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Research Article

Investigation of Campylobacteriosis in Abort Cases in Kars Province by Pathological, Immunohistochemical, PCR and Microbiological Methods

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ABSTRACT

Campylobacteriosis is an infectious, zoonotic infection characterized by offspring and infertility, leading to economic losses in cattle and sheep breeding. In this study, we aimed to investigate the incidence of cattle and sheep abortion in Kars region in terms of Campylobacteriosis and evaluate the results by PCR, immunohistochemical, histopathological and microbiological methods. In this context, liver and lung tissue examples and abomasum contents of 444 abort cases brought from Kars Center and districts to Kafkas University Faculty of Veterinary Medicine Pathology Department between 2013-2019 years were examined. Tissue examples from animals were fixed in % 10 buffered formaldehyde solutions. After routine procedures, paraffin blocks were prepared and sections with a thickness of 5 µm were taken for Hematoxylin & Eosin staining and 4 µm were taken for immunohistochemical staining . Sections were examined under light microscope to determine histopathologic changes. Organs belonging to aborted fetuses and abomasum contents were inoculated into the Preston Campylobacter Enrichment Broth containing microbial study selective supplement, and then enriched by pre-enrichment and then passed through Preston Campylobacter Selective Agar. Cultures in which the culture was incubated after incubation were examined for colony morphology and microscopic appearance and *Campylobacter spp.* suspicious colonies were evaluated by biochemical tests. As a result of histopathologic studies, characteristically, 7 of 17 abortion cases with multifocal necrotic hepatitis pattern and yellow abomasum contents were blurred and clotted, PCR, immunhistochemical and microbiological methods detected as *Campylobacter spp.* positive towards the direction. As a result, we thought that Campylobacteriosis is an important place in the abortion cases from Kars region and should be taken into consideration in breeding.

Kars İlinde Gözlenen Atık Vakalarında Kampilobakteriozisin Patolojik, İmmunohistokimyasal, PCR ve Mikrobiyolojik Yöntemler ile Araştırılması

ÖZ

Kampilobakteriyozis, sığır ve koyun yetiştiriciliğinde ekonomik kayıplara yol açan yavru atımı ve infertilite ile karakterize, bulaşıcı ve zoonotik bir infeksiyondur. Bu çalışmada, Kars yöresinde meydana gelen sığır ve koyun abort vakalarını Kampilobakteriyozis yönünden incelemek ve sonuçları PCR, immunohistokimyasal, histopatolojik ve mikrobiyolojik olarak değerlendirmek amaçlanmıştır. Bu kapsamda, 2013-2018 yılları arasında Kafkas Üniversitesi Veteriner Fakültesi Patoloji Anabilim Dalına Kars merkez ve ilçelerinden getirilen 444 adet atık vakasına ait karaciğer ve akciğer doku örnekleri ile abomazum içerikleri incelenmiştir. Hayvanlardan alınan doku örnekleri % 10'luk tamponlu formaldehit solüsyonunda tespit edildi. Rutin işlemlerin ardından hazırlanan parafin bloklardan, Hematoksilen Eozin boyaması için 5 µm, immunohistokimyasal boyama için kalınlığında 4 µm kesitler alındı. Histopatolojik değişikliklerin belirlenmesi amacıyla kesitler ışık mikroskobunda incelendi. Abort vakalarına ait organlar ve abomasum içerikleri mikrobiyolojik inceleme amacıyla selektif supplement içeren Preston Campylobacter Enrichment Broth içerisine inoküle edilerek ön zenginleştirmeye ve daha sonra Preston Campylobacter Selektif Agara geçilerek inkübe edildi. İnkübasyon sonrası üremenin olduğu kültürler koloni morfolojisi ve mikroskobik görünüm yönünden incelendi ve *Campylobacter* spp. yönünden şüpheli görülen koloniler biyokimyasal testlere tabi tutularak değerlendirildi. Yapılan histopatolojik incelemeler sonucunda karakteristik olarak hedef tahtası görünümünde multifokal nekrotik hepatitis tablosu gözlenen ve abomazum içeriği sarı renkte, bulanık ve pıhtı içeren 17 adet abort vakasının 7'si *Camphylobacter* ssp. yönünden PCR, immunohistokimyasal ve mikrobiyolojik yöntemlerle pozitif bulundu. Sonuç olarak Kars yöresinde meydana gelen atık vakaları içerisinde Kampilobakteriyozis'in önemli bir yeri olduğu ve yetiştiricilikte dikkate alınması gerektiği düşünmekteyiz.

INTRODUCTION

Abortion in sheep and cattle can occur due to many reasons. These reasons can be infectious or non-infectious (1,2). Among the causes of abortion due to infections, Campylobacteriosis is one of the main factors in many countries and causes significant economic losses in infected flocks. (3,4). In addition to cattle and sheep abortions, Campylobacteriosis leads to abnormal

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oestrus cycle and decreased fertility (5). *Campylobacter fetus* (formerly *Vibrio fetus*) is a microaerophilic and gram negative organism (6,7). C.fetus originally divided into three subspecies: *C. fetus subsp. venerealis, C. fetus fetus* and *C. fetus subsp. testudinum* (8,9). Of these species, *C.fetus subsp. venerealis* causes enzootic infertility and abortion in cattle, while *C. fetus fetus* is associated with epizootic abortion in cattle and sheep (10). *Campylobacter fetus* species are important veterinary pathogens as well as infect humans (11). In this study, we aimed to present the cases of cattle and sheep abortion in Kars region between 2013-2019 years in terms of Campylobacteriosis and to evaluate the results by PCR, immunohistochemical, histopathological and microbiological methods.

MATERIALS AND METHODS

Animals

The material of the study consisted of liver and lung tissue samples and abomasum contents of 444 (188 cattle and 261 sheep) abort cases that were brought from Kars center and districts to Kafkas University Veterinary Faculty Pathology Department between 2013-2019 years.

Bacterial isolation and phenotypic identification

In this study, tissue samples taken from cattle and sheep abortions were examined. For isolation purposes, blood agar from various organs of abortion cases was transferred to Preston Campylobacter Selective Agar and incubated at 37^{0}C and 42^{0}C for 48-72 hours. Breeding cultures were examined for colony morphology, microscopic appearance, catalase, oxidase and aerobic reproduction. (12,13). Campylobacter spp. The colonies that were suspected in terms of blood were purified by switching to Blood agar base no: 2 (CM271, Oxoid) medium (12,13). The purified colonies were transferred to Brucella broth containing 20% glycerol for subsequent molecular identification and stored at -20°C.

DNA Extraction and Multiplex PCR

The classical phenol-chloroform extraction method (14) was used for DNA extraction from the isolates and then multiplex PCR technique was applied on for *Campylobacter* spp. The primer sets targeting the 23S rRNA gene of *Campylobacter* spp.

Each PCR tube for *Campylobacter* spp. contained 12,5 µl Taq DNA Polymerase 2x Master Mix 1.5 µl 23S rRNA primer and 3 µl of whole-cell template DNA. The volume was adjusted with sterile distilled water to give 25 µl. DNA amplification was carried out in a thermocycler using an initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), and was finalized with an extension at 72°C for 7 min.

The PCR reaction is accompanied by the *Campylobacter* reference strains and the amplified products were visualised by 1.5% agarose jel electrophoresis and the images were photographed under UV transilluminator (UVP, CA 91786, U.S.A.).

Histopathological Investigations

Tissue samples from animals were fixed in % 10 buffered formaldehyde solution (Merck). Paraffin blocks were prepared ater routine tissue procedures and 5 µm thick sections were obtained for Hematoxylin & Eosin (H&E) staining. In order to determine the histopathological changes, the sections were examined by light microscope (Olympus Bx53) and photographed with Cell ^P Program (Olympus Soft Imaging Solutions GmbH, 3,4).

Immunohistochemical Investigations

Avidin-Biotin Peroxidase method was used as immunohistochemical stain. For immunohistochemical staining, sections of 4 µm thick from paraffin blocks were rehydrated. In order to prevent endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide solution for 15 minutes. Then, the microwave method was applied to the sections to reveal the antigenic receptors (Citrat Buffer Solution pH 6 for 25 min). In order to prevent nonspecific staining, the sections were incubated for 30 min with non-immune serum (Genemed Biotechnologies REF 54-0003). Following treatment with Phosphate Buffered Salt Solution (PBS) with 1/50 of diluted antibody (Accurate Chemical & Scientific Corporation; Cat No: QRL01-92-93) were incubated for over night (+ 4 °C in refrigerator). The sections were washed 3 times in PBS solution for 5 minutes, and the biotinised secondary antibody (Genemed Biotechnologies REF 54-0003) were applied to them at room temperature for 30 minutes. After washing in PBS (3-5 min), all sections were incubated with peroxidase-bound Strep Avidin (Genemed Biotechnologies REF 54-0003) for 30 minutes. A solution of 3.3-diaminobenzidine tetra hydrochloride (DAB) (Genemed Biotechnologies REF 10-0048) were used as colour revealing substrate. The sections were stained with Mayer Hematoxylin and coated with immune mount.

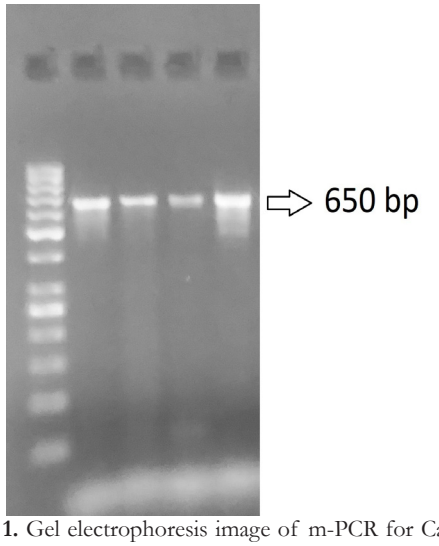

Figure 1. Gel electrophoresis image of m-PCR for Campylobacter spp. 1: DNA marker (Gene ruler 50 bp DNA Ladder, Fermentas); 2-4: Positive samples; 5: Positive control for Campylobacter spp.

RESULTS

Isolation Results

As a result of cultural examination colonies of the *Campylobacter* spp. were isolated showing microscopic characteristics such as small size, pinpoint morphology, non-hemolytic, and Gram-negative "gull-wing" shaped bacilli. Suspected isolates were subjected to biochemical tests. Thus, *Campylobacter* spp. was isolated in 7 (%1.58) of the 444 aborted fetuses. Of the 7 positive Campylobacter cases, 6 were sheep (%2.30, total 261 cases) and only 1 were cattle (%0.55, total 183 cases) specimens.

Macroscopic Results

A small amount of fluid was observed in the abdominal and thoracic cavities with gelatinous and sometimes serous subcutaneous edema. Abomasum contents were determined to be fuzzy and clotted. From 1-2 mm to 1-2 cm, a large number of grizzly white foci are detected in liver. We detected that the central part of these lesions was light brown and collapse,while the outer part was slightly raised and pale (Figure 2a, 2b, 2c).

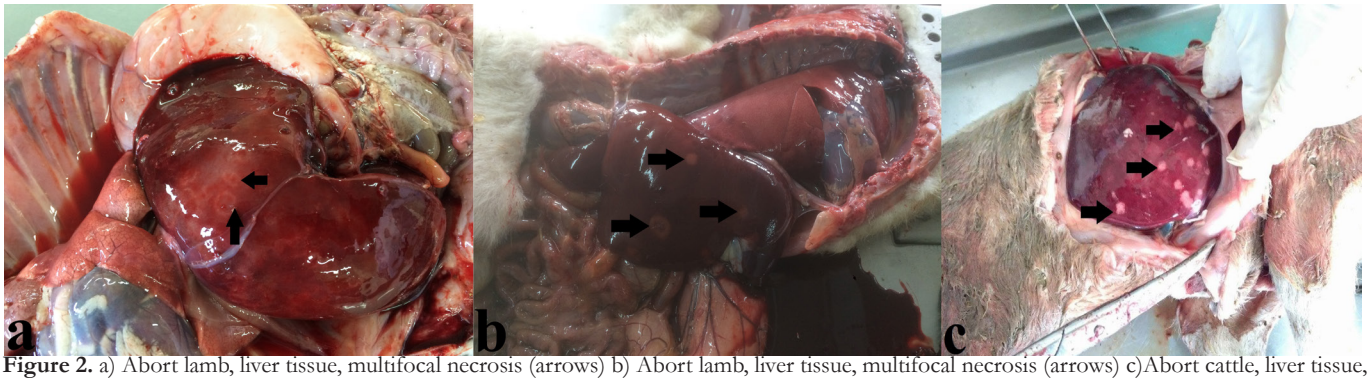

Figure 2. a) Abort lamb, liver tissue, multifocal necrosis (arrows) b) Abort lamb, liver tissue, multifocal necrosis (arrows) c)Abort cattle, liver tissue, multifocal necrosis (arrows)

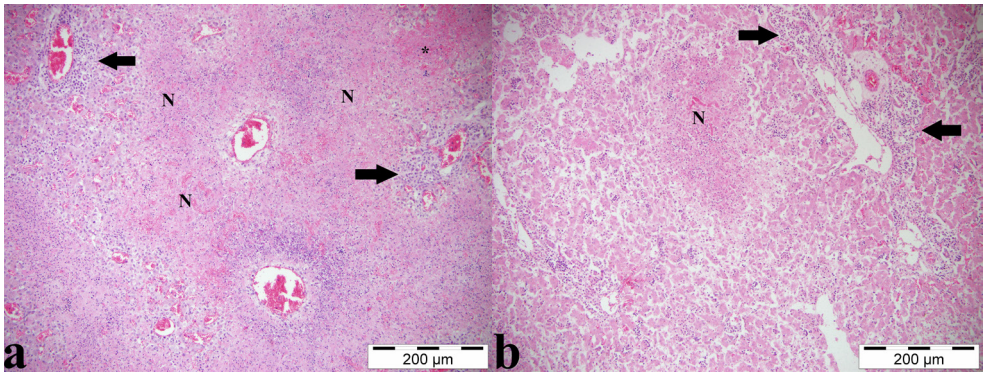

Figure 3. a: Liver tissue, multifocal necrosis (N), hemorrhage (*), mononuclear cell infiltration (arrows), Bar: 200 µm, H&E b: Liver tissue, necrosis (N), hemorrhage (*), mononuclear cell infiltration (arrows), Bar: 200 µm, H&E

Histopathological Results

We observed that multiple necrotic foci in the liver. In addition to these necrosis foci, a severe mononuclear cell infiltration was detected around the vessels. Hemorrhage was another finding in addition to multiple necrosis foci and perivascular mononuclear cell infiltrations (Figure 3a, 3b).

Immunohistochemical Results

We determined *Campylobacter spp.* immunoreactivity, especially in the cytoplasm of hepatocytes around multifocal necrosis areas in the liver. We also observed brown-stained positive reactions in hepatocytes in the middle of necrotic areas (Figure 4a, 4b, 4c, 4d).

PCR Results

7 isolates (6 sheep and 1 cattle), which were phenotypically characterized, were identified as *Campylobacter* spp. by using PCR. (Figure. 1).

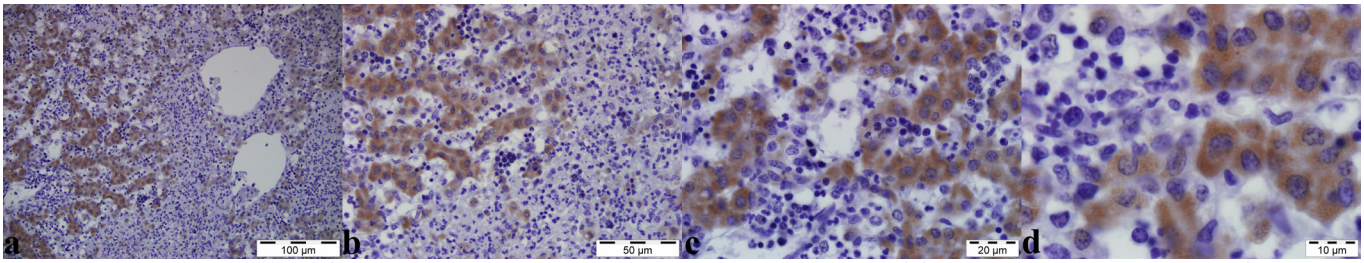

Figure 4. a: Liver tissue, Campylobacter spp. immunoreactivity, Bar: 100 µm, IHC b: Campylobacter spp. immunoreactivity in hepatocytes around the necrosis, Bar: 50 µm, IHC c: Brown positive reaction in the cytoplasm of hepatocytes, Bar: 20 µm, IHC d: Higher magnification, brown positive reaction in the cytoplasm of hepatocytes, Bar: 10 µm, IHC

DISCUSSION

Campylobacter fetus is a curved, motile, non-spore-forming Gram-negative bacillus with one or two polar flagella, and is highly contagious zoonosis causing abortions in cattle and sheep (%5-10) and diarrhea and systemic diseases in elderly and immunocompromised humans (2, 4, 5).

Bovine genital campylobacteriosis is characterized by temporary infertility with mild endometritis, repetition of oestrus, early embryonic deaths and to a lesser extent abortions in the female (15-17). Campylobacteriosis is economically important for cattle breeding worldwide (18). Bovine venereal campylobacteriosis is arises from carrier bulls, but infection can also spread during artificial insemination (6). Campylobacteriosis causes endometritis and salpingitis in cows and heifers as a result of bacteria spreading to uterus and oviducts (19-20). Abortions may occur at any time during pregnancy, but are most commonly observed within a period of 6 to 8 months (21). Variable postmortem changes and histopathological changes such as neutrophilic bronchopneumonia, neutrophilic serositis, fibrinous peritonitis and rarely abomazitis may be observed in aborted fetuses (7,15,22,23). Fetuses in the liver ranging from 1-2 mm to 1-2 cm, randomly spread, varying number of light and colored foci are encountered. The inner parts of these lesions are light brownish decadent and the outer parts are slightly puffy and pale. Microscopic investigations show single cell necrosis with karyomegaly in hepatocytes or necrosis foci in severe cases and mononuclear cell infiltration around these foci (21,22). Campylobacteriosis is highly contagious in sheep. It is characterized by abortions, stillbirths, weak and premature births in the last trimester of pregnancy (24,25). Animals are infected by ingesting contaminated feces on feed and water from infected carrier animals (4). The organism colonizes the intestinal tract of animal, usually without clinical symptoms of diarrhea. A bacteremia may occur in susceptible pregnant sheep causes metritis and placentitis. Placental infections usually lead to fetal septicemia previous to abortions (10,26). Macroscopically, there is gelatinous, sometimes serous subcutaneous edema in the abort fetuses and a slightly bloody fluid in the abdominal and thoracic cavity. The fetus is usually autolytic (10). Similar to previous studies (10,15), we also observed a small amount of fluid in the abdominal and thoracic cavities with gelatinous and sometimes serous subcutaneous edema. In addition, abomasum contents were determined to be fuzzy and clotted. However, the findings in the fetal liver are characteristic (27). Multifocal, pale-white, circular to targetoid necrotic foci in the liver up to 2 cm in diameter is the most diagnostic gross lesion but this lesion is not pathognomonic for Campylobacter abortions (17). Consistent with literature data (17,27), we also detected from 1-2 mm to 1-2 cm, a large number of grizzly white necrotic foci in fetal liver. We found that the central part of these lesions was light brown and collapse, while the outer part was slightly raised and pale. Microscopically, lesions characterized by widespread coagulation necrosis foci, mononuclear cell infiltrations, sinusoidal dilatation and hemorrhage were detected in the abort fetus liver. Lighter lesions were observed in the lung (26,28). Similar to literature data (21,22,26,28), we also observed that multiple necrosis foci in the liver. In addition to these necrotic foci, a severe mononuclear cell infiltration was detected around the vessels. Hemorrhage was another finding in addition to multiple necrosis foci and perivascular mononuclear cell infiltrations. Incompatible with literature data (7,15,22,23) we didn't observe neutrophilic bronchopneumonia in the fetal lung. Consistent with previous studies (21) we determined *Campylobacter spp.* immunoreactivity, especially in the cytoplasm of hepatocytes around multifocal necrosis areas in the liver. We also observed brown-stained positive reactions in hepatocytes in the middle of necrotic areas.

Arda et al. (1987) isolated %7.5 *Campylobacter fetus* from aborted sheep in Central Anatolia Region (29). Erdoğan et al. (1993) isolated %2.7 *Campylobacter fetus* from aborted sheep and goats in Thrace Region (30). Güler et al. (1998) isolated %8.51 *Campylobacter fetus subsp. fetus* from aborted sheep in Konya (31). Sağlam et al. (1998) found % 3.57 *Campylobacter fetus subsp.* from aborted sheep fetus in Erzurum and %1.04 from aborted bovine fetuses and % 5.70 from aborted sheep fetuses in Kars (23). Muz et al. (1999) isolated and identified *Campylobacter fetus subsp. fetus* from aborted sheep and goats in Elazığ and its borders (28). Karaman and Küçükayan (2000) isolated %1.3 *Campylobacter fetus subsp. fetus* from aborted sheep in 17 different provinces (32). Gürtürk et al. (2000) found 23.5% Campylobacter antibodies from sheep blood sera in Van (33). Küçükayan et al. (2007) found 7.44 % in sheep blood sera and 01.29 % in fetuses as *Campylobacter spp.* in Ankara (34). Yeşilmen and Gül (2007) investigated % 10 *Campylobacter spp.* from aborted sheep fetuses in Diyarbakır and its borders (24). Tuzcu et al. (2011) found %6.6 Campylobacteriosis from abort bovine fetuses by immunohistochemical, microbiological and Real Time PCR in Adana and its borders (21). Büyük et al. (2011) found in %10.25 in sheep and goat fetuses as *Campylobacter coli* in Kars (35). In our study, In our study, we found *Campylobacter spp.* to be positive by immunohistochemical, microbiological and PCR methods in 7 (%1.58) of 444 cattle and sheep abort fetuses. Of the 7 positive Campylobacter cases, 6 were sheep (%2.30, total 261 cases) and only 1 were cattle (%0.55, total 183 cases) specimens.

The most important problem of sheep and cattle breeding is abortion (35). Infectious ovine abortions occur due to various bacterial, viral and protozoal agents (10). Most of the factors causing abort in sheep and bovine are of bacterial origin (21,34). It has been shown that the majority of abortions in sheep and cattle breeding are related to Brucellosis, Campylobacteriosis, Listeriosis, Salmonellosis, Leptospirosis, Chlamydiosis (21,33). In particular, most of these diseases are zoonosis and pose an important threat to human health (28). Campylobacteriosis is a highly contagious and zoonotic disease (24). It is known that the source of infection in humans is products from sheep and cattle. In order to prevent and control the disease, the causative agent must be diagnosed quickly and reliably (4). Many different techniques such as serology, PCR, immunohistochemistry and immunofluorescence staining are used in the diagnosis of this disease (24). The old methods used in the diagnosis of campylobacteriosis are time-consuming and partly difficult and do not always give correct results. In particular, PCR has been reported to be used in current studies

for the diagnosis of campylobacteriosis and provides reliable results (4). In our study, we aimed to evaluate old and new methods together. Although there is no difference in molecular and immunohistochemical results, it is more advantageous to use PCR in the diagnosis of this disease for faster and more reliable results (4,21). Only 7 of 17 abortion cases with multifocal, pale-white, circular to targetoid necrotic foci in the liver up to 2 cm in diameter is positive for Campylobacter spp. We thought that the remaining 10 cases gave negative results because of autolysis, placenta was not brought with abort or other enfectious agents such as Flexispira rappini (17).

Kars is an important sheep and cattle breeding region. We believe that reliable and rapid methods such as PCR should be used in the diagnosis of this disease in order to eliminate the economic losses due to abort and to protect human health. In addition, it is obvious that the disease will be detected more effectively if the specimens are delivered to us correctly and on time. For this reason, it is essential to inform the persons dealing with animal husbandry about abortive diseases. In conclusion according to the data of our study; we noted that Campylobacteriosis infection has an important role in the abortion cases in Kars.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest with respect to the publication of this manuscript.

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