia abortus|USA|Sheep
42|S95 3|Chlamydia abortus|UK|Sheep
2|T20|Chlamydia abortus|UK|Sheep
19|S152 3|Chlamydia abortus|UK|Sheep
4303|09 03|Chlamydia abortus|UK|Sheep
4306|86 33|Chlamydia abortus|UK|Sheep
4329|C13 98|Chlamydia abortus|Unknown|
4335|S82/3|Chlamydia abortus|Unknown|
4336|8630|Chlamydia abortus|Unknown|Sheep
4337|8312|Chlamydia abortus|UK|Sheep
4338|09 04|Chlamydia abortus|UK|Sheep
4339|C20 98|Chlamydia abortus|UK|Sheep
4340|03DC36|Chlamydia abortus|Germany|cow
4342|09 05|Chlamydia abortus|UK|Sheep
4343|C18 98|Chlamydia abortus|Unknown|
4345|S124 3A|Chlamydia abortus|UK|Sheep
4433|GIMC2006:CabB577|Chlamydia abortus|USA|sheep
48|FAG|Chlamydia abortus|Greece|goat
180|C2 98|Chlamydia abortus|Germany|Sheep
4276|C1 98|Chlamydia abortus|Germany|Sheep
4284|99DC1|Chlamydia abortus|Germany|Sheep
4285|99DC2|Chlamydia abortus|Germany|Sheep
4287|99DC7|Chlamydia abortus|Germany|Sheep
4291|09DC76|Chlamydia abortus|Germany|Sheep
4292|10DC82|Chlamydia abortus|Germany|Sheep
4293|10DC83|Chlamydia abortus|Germany|Sheep
4294|10DC85|Chlamydia abortus|Germany|Sheep
4295|10DC86|Chlamydia abortus|Germany|Sheep
4296|10DC87|Chlamydia abortus|Germany|Sheep
4278|C11 98|Chlamydia abortus|Germany|cow
4297|10DC89|Chlamydia abortus|Germany|Sheep
4308|10DC90|Chlamydia abortus|Germany|Sheep
4326|GN6|Chlamydia abortus|China|yak
4330|03DC34|Chlamydia abortus|Germany|cow
4334|03DC37|Chlamydia abortus|Germany|cow
4349|162STDY5437304|Chlamydia abortus|Unknown|
4281|C21 98|Chlamydia abortus|Namibia|goat
4327|1H|Chlamydia abortus|Unknown|
4328|1B cevac|Chlamydia abortus|Unknown|
4331|162STDY5437300|Chlamydia abortus|Unk|own|
4333|C22 98|Chlamydia abortus|Namibia|goat
4344|15a|Chlamydia abortus|Unknown|
4346|162STDY5437296|Chlamydia abortus|Unknown|
4347|162STDY5437297|Chlamydia abortus|Unknown|
4348|162STDY5437298|Chlamydia abortus|Unknown|
4350|162STDY5437303|Chlamydia abortus|Unknown|
4351|162STDY5437302|Chlamydia abortus|Unknown|
4352|162STDY5437294|Chlamydia abortus|Unknown|
MK643153 Chlamydia abourtus Sul/2017 Iraq Sheep
LN554882.1 Chlamydophila abortus CAAB7
LN554883.1 Chlamydophila abortus 1H
KY399850.1 Chlamydia abortus isolate sul/014 Iraq sheep
HQ622431.1 Chlamydophila abortus strain Krauss-15 Tunisia
62|POS|Chlamydia abortus|Unknown|Sheep
214|LLG|Chlamydia abortus|Greece|goat
202|VS225|Chlamydia psittaci|Unknown|parakeet
194|WC|Chlamydia psittaci|Unknown|Bovine
201|NJ1|Chlamydia psittaci|Unknown|turkey
199|M56|Chlamydia psittaci|Unknown|muskrat
197|CP3|Chlamydia psittaci|Unknown|pigeon
4282|Horse pl|Chlamydia psittaci|Australia|horse
193|C19/98|Chlamydia psittaci|Unknown|Sheep
216|01DC11|Chlamydia psittaci|Unknown|Pig
217|02DC15|Chlamydia psittaci|Unknown|cattle
218|08DC60|Chlamydia psittaci|Unknown|human
GPIC Chlamydia caviae
AY184290 Chlamydia felis
Fe/C-56 Chlamydia felis
C. avium P4 Chlamydia aviam
10DC88 Chlamydia aviam
L1 chlaydia pecorum
L17 chlaydia pecorum
K7 Chlamydia Pneumoniae
CH-1 Chlamydia Pneumoniae
CWL-011 Chlamydia Pneumoniae
66|A/HAR-13|Chlamydia trachomatis|Saudi Arabia|human
227|B/Jali20/OT|Chlamydia trachomatis|The Gambia|human
269|Sweden2|Chlamydia trachomatis|Sweden|human
63
56
82
100
62
99
77
85
89
40
100
100
96
90
66
100
100
100
65
100
32
100
100
Group 1.1
Group 1
Chlamydia abortus
Group 2
Group 3
Chlamydia psittaci III
Chlamydia psittaci II
Chlamydia psittaci I
Chlamydia felis
Chlamydia aviam
Chlamydia pecorum
Chlamydia Pneumoniae
Chlamydia trachomitis

that the field isolate Sul/2017 had a common ancestor with Sul/2014, CAAB7, 1H, and Krauss-15 isolates in group 2 of *C. abortus*.

## Discussion

Abortions by infectious agents in ewes and goats cause considerable economic loss. In addition to *Brucella* species, *Campylobacter fetus* subspecies *fetus*, *C. abortus*, *Salmonella,* and *Listeria monocytogenes* are responsible for abortion in small ruminants (Türütoğlu et al. 2000). In previous surveys, the less-sensitive and -specific complement fixation test had been used, which was described by the World Organization for Animal Health (OIE) as the most commonly used method for serodiagnosis of animals chlamydiosis. However, the technique is tedious, has limited sensitivity, and frequently afflicted by cross-reactions between chlamydial species (McCauley et al. 2007). The recently evolved serodiagnostic tests are primarily based on two main cross-reactive antigens present in all chlamydial species, lipopolysaccharide and the major outer membrane protein (MOMP). Thus, these tests are not species-specific for diagnosing animals infected by OEA. Possibilities for diagnostic detection of chlamydia are better after the introduction of DNA-based methods, particularly the PCR, which permit direct identification from clinical samples and differentiation of species (Longbottom et al. 2001).

In the current study, primers of the *C. abortus ompA* gene were used; only one sample (3.33%) from aborted ewes was found positive for *C. abortus* both by yolk sac inoculation and PCR. This result represents the first insight into the presence of *C. abortus* infection of sheep in Sulaimani province, Iraq, using molecular methods.

Embryonated egg inoculation is considered the gold standard for the isolation of chlamydia. However, the necessity of long-time incubation is its only disadvantage (Condon and Oakey 2007). The findings in this study showed that only one smear from all the impression smear of yolk sac membranes of chicken eggs was found positive for *C. abortus* by Giemsa stain. Bacterial isolation in embryonated eggs depends on the viable *C. abortus* elementary bodies in the infected material, which are small and dense. This result was following a study done by Kalender et al. (2013). In this study, the cytological examination of the inoculated egg revealed the typical vascular congestion of yolk sac membranes. The variations in the results of our study and other studies may be attributed to many factors. For example, the geographical location, size and type of samples taken, animal breed, grazing and management strategies, nutritional deficiency, and uncontrolled restriction of diseased animal movement from infected areas are factors that may affect the incidence rate of *C. abortus*.

The result of this study showed that 29 out of 30 (96.66%) samples were infected with *B. abortus* using the genus-specific (B4/B5) primers. This result is higher than the outcome of Mohammed et al. (2013), nine out of 15 (60%), and lower than the result of Mukherjee et al. (2007), 19 out of 19 (100%), who detected the *Brucella* genus using the same primers. This variation may be due to the sampling time from aborted animals (Dağ et al. 2012).

Molecular analysis reveals that *C. abortus* Sul/2107 strain was the causative agent of abortion in the sheep herd. There was not enough genetic information about *C. abortus* in Iraq before this study in the GenBank database, except for one isolate (Sul/2014). Genetic analyses indicated that the strain in the present study differed from the previous strain in the region. The number of *C. abortus* isolates worldwide were 6,718 variable locations documented within the complete phylogeny (Seth-Smith et al. 2017). On the other hand, there were 17,163 variable locations recognized within the phylogeny of *C. trachomatis* (Harris et al. 2012), and 47,710 variable locations were identified within the strains of *C. psittaci* (Read et al. 2013). This variation was evident in our study, in which each species of *Chlamydia* was in a different cluster (Fig. 3). Because of low diversity in *C. abortus* (Seth-Smith et al. 2017), there were no apparent differences among the strains according to the geographical region or host range within the groups of *C. abortus* cluster. This outcome made Sul/2017 share a common ancestor with a wide range of strains that originated from different hosts and countries. The grouping within *C. abortus* was not clear due to a low rate of diversity in the species. Therefore, to make the phylogram more prominent, we used cutoff value with the condensed tree option in the phylogenic tree constriction. Further research is recommended for the whole genome analysis of all *Chlamydia* strains in Iraq and the neighboring countries due to insufficient genetic information about *C. abortus* in those countries.

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**Author contribution**
All the authors equally participated in the study.

**Conflict of interest**
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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