Molecular evidence of Anaplasma phagocytophilum *in aborted goat fetuses and placenta*

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Summary
Anaplasma phagocytophilum, transmitted by *Ixodes* ticks, is an intracellular pathogen of zoonotic interest. Regarding animals of veterinary importance, infection by this agent has been linked mainly to high fever, neutropenia, reduced milk production, but hemorrhagic diathesis, abortion and impaired spermatogenesis have also sporadically been reported. In Greece, *A. phagocytophilum* has been detected in dogs, ticks and humans, while so far only *A. ovis* had been detected in farm animals. Following the occurrence of multiple abortions in two goat farms in Northern Greece, samples were collected from aborted animals. Stomach contents and placental tissue from aborted animals tested positive for *A. phagocytophilum* by molecular assays and negative for other infectious and parasitic agents. Treatment with oxytetracycline LA stopped the abortions. In tick risk areas clinicians should consider *A. phagocytophilum* as a cause of abortion in goats.

Anaplasma phagocytophilum is an obligate intracellular zoonotic bacterium transmitted by Ixodid ticks which can cause high fever, neutropenia (due to unique tropism for granulocytes), reduced milk production and hemorrhagic diathesis in ruminants (Woldechiwet 2006, Giadinis *et al.* 2011). In Europe the *Ixodes ricinus* ticks is the most important vector. In a recent study the presence of *A. phagocytophilum* infection in a bovine herd was associated with depression, nasal and eye discharge, anorexia, coughing, diarrhea, recumbency, weakness, swelling of the hind limbs, decrease of milk production (Aktas and Ozubek 2015); co-infection with other *Anaplasma* spp. did not seem to worsen the symptoms. The disease in ruminants is called tick-borne fever (TBF). Reproductive disorders, such as impaired spermatogenesis and abortion have been observed in sheep but not in goats (Jones and Davies 1995, Garcia-Perez *et al.* 2003, Chianini *et al.* 2004, Lillini *et al.* 2006, Stuen 2007).

In Greece, *A. phagocytophilum* has been detected by PCR in humans (Psaroulaki *et al.* 2008) and ticks (Kachrimanidou *et al.* 2011, Papa *et al.* 2017). By PCR *A. ovis* has been also detected in small ruminants (Giadinis *et al.* 2012), while serological evidence of the presence of *A. phagocytophilum* has been recorded in an ill ram (Giadinis *et al.* 2011).

This manuscript describes sporadic abortions in two goat herds in Northern Greece, in which *A. phagocytophilum* was the only pathogen detected in placenta or aborted fetuses.

The 1st goat herd (Herd 1) was located in Chalkidiki Prefecture (Northern Greece) and consisted of 800 dairy goats of local breeds that were reared under the semi-intensive feeding system. In 2011, the farmer observed sporadic abortions in goats of different ages, and about 30 abortions were recorded during the last 45 days of pregnancy. Two aborted fetuses (from different mothers) without fetal membranes were submitted to the Farm Animal Clinic of the Aristotle University in Thessaloniki. Both fetuses were normal in appearance and no specific lesions were found by post mortem investigation. Whole blood from the mothers, brain tissue and stomach content from the fetuses were collected.

The 2nd farm (Herd 2) was situated in Thessaloniki

Prefecture (Northern Greece) and consisted of 300 dairy goats of local breeds that were reared under the semi-intensive feeding system. In 2014, the farmer observed sporadic abortions in goats of different ages, and referred about 15 abortions at the 3rd-4th month of pregnancy. Two aborted fetuses (from different mothers) and fetal membranes were submitted to the Farm Animal Clinic of the Aristotle University in Thessaloniki. Both fetuses and fetal membranes were normal in appearance and no specific lesions were detected at autopsy. Whole blood from the aborting mothers along with fetal spleen, brain, liver, placental tissues and stomach contents were collected.

All samples were tested by PCR or cultures for *Chlamydia* spp., *Brucella* spp., *Campylobacter* spp., *Salmonella* spp., *Escherichia coli*, *Listeria* spp., *Mycoplasma* spp., *Toxoplasma* spp., *Neospora* spp., and Schmallenberg virus at the Aristotle University and they were found all negative for the above pathogens (Giadinis *et al.* 2013).

DNA was extracted from the sampled tissues using a commercial kit (Tissue kit, Qiagen, Hilden Germany); portions of DNA samples were sent at the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of the University of Crete, where they were tested by PCR for *Babesia* species (targeting 18s rRNA) and by a multiplex real-time PCR for *Coxiella burnetii* (targeting com1), *Anaplasma* species (targeting msp4 of *A. centrale, A. marginale* and *A. ovis*) and *A. phagocytophilum* (targeting msp4). DNA from positive samples against the pathogens of interest was used as positive marker; sterile water was used as negative marker. All samples were negative for *Babesia* species, *Anaplasma* species and *Coxiella burnetii*, while DNA from the stomach contents of the 1st herd and placental tissue from the 2nd herd were tested positive for *A. phagocytophilum*. Also, blood samples from the examined mothers were found positive for *A. phagocytophilum*.

Positive samples were further amplified using primers ehr16SR and ehr16SD (targeting a 345 bp portion of the 16s rRNA (Parola *et al.* 1998) and HS1 and HS6 (targeting a 1300-1450 bp portion of groesl) (Sumner *et al.* 1997).

PCR amplicons (345 bp for 16s rRNA and 1350 bp of groesl) were purified using a commercial kit (QIA quick PCR purification spin kit, Qiagen, Germany). Sequencing was performed twice using the above described primers at a CEQ 8000 Beckman Coulter sequencer. The sequences revealed were processed using Chromas v1.49 and Lasergene ver.7.1 software for viewing the chromatograms and editing of the retrieved nucleotide sequences, and nucleotide BLASTn for investigating the homology of the obtained sequences compared to the NCBI databases.

Sequences revealed were 100% identical to each other and 100% identical to already published sequences (16S rRNA: Genbank accession number EU090186) and 99% identical to the 16S rRNA (M73220) and groESL (AF548385) of the already published variant known to cause a more severe form of the disease (Stuen *et al.* 2003).

In both herds, treatment with oxytetracycline LA of the rest pregnant animals (two injections with three days a part at a dose of 20 mg/kg) following *A. phagocytophilum* detection, was effective and no other cases of abortion were observed. According to the owners, the animals in both herds were grazing during large periods of the year, consequently tick infestation was not unusual. At the intervention time, the animals didn't have ticks, because few days earlier they had been treated for ectoparasites.

Abortions, which often are determined by zoonotic agents, are a serious economic threat in goat herds, as they can reduce the meat and milk productions (Smith and Sherman 2009, Giadinis *et al.* 2013).

Anaplasma phagocytophilum has been demonstrated as a possible cause of reproductive failures in sheep flocks in Italy, Norway, Spain and UK (Jones and Davies 1995, Garcia-Perez *et al.* 2003, Chianini *et al.* 2004, Lillini *et al.* 2006, Giudice *et al.* 2011). However, this is the first time that *A. phagocytophilum* has been associated to abortion cases in goats.

As regards the pathophysiology of abortions caused by *A. phagocytophilum*, they seem to occur due to placental dysfunction (Chianini *et al.* 2004). It has recently been found that transplacental transmission of *A. phagocytophilum* can occur in sheep without abortion (Reppert *et al.* 2013). It is interesting, that in the previous study, *A. phagocytophilum* was detected in many tissues of an infected lamb during pregnancy. In the present study, the pathogen was detected in the stomach contents of the goat kids of the 1st herd and in placental tissue from the 2nd herd, while it was not found in other tissues of the aborted goat kids. This suggests that abortion might be a consequence of placental dysfunction. Unfortunately, histopathology was not conducted in the fetal membranes. In any case, it would be interesting to investigate the distribution of *A. phagocytophilum* in various tissues.

Since the number of examined herds and fetuses was low, these data should be treated with caution. However, in case of *A. phagocytophilum* infection in goat herds it is likely that abortion is a sporadic event (Giadinis *et al.* 2011, Giadinis *et al.* 2013). Abortion storm with ewe mortality has been reported once in sheep in UK (Jones and Davies 1995). However, this outbreak was not thoroughly investigated and oxytetracycline administration was not effective. In

the present study, oxytetracycline administration was very effective in controlling the abortions.

Regarding the responsible strain, the sequences revealed a 99% similarity with those of *A. phagocytophilum* variant 1 (Stuen *et al.* 2003). The incidence and severity of the disease depend on geographic area, species and host breed, as well as the variant of *A. phagocytophilum* involved (Stuen *et al.* 2003, Teglas and Foley 2006, Granquist *et al.* 2010). *Anaplasma phagocytophilum* infection may persist in different animal species, depending on individual variations, genetic variants and host species involved. However, persistent infection in goats has not been reported yet (Stuen 2007, Thomas *et al.* 2012).

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