An outbreak of abortion in Afshari sheep with probable involvement of *Campylobacter fetus*

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Abstract:

BACKGROUND: Abortion is one of the most important factors reducing lambing rate and consequently profitability of sheep farms. In addition to financial losses, it is also important from a zoonotic point of view. **OBJECTIVES:** The aim of this study was to investigate bacterial abortifacient agents in an outbreak of abortion occurring in Afshari sheep in the northwest of Zanjan province. **METHODS:** Vaginal swab samples were collected from 217 Afshari ewes (129 samples were taken from aborted ewes, 3 samples from ewes with crippled and deformed lambs, and 85 samples from animals that had given birth to healthy lambs) from reported flocks involved in outbreak. Swabs were examined by PCR assay to detect DNA from *Coxiella burnetii*, *Chlamydophila abortus*, *Salmonella enterica*, *Yersinia enterocolitica*, *Campylobacter fetus*, *Brucella ovis* and *Leptospira interrogans*. **RESULTS:** Based on the results, only DNA of *Campylobacter* was detected in the samples. A 266 bp fragment specific for *Campylobacter* was amplified from 51.52% and 34.12% samples belonging to aborted and non-aborted ewes, respectively. **CONCLUSIONS:** Significant presence of the bacterium in aborted ewes ($p<0.001$) compared to the non-aborted groups with odd ratio of 3, emphasizes that *Campylobacter* could be involved in the outbreak of the abortion. Considering the importance of the disease, prophylactic measures are needed to reduce the disease. However, further investigations are required to determine the impact of this bacterium in prevalence of abortion in sheep in other areas.

Introduction

Two major groups of abortive agents are infectious and noninfectious factors. There are various infectious agents which cause abortion in sheep and goats. The most common are *Campylobacter fetus* (also called Vibrio), *Chlamydophila abortus* (Abortion due to *Chlamydophila abortus* also called EAE (Enzootic Abortion in Ewes)), *Coxiella burnetii*, *Salmonella enterica*, *Yersinia enterocolitica*, *Brucella* spp., *Leptospira interrogans* and Bluetongue virus. In Iran, attention is focused on abortion caused by *Brucella* and most flocks are vaccinated against this bacterium. An epidemiologic survey from Fars, Chaharmahal bakhtiari and Yazd provinces indicated that 20 to 48% of abortion in sheep and goats is caused by *Brucella* melitensis in those areas (Firouzi, 2005 and Ghosian moghadam et al., 2008). *Campylobacter* is one of the most important agents in ovine and bovine abortion after brucellosis (Tajbakhsh et al.,2000).Campero et al. (2003) reported *Campylobacter* to be the second most important abortifacient agent in beef cattle. Infection with *Campylobacter* is known as zoonosis (Ertas et al., 2003 and Forouhesh Tehrani et al., 2003) and the organisms can be transmitted to human via food,

water and through contact with farm animals and pets (Salihu et al., 2009). When infection with *Campylobacter* occurs most of the bacteria can be found in lungs and gastrointestinal tract (Campero et al., 2005). Ewes infected with *Campylobacter* often abort during the last weeks of gestation or give birth to weak, stillborn or dead lambs (Salihu et al., 2009). Abortions due to *Campylobacter* often emerge in a flock by a purchased infected carrier animal (Hum et al., 1997). Although there is no complete information about epidemiology of this agent in sheep flocks in Iran, the infection is possible in most areas (Firouzi, 2005). *Campylobacter* is one of the most common bacterial agents of ewe abortion in the United States with an overall abortion rate of 5 to 50% (average, 23.2%) in affected herds (Sahin et al., 2008). The majority of Campylobacterial sheep abortion in New Zealand is associated with *Campylobacter fetus* while *Campylobacter jejuni* is more important in United States (Mannering et al, 2006 and Sahin et al., 2008). Firouzi (2005) has reported a prevalence of 7.5% for the infection in aborted fetuses of sheep around Shiraz (Iran). Most of the epidemiological studies performed previously were based on serologyical assays and culture methods, which are difficult and time consuming procedures. Especially in the case of *Campylobacter* spp. being biochemically less active, molecular techniques has become the method of choice in diagnosis of the diseases in recent years (Stoyanchev, 2004). In December 2010, an outbreak of abortion was reported in sheep flocks of Mahneshan areas in the Zanjan province. The initial surveys indicated that in some flocks over 50% of pregnant ewes were aborted (Half the pregnant animals in each herd had abortion). The main objective of this study was to determine the cause(s) of the outbreak in Mahneshan.

Materials and Methods

Sampling: Samples were taken from 217 ewes (129 samples were taken from aborted ewes, 3 samples from ewes with the crippled and deformed lambs, and 85 samples from animals that had given birth to healthy lambs) from 11 flocks of villages around Zanjan province where high prevalence of abortion was reported by farmers in December 2010 (see Table 2). In the area, animals were reared indoors and with high density in this time of year. Sterile, dry and cottontip swabs were used for sampling of aborted or ewes with alive and apparently healthy delivered lambs. Vaginal and blood samples were taken for nucleic acid and serological detections, respectively. Sera and swab samples were stored at -20°C until tested. During sampling, information including ear tag number, age, time of abortion, vaccination status, and pregnancy time of sampled animals and type of diet were collected. Most farmers vaccinate their herds against *Brucella* in this province every year.

DNA extraction: First, DNA extraction for swab samples was optimized using a gram-negative bacterium, Pectobacterium carotovorum, which is not a pathogen for animals. This bacterium was cultured in Nutrient agar medium. Several protocols were tested on samples spiked with Pectobacterium carotovorum to evaluate the efficiency of DNA extraction and test for the presence of inhibitors. Extractions were evaluated by PCR and using specific primers. Finally, a modified phenol-chloroform extraction method was used for DNA extraction from vaginal swabs. Briefly, swab samples were defrosted and one mL of STD buffer ((0.01 M Tris-HCl [pH 8.3], 0.05 M KCl, 0.0025 M MgCl2.6H20, 0.5% Tween20) was added in a 2 mL microcentrifuge tube. Samples were shaken for one minute and centrifuged at 16000 x g and pellet was dissolved in 200 µL of TE (Tris- EDTA) containing 10% SDS (sodium dodecyl solfate). The specimens were incubated at 56°C for one hour and subsequently conducted to phenol-chloroform DNA purification procedure.

PCR assay: Specific primers were designed for *Coxiella burnetii*, *Yersinia enterocolitica*, *Campylobacter fetus*, *Brucella ovis* and *Leptospira interrogans*. The primer sequences for *Chlamydophila abortus* and *Salmonella enterica* were obtained from previous studies (Table1). The primers were designed in a way whereby they can produce amplicons with different length (except for *Brucella* and *Leptospira*) that can be easily distinguished on agarose gel electrophoresis. Furthermore, the primers can be used for detection of DNA of these bacteria by real-time PCR. Sensitivity and specificity of the primers were tested using the abovementioned pure bacteria purchased from Persian Type Culture Collection (PTCC), swab samples and sequencing of PCR products. However,

Chlamydophila and *Leptospira* organisms were not available to be used as positive control or for testing the sensitivity. PCR assay was performed in a final volume of 25 µL containing 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2 mM MgCl2, 0.1% Tween20, 200 µM each dNTP, 1.25 µM each primer (Table 1) and 0.8 U Taq polymerase. After an initial denaturation at 95°C for 5 minutes, 30 cycles of one min for each temperature at 95°C, 52-55°C and 72°C, with a final extension at 72°C for 5 min were performed. Annealing temperature was approximately the same for all primers pairs in multiplex PCR. Amplification products were then visualized by electrophoresis in 1.5% agarose gel in 1X TBE buffer (Tris-borate-EDTA) and ethidium bromide staining. Purification of PCR products on agarose gel was done using phenolchloroform extraction procedure and sequencing was performed on the ABI 3730XL DNA Analyzer (Bioneer, South Korea).

Test of sera: The sera samples of 20% of aborted ewes (25 samples from 129 aborted ewes randomly) were tested for *Brucella* infection using Rose Bengal Test (RBT) and any visible agglutination and/or the appearance of a typical rim was taken as a positive result.

Statistical analysis: Based on type of data (categorical) in this study, non-parametric binary logistic regression and Mann-Whitney test were employed for statistical analyses. The results were considered significantly different at the level of $p<0.05$.

Results

PCR: In some cases reported abortion in sheep flocks of villages around Zanjan province exceeded more than 50% of pregnant ewes. PCR based nucleic acid detection of the bacteria revealed presence of *Campylobacter* DNA in 68 vaginal swab samples of 132 aborted ewes (51.52 %). None of the samples were detected for DNA from the other six bacteria tested in this study. However, 29 samples of 85 non-aborted ewes (34.12 %) were positive by PCR using *Campylobacter* specific primers. In positive samples an expected DNA band with correct molecular size product of 266 bp was observed on agarose gel stained by ethidium bromide (Figure 1). Sequencing results of this DNA band confirmed our finding as *Campylobacter* when blasted in biological databases and aligned against sequences of 16s rRNA gene of *Campylobacter*. The presence of this bacterium in samples from aborted ewes was significantly ($p<0.001$) higher than those of non-aborted group with odd ratio (OR) of 3.

Young animals at first till third time of pregnancy were more likely to be aborted due to *Campylobacter* infection than elder animals (OR= 1.15). Of 68 PCR positive samples for *Campylobacter* in different ages, 32.35 %, 20.59% and 17.65% were animal at third, first and second time of pregnancy, respectively. However, number of the aborted ewes and contaminated with *Campylobacter* at fourth and higher time of pregnancy was 19 for 4th and 5th time of pregnancy while those for animal at first, second and third time were 14, 12 and 34 respectively (see diagram 1).

The sera of 20% of aborted ewes were randomly tested for presence of *Brucella* antibody and all were negative in RBT. This result was in accordance with PCR results where DNA of *Brucella* was not detected in the tested samples.

Discussion

The most important source of income for sheep farmers comes from selling the lambs, while abortion which occurred mostly in the last months of gestation caused reduction in total number of lambs per farm. In Mahneshan, where the outbreak of abortion in some flocks was more than 50% of pregnant ewes caused a serious economic loss to sheep rearing farmers. From 85 samples of aborted ewes 61 (71.76%) were PCR positive for *Campylobacter* while from other bacteria that have been tested for in this study no DNA were detected. Sampling of these aborted ewes was done by the investigator of this study at the time of visiting the flocks, therefore all the animals were not at immediate day(s) of post abortion. Consequently, it was not expected that all the animals would shed the bacterium, so this percentage of detection may be reasonable. Furthermore, it is possible that the number of the bacterium was very low or presence of inhibitor might prevent PCR reactions. However, several vaginal swabs were spiked with different number of *P. carotovorum* and in all cases DNA of this bacterium was detected by

Table1. Primer sequences used in PCR amplification. bp*: base pair.

Primers	Gene	Product length	Microorganism	Reference
ATAATGACTTCGGTTGTTATT F: R: TGTTTTAGATGCCTAAACAT	16s rRNA	127 bp*	*Chlamydophila abortus*	Messmer et al, 1997
F: GACGCCATCCAGGAACAG R: GTATACGATCTGGTCCTGC	ompA	200 bp	*Brucella ovis*	this study
F: ACGGTAACAGGAAGMAG R: TATTAACCACAACACCT	16s rRNA	402 bp	*Salmonella enterica*	Trkov and Avgus?tin ,2003
F: ACTGTCGAATAACCGGCTTC R: GTTACAGTTACTACTAATGGG	ompA	bp 304	*Coxiella burnetii*	this study
F: TTTGTTAGGGAAGAACCATG R: CGCAATGGGTATTCCTGGT	16s rRNA	265 bp	*Campylobacter fetus*	this study
F: CTCTTCTACTTATGATATCGG R: ACATTACAGCCAGGTATACGT	ompA	166 bp	*Yersinia enterocolitica*	this study
F: GGTCCCGAGAGATCATAAG	16s rRNA	199 bp	*Leptospira*	this study

Table 2. The number of aborted (A) and not aborted (NA) samples the separation of positive (C+) and negative (C-) *Campylobacter* for villages in the province.

Name of village	Non aborted samples (NA)		Aborted samples (A)	
	NA- C-	NA- C+	A- C-	A- C+
Ebrahim Abad	0	0	4	20
Ghale jugh Sadat	0	2	6	9
Mianaj	14	4	4	4
Haji inak	0	0	4	10
Angouran	0	1	6	24
Arze khuran	15	10	1	1
Zanjan	10	0	0	0
Sansyz	10	0	20	0
Eich	0	0	17	0
Aspirin	6	3	2	0
Hasan abad	8	0	0	0

PCR. The presence of the infection in several non-aborted ewes may be the result of postpartum contamination that in turn resulted from close contact with aborted animals' secretions because animals are kept indoors in the season of lambing and they are more often closely confined in unhygienic conditions in such situations.

The step of pregnancy plays an important role in the prevalence of infection in herd. For example, it has been reported that if the infection happens at 105 the day of gestation abortion will happen in all of the ewes, but if infection happens at 126 the day of gestation abortion will be found in only about 20 percent of ewes (Skirrow et al, 1994). It is therefore possible that non aborted animals be contaminated with *Campylobacter* after 126 days of gestation (in this study). In addition, only in infected herds have infected ewes without abortion been observed, which probably happened due to contact with infected animals or contamination after 126 days of gestation. But in healthy herds no case of the *Campylobacter* infection was found. In the case where the *Campylobacter* was present naturally in the vagina of pregnant animals, it was expected that in healthy herds cases of *Campylobacter* nucleic acid must be detected, but not even one case of infection was found in these flocks.

When an abortion occurs it is essential that aborted ewes be separated immediately and any excretion of the abortion disposed of to avoid further contamination of the environment, but this is something that is rarely done accurately in these sheep farms. In addition, there are immunological differences among animals and not all infections lead to abortion. Gürtürk et al. (2002) in an epidemiological study using serological tests (Elisa and Complement fixation test) found that *Campylobacter* caused 38% abortion in a sheep flock in Turkey. Prevalence of *Campylobacter* infection is also reported in 23.2 % of all aborted animals in United State (Sahin et al., 2008), whereas the prevalence of the infection was even higher in previous years and Ertas et al. (2003) reported that 66 % of all abortions in the United States are due to contamination with this bacterium. Zahra'i Salehi (1999) reported *Campylobacter fetus* as a cause of abortion in one of the farms around Tehran. Fenwick et al. (2000) reported that vaccination with a vaccine strain of *Campylobacter* cannot essentially protect animals against all other strains and abortion may occur. Results of this study showed that younger animals were more likely to be aborted due to infection with this bacterium than elders. It is possible that elder animals had experienced the infection previously and there was some level of immunological protection (in this study). Nonetheless, this is

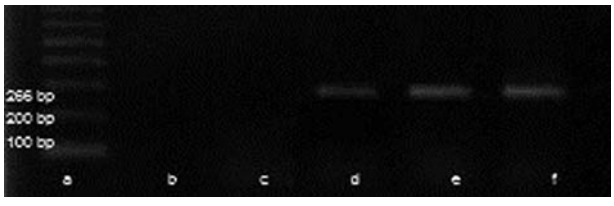

Diagram 1. the percent of animals with different ages of the total sample, the percentage of abortion in each age group (A) and the percentage of positive samples for *Campylobacter* in aborted ewes at any age (A+).

Figure 1. Specific amplification of target DNA from *Campylobacter* by PCR using specific primers, and Lanes: a: DNA Ladder 100 bp (company), b: negative control (no DNA in the PCR reaction mix), c: sample of healthy animal, d, e and f: aborted ewes samples.

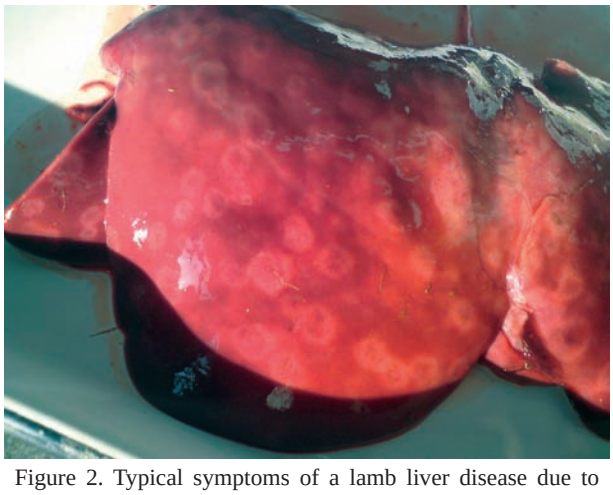

Figure 2. Typical symptoms of a lamb liver disease due to abortion is campylobacteriosis.

something to be tested to address the question. This outbreak caused a high economic loss to sheep farms of the area and tracing the cause of the outbreak is the first step in preventing such events. The results of this study indicated that infection with this organism as reported in other countries can cause outbreak of abortion in sheep farms in Iran. Therefore, it is essential that hygienic and prophylactic measures be implanted by farmers to avoid these kinds of infections. Especially in closed places, sick and aborted ewes must be isolatedfrom healthy animals and pregnant ewes have to deliver their lambs in a separate place. Also, delivery fluid (discharges) and dead fetus have to be immediately removed and be disposed of in a lime pit. None of the ewes showed any specific clinical signs of campylobacteriosis at the time of sampling but after dissection of a few lamb carcasses by veterinary staff, they diagnosed abnormally enlarged liver and yellow spots on the liver that indicated vibriosis (Figure 2).

In this investigation, to examine whether ewes in early ages have a higher number of abortions they were split into two groups and the results were compared. One group was three years old and less and the other group four and five years. The ratio of aborted ewes when the positive and negative results were considered separately, indicated the two groups Odd ratio=1.15 with 95 % confidence interval between 0.5-2.5 which was not statistically significant.

However, further investigations on outbreaks of other areas and provinces are necessary to have comprehensive information about epidemiology of the infection with this bacterium.

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References

1. Campero, C.M., Anderson, M.L., Walker, R.L., Blanchard, P.C., Barbano, L., Chiu, P. Marti´nez, A., et al. (2005) Immunohistochemical identification of

Campylobacter fetus in natural cases of bovine and ovine abortions. J Vet Med. 52: 138- 141.

2. Campero, C.M., Moore, D.P., Odeon, A.C., Cipolla A.L., Odriozola, E. (2003) Aetiology of bovine abortion in argentina. J Vet Res Com. 27: 359- 369.
3. Donkersgoed, J.V., Janzen, E.D., Chirino-Trejo, M., Berry, C., Clark, E.G., Haines, D.M. (1990) *Campy lobacter jejuni* abortions in two beef cattle herds in Saskatchewan. J Can Vet. 31: 373-377.
4. Ertas, H.B., Ozbey, G., Kilic, A., Muz, A. (2003) Isolation of *Campylobacter jejuni* and *Campylobacter* coli from the gall bladder samples of sheep and identification by polymerase chain reaction. J Vet Med. 50: 294-297.
5. Fenwick, S.G., West, D.M., Hunter, J.E., Sargison, N.D., Ahmed, F., Lumsden, J.S., et al. (2000) *Campylobacter* fetus fetus abortions in vaccinated ewes. J N Z Vet. 48: 155-157.
6. Firouzi, R. (2005) Study of Bacterial causes of abortion in sheep around Shiraz. J Vet Uni. 61: 15-17. (in Persian)
7. Ghosian Moghadam, M.H., Kayvani Amine, H., Zahra'i Salehi, T., Khazrayi Nia, P., Fallah, N. (2008) Comparison of culture and polymerase chain reaction (PCR) with Wright test in the diagnosis of brucellosis. J Med Tec. 74: 51. (in Persian)
8. Gürtürk, K., Ekin, I.H., Aksakal, A., Solmaz, H. (2002) Detection of *Campylobacter* antibodies in sheep sera by a Dot-ELISA using acid extracts from *C. fetus* ssp. fetus and *C.jejuni* strains and comparison with a complement fixation test. J Vet Med. 49: 146-151.
9. Hum, S., Quinn, K., Brunner, J. (1997) Evaluation of a PCR assay for identification and differentiation of Campylobacter fetus subspecies. J Aust Vet. 75: 827-831.
10. Mannering, S.A., West, D.M., Fenwick, S.G., Marchant, R.M., Connell, K.O. (2006) Pulsed-field gel electrophoresis of *Campylobacter jejuni* sheep abortion isolates. J Vet Microbiol. 115: 237-242.
11. Messmer, T.O., Skelton, S., Moroney, J.F., Daugharty, H., Fields B. (1997) Application of a nested, multiplex PCR to psittacosis outbreaks. J Clin Microbiol. 35: 2043-2046.
12. Salihu, M.D., Junaidu, A.U., Oboegbulem, S.I., Egwu, G.O. (2009) Prevalence and biotypes of *Campylobacter* species isolated from sheep in sokoto state, nigeria. J Anim & Vet. Adv. 1: 6-9.
13. Skirrow, M.B. (1994) Diseases due to *Campylobacter*, *Helicobacter* and related bacteria. J Comp Pathol. 111: 113-149.
14. Stoyanchev, T. (2004) Detection of *Campylobacter* using standard culture and PCR of 16S rRNA gene in freshly chilled poultry and poultry products in a slaughter house. Trakia J Sci. 3: 59-64.
15. Tajbakhsh, H., Ahmadi, M., Fakhrzadgan, F., Nadalian, M. (2000) Surveys of infections due to *Campylobacter fetus* spp. fetus in flocks of sheep around Tehran and Isfahan. J Vet Teh Uni. 3: 69-71. (in Persian)
16. Trkov, M., Avgustin, G. (2003) An improved 16S rRNA based PCR method for the specific Detection of *Salmonella enteric*. J Food Mic. 80: 67-75.
17. Zahra'i Salehi, T. (1999) Prevalence of abortions due to *Campylobacter fetus* ssp. fetus in one of Tehran farms. J Vet Uni. 2: 11-14. (in Persian)

مجله طب دامی ایران، ۱۳۹۲، دوره ۷، شماره ۱، ۵۶-۵۱

شیوع سقط جنین در نتیجه آلودگی احتمالی کمپیلوباکتر فتوس در گوسفند افشاری

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چکیده

زمینه مطالعه: سقط جنین یکی از مهمترین عوامل کاهش تعداد بره و در نتیجه کاهش سوددهی در دامداری ها می باشد. علاوه بر خسارات مالی، سقط جنین از جنبه سلامت عمومی نیز حایز اهمیت است. **هدف:** هدف از این مطالعه تشخیص عوامل باکتریایی سقط جنین گوسفند افشاری در یک شیوع ناگهانی در شمال غربی استان زنجان بود. **روش کار:** نمونه سواب های واژینال از ۲۱۷ میش افشاری (۱۲۹ نمونه از میش هایی که سقط کرده بودند، ۳ نمونه از میش های که بره نارس به دنیا آورده بودند و ۸۵ نمونه از میش هایی که بره سالم داشتند) از گله های گزارش شده ی درگیر در شیوع جمع آوری گردید. سواب ها به منظور تعیین DNA باکتری های کوکسیلا بورنتی، کلامیدوفیلا آبورتوس، سالمونلا انتریکا، یرسینیا انتروکولیتیکا، کمپیلوباکتر فتوس، بروسلا اویس و لپتوسپیرا اینتروگانس به وسیله PCR، مورد آزمون قرار گرفتند. **نتایج:** براساس نتایج فقط DNA کمپیلوباکتر در نمونه ها تشخیص داده شد. یک قطعه اختصاصی ۲۶۶ bp مربوط به کمپیلوباکتر به ترتیب در ۵۱/۵۲٪ و ۳۴/۱۲٪ از میش های سقط کرده و آنهایی که بره های سالم بدنیا آورده بودند تکثیر شد. **نتیجه‌گیری نهایی:** وجود معنی دار این باکتری در میش های سقط کرده ($p<۰/۰۰۱$) در مقایسه با گروه دارای بره های سالم با odd ratio ۳ تاکید بر این دارد که کمپیلوباکتر می تواند در شیوع سقط جنین در این شیوع درگیر باشد. با توجه به اهمیت این بیماری به ابزارهای پیشگیری کننده برای کاهش بیماری نیاز است. به هر حال برای تعین تاثیر این باکتری بر شیوع سقط جنین در گوسفندان مناطق دیگر نیاز به بررسی های بیشتری است.

واژه های کلیدی: سقط جنین، کمپیلوباکتر، گوسفند افشاری، PCR

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