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CASE REPORT

Multi-Etiological Abortion due to *Campylobacter* spp. and *Chlamydia abortus* in a Sheep

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ABSTRACT

In this case, multi-etiologic abortion due to *Campylobacter* spp. and *Chlamydia abortus* (*C. abortus*) was reported from an aborted sheep fetus sent to Konya Veterinary Control Institute (KVKE) from a sheep farm operating in Aksaray province in 2020. The presence of *Campylobacter* spp. was determined by the bacterial isolation method. *C. abortus* was identified by qPCR and immunohistochemistry (IHC) methods. In this study, it was aimed to indicate that multi-etiological abortions involving multiple factors should be taken into consideration in the fight against sheep abortions, that it would be appropriate to use a simultaneous multidisciplinary approach in diagnosis to identify abortion factors, and that it could contribute to a more effective fight against abortions.

Keywords: *Campylobacter* spp., *Chlamydia abortus*, Immunohistochemistry, Multi-etiological abortion, PCR.

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Bir Koyunda *Campylobacter* spp. ve *Chlamydia abortus* Tarafından Oluşturulan Multi-Etiyolojik Abort

ÖZ

Bu vakada, Konya Veteriner Kontrol Enstitüsü'ne (KVKE) 2020 yılında, Aksaray ilinde faaliyet gösteren bir koyun işletmesinden gönderilen aborte koyun fetusunda *Campylobacter* spp. ve *Chlamydia abortus* (*C. abortus*) etkenlerine bağlı multi-etiyolojik abort belirlendi. *Campylobacter* spp. varlığı bakteriyel izolasyon yöntemi ile belirlendi. *C. abortus* ise qPZR ve immunohistokimya (İHK) yöntemleri ile tanımlandı. Bu çalışmayla, koyun abortlarıyla mücadelede birden çok etkenin karıştığı multi-etiyolojik abortların dikkate alınması, abort etkenlerini tespit etmek için teşhiste eş zamanlı multidisipliner bir yaklaşımın kullanılmasının uygun olacağı ve abortlarla daha etkin bir mücadeleye katkıda bulunulabileceğini belirtmek amaçlanmıştır.

Anahtar kelimeler: *Campylobacter* spp., *Chlamydia abortus*, İmmunohistokimya, Multi-etiyolojik abort, PCR.

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INTRODUCTION

Pregnancy and birth rates are relatively high in sheep during the mating period. To maintain profitability in ovine breeding, the aim is to have at least one offspring per year. There are significant problems in the birth of healthy lambs in sheep. The main problem affecting this goal in both ovine and bovine animals is abortion, which can cause severe economic losses (Erdem and Sarıbay 2019). Abortion factors that can emerge at any stage of pregnancy are classified as infectious or noninfectious causes. Noninfectious causes include care and nutritional disorders, environmental conditions, and misuse of hormones and drugs (Ay 2017). Infectious causes, which play a much more significant role in the aetiology of abortion, include viral, parasitic, bacterial, and fungal agents. Infectious abortions are often herd-based rather than sporadic cases. Despite the differences in prevalence among countries, the most significant infectious abortion agents in sheep of Türkiye are *Brucella melitensis* and *Chlamydia abortus* (*C. abortus*), *Campylobacter fetus subsp. fetus*, *Salmonella abortusovis*, *Akabane disease virus (Arbovirus)*, *Border disease virus* (*Pestivirus*), *Bluetongue disease virus (Orbivirus)*, *Coxiella burnetii*, *Toxoplasma gondii*, and *Neospora caninum*. Some of these pathogens are zoonotic agents that cause miscarriage and stillbirth in domestic animals and humans (Gulaydın et al. 2023).

Campylobacter spp. colonize the intestines of sheep and cattle. It can cause sporadic abortions in both species (Lastovica and Allos 2008). *Campylobacter* spp. are recognized as critical causative agents of ovine abortions in Türkiye and worldwide. *Chlamydiae*, an obligate intracellular and gram-negative bacterium, causes many diseases in cattle, sheep, goats, pigs, and humans. *Chlamydiae* causes kerato-conjunctivitis, pneumonia, enteritis, hepatitis, mastitis, polyarthritis, sporadic encephalomyelitis, vaginitis, endometritis, fertility problems, and abortion in ruminants (Otter et al. 2003).

This study aimed to describe a case of multi-ethiologic abortion due to *Campylobacter* spp. and *C. abortus* diagnosed in a sheep abortion and to draw attention to multi-ethiologic abortions in the fight against abortions.

CASE HISTORY

Informed consent was obtained from the flock owner for this case report. In 2020, an aborted fetus from a sheep flock in Aksaray province was submitted to the KVCI. According to the information provided by the owner of the herd, there was a small number of abortions in each parturition period in the herd, but the abortion rate reached 30-35% in 2020. It was reported that only the vaccines against *Brucella melitensis* Rev-1 and *Peste Des Petits Ruminants Virus* (PPRV) were applied in the herd within the framework of the programmed vaccinations of the official institutions. The aborted fetus was necropsied, and stomach contents, lung, liver, heart, and umbilical cord were taken for laboratory studies.

Macroscopically, autolytic changes were detected in the aborted sheep fetus at the first examination. In addition, typical macroscopic findings were not observed during necropsy, but the fetal stomach contents were egg-white in colour and consistent. (Figure 1A).

For bacteriological examination, fetal stomach contents and fetal liver tissues were inoculated on *Campylobacter* agar (CM0689, UK) enriched with 7% sheep blood by adding *Campylobacter* selective supplement (Oxoid, Skirrow, SR0069E, UK). Then, the mixture was incubated for 2-3 days at 37 °C in a 10% CO_2 (microaerophilic) oven (Thermo Heracell 150, Germany). The colonies observed after incubation were classified according to their morphological characteristics by Gram staining (Martin et al. 2002). As a result of bacteriologic investigations, *Campylobacter* spp. was isolated from the stomach contents and liver of the aborted fetus. A Gram-stained image of the isolated *Campylobacter* spp. is presented in Figure 1B.

Chlamydiae, which are obligate intracellular bacteria, require a living organism for isolation and multiplication. Therefore, they cannot be isolated using the classical bacteriological culture method. For this purpose, cell culture or inoculation of embryonic chicken eggs are the preferred standard methods for isolating the pathogen. However, these methods may not be considered practical because they require a suitable laboratory environment and involve a laborious process (Woah 2018). In this case, qPCR and IHC methods were used to diagnose *C. abortus*.

The lung, liver, heart, and umbilical cord were frozen at -20 °C after necropsy for DNA extraction. Tissues were then dissected. DNA was extracted from the supernatant after centrifugation via an automatic extraction device (QIAcube, Qiagen, Germany) according to the manufacturer's protocol (IndiSpin Pathogen Kit, Indical Bioscience, Germany). qPCR analysis was performed with Qiagen Rotor-Gene Q (Qiagen, Germany) according to the kit protocol using a primer/probe set targeting the *ompA* gene (Pantchev et al. 2009) and a LightCycler 480 Probe Master Kit (Roche, USA) as indicated in Table 1. qPCR analysis was used to detect *C. abortus* DNA in fetal tissues.

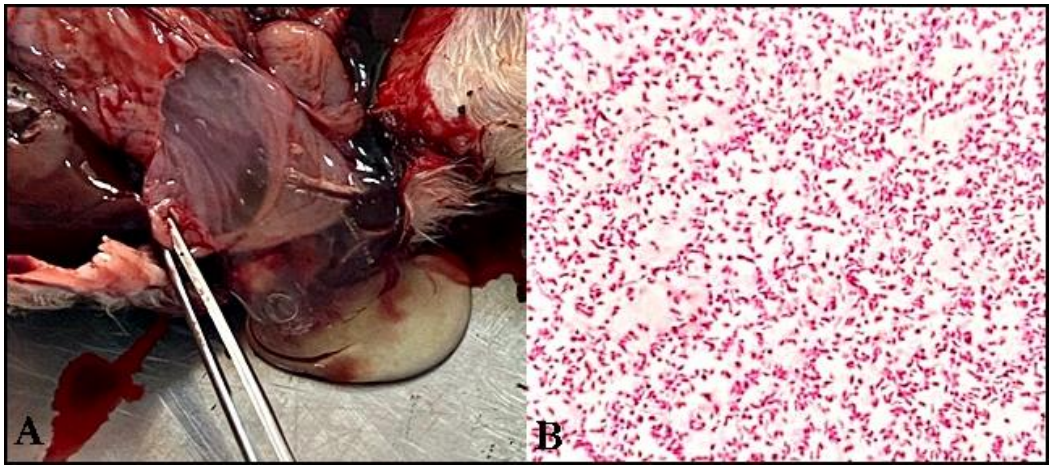

Figure 1: A. Fetal stomach contents. B. Gram stained image of spiral shaped *Campylobacter* spp. bacteria. Original magnification. 1000X.

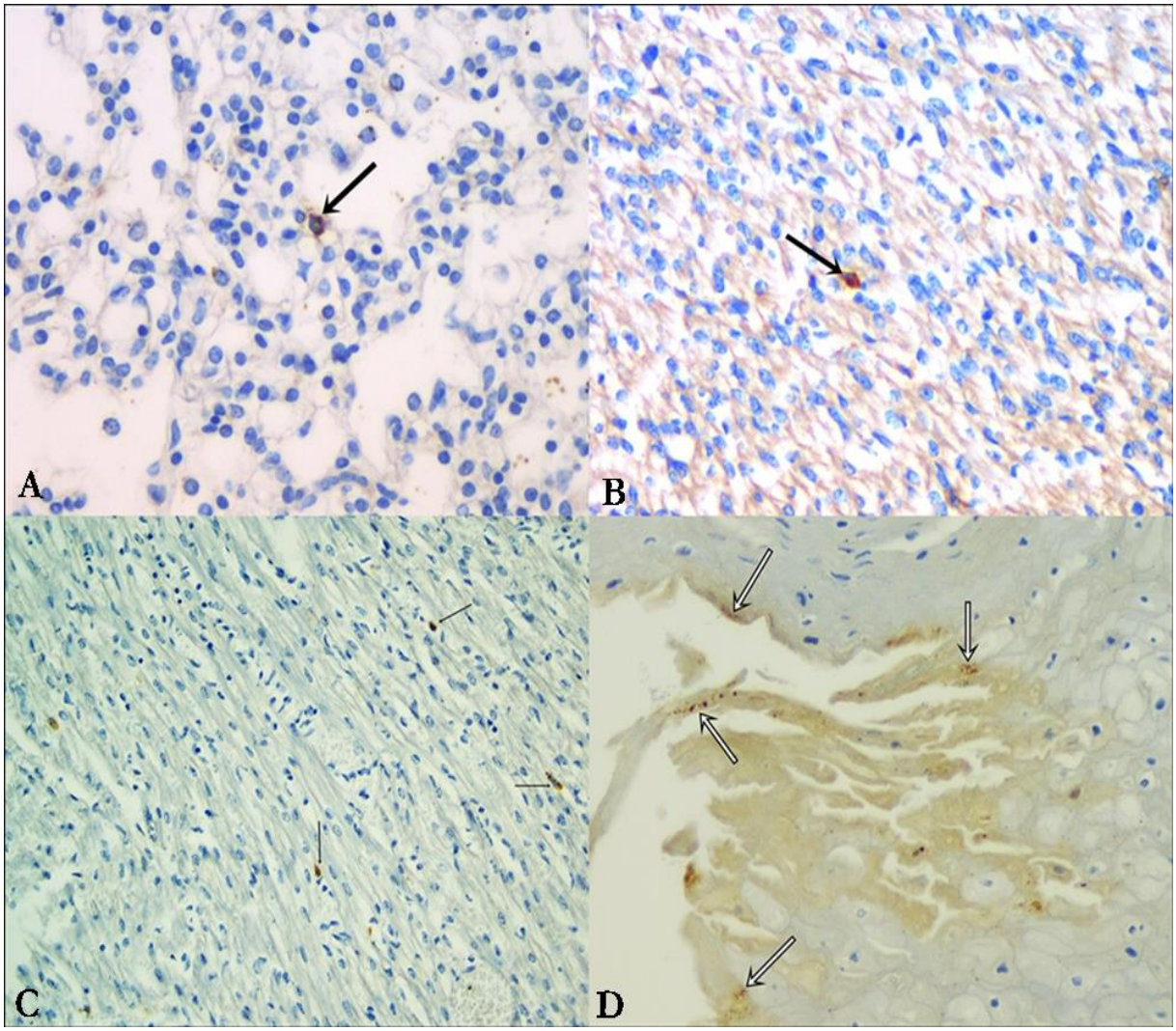

Figure 2: A. Lung. *Chlamydia* spp. positivity in alveolar macrophages (black arrow). Indirect IHC. 400X. B. Liver. *Chlamydia* spp. positivity in macrophages (black arrow). Indirect IHC. 400X. C. Heart. *Chlamydia* spp. positivity in inflammatory infiltrates (black arrows). Indirect IHC. 200X. D. Umbilical cord. *Chlamydia* spp. positivity (white arrows) in epithelial cells. Indirect IHC. 200X.

Table 1. Primer/probe sets are used in PCR.

Abort Agent	Diagnostic Method	Primer/Probe Index	Amplicon Size (bp)	Reference
Akabane Disease Virus	One-Step RT-PCR	AKSF19: 5'-TAA CTA CGC ATT GCA ATG GC-3' AKSR740: 5'- TAA GCT TAG ATC TGG ATA CC-3'	-	Akashi et al. 1999
Border Disease Virus	One-Step RT-PCR	PBD1: 5′-TCGTGGTGAGATCCCTGAG-3′ PBD2: 5′-GCAGAGATTTTTTATACTAGCCTATRC-3′	-	Vilbek and Paton 2000
Bluetongue Disease Virus	RT-PCR	F:5'-TGGAYAAAGCRATGTCAAA-3' R:5'-ACRTCATCACGAAACGCTTC-3' P:5'FAM-ARGCTGCATTCGCATCGTACGC-3' BHQ1	-	Hofmann et al. 2008
C. abortus	qPCR	F: 5'-GCAACTGACACTAAGTCGGCTACA-3' R: 5'-ACAAGCATGTTCAATCGATAAGAGA-3' P:(FAM-AAATACCACGAATGGCAAGTTGGTTTAGCG-TAMRA)	82	Pantchev et al. 2009
Neospora caninum	Nested PCR	JB1: 5′AGGAGGAGAAGTCGTAAGG3′ JB2: 5′GAGCCAAGACATCCATTGC3′	500	Barratt et al. 2008
Peste Des Petits Ruminants Virus (PPRV)	One-Step RT-PCR	NP3: 5'-GTCTCGGAAATCGCCTCACAGACT-3' NP4: 5'-CCTCCTCCTGGTCCTCCAGAATCT-3'	-	Couacy-Hymann et al. 2002
Toxoplasma gondii	qPCR	F: 5'-GGAGGACTGGCAACCTGGTGTCG-3' R: 5'-TTGTTTCACCCGGACCGTTTAGCAG-3' P-1: 5'-ACGGGCGAGTAGCACCTGAGGAGAT-3' P-2: 5'-CGGAAATAGAAAGCCATGAGGCACTCC-3'	126	Costa et al. 2000
Brucella spp.	Bacteriological Culture	-	-	Alton et al. 1988
Campylobacter spp.	Bacteriological Culture	-	-	Martin et al. 2002
Listeria spp.	Bacteriological Culture	-	-	Jinneman et al. 2003
Salmonella spp.	Bacteriological Culture	-	-	Woah 2022

For differential diagnosis, bacteriological cultures were performed from stomach contents and lung, liver, and heart samples to detect the presence of other microaerophilic and aerobic bacterial abortion agents (*Brucella* spp., *Listeria* spp., and *Salmonella* spp.) other than *Campylobacter* spp. Molecular tests were performed to investigate the presence of *Akabane disease*, *Border disease*, *Bluetongue disease viruses*, *Peste Des Petits Ruminants Virus* (PPRV), *Toxoplasma gondii*, and *Neospora caninum* (Table 1). Bacteriological culture and molecular analyses of fetal tissues were negative for *Brucella* spp., *Salmonella* spp., *Listeria* spp., *Akabane virus*, *Border disease virus*, *Bluetongue virus, PPRV*, *Neospora caninum*, and *Toxoplasma gondii.*

Tissue samples taken after necropsy and fixed in 10% buffered formalin were processed according to routine methods and blocked in paraffin. Then, five µm thick sections from the tissue blocks were subjected to IHC staining. Immunohistochemistry was performed on 4-5 µm paraffin sections on poly-L-lysine slides (Isotherm, Türkiye). Staining was then performed by biotinylated indirect IHC staining on a Ventana Benchmark XT using 125 µl of 1/200 diluted *Chlamydiaceae* specific mouse monoclonal

antibody (Cat. No. ACI-P, Progen Biotechnik GmbH, Germany) for each specimen according to the manufacturer's procedure (Ref. No. 760-500, UltraVIEW Universal DAB Detection Kit, USA). All sections of the tissues were examined under a light microscope. *Chlamydia* spp. IHC positive control preparations from the Pathology Laboratory of the Faculty of Veterinary Medicine, University of Zurich, Switzerland, were used. Immunohistochemical staining with sterile PBS was used as a negative control instead of the primary antibody in sections. As a result of IHC staining, granular or homogeneous brown staining with cellular association on a blue background was considered positive. *Chlamydia* spp. immunopositivity was detected in the lungs, liver, heart, and umbilical cord of the aborted fetuses. *Chlamydial* antigens were localized to alveolar macrophages in the lung (Figure 2A), macrophages in the liver (Figure 2B), inflammatory infiltrates in the interstitial space in the heart (Figure 2C), and epithelial cells in the umbilical cord (Figure 2D).

DISCUSSION

Species belonging to the genus *Campylobacter* are responsible for many sheep abortions. Goats are more resistant to this infection, and the abortion rate is lower (Buyuk et al. 2011). *C. abortus* is also a critical abortion agent in Türkiye (Malal and Turkyilmaz 2021). In Türkiye, as in other countries, abortions continue due to the inability to obtain healthy offspring despite strict vaccination programs and severe economic losses that occur in animal husbandry. This situation suggests that in addition to abortions due to a single etiological agent, multiple etiological abortions may be more common than estimated. The introduction mentions that abortion cases in which different etiological agents are detected together have been reported in recent years (De Angelis et al. 2022; Ramo et al. 2022; Şevik et al. 2017a; Şevik et al. 2017b; Deniz and Oruc 2023). In the present study, a case of multi-etiological abortion caused by *Campylobacter* spp. and *C. abortus* in a sheep abortion was described.

Recent studies on abortion in Türkiye have shown positivity for different etiologic agents. Gulaydın et al. (2023) examined a total of 113 samples from 85 several sheep flocks by qPCR for bacterial abortion agents. They found that 42.8 % were positive for *C. abortus*, 25.7 % for *B. melitensis*, 14.2 % for *S. abortusovis*, 11.4 % for *Coxiella burnetii*, 2.8 % for *L. monocytogenes* and 2.8 % for *Campylobacter* spp. Although 42.8% *C. abortus* and 2.8 % *Campylobacter* spp. positivity was detected in the study of Gulaydin et al. (2023); it is understood that these results were obtained from different animals, and these agents were not multi-etiologically detected in a single aborted fetus. In this case, the presence of *Campylobacter* spp. and *C. abortus* in a single aborted fetus was demonstrated simultaneously with a multidisciplinary approach. Although 42.8 % C. abortus and 2.8 % *Campylobacter* spp. positivity was detected in the study by Gulaydin et al. (2023); it is understood that these results were obtained from different animals, and these agents were not detected multi-etiologically in a single aborted fetus. Sakmanoglu et al. (2019) investigated the epidemiology of pathogenic bacteria in 250 stomach contents of aborted fetuses of cattle, sheep, and goats from different regions of Türkiye by PCR and found 155 positive samples for bacterial agents. Of these positive samples, 58.88 % were found in sheep, 43.47 % in goats, and 67.15 % in cattle samples. The five most common bacteria associated with abortion were *Brucella melitensis* at 20.9 %, *B. abortus* at 5.2 %, *Leptospira* spp. at 13.6 %, *Campylobacter fetus* at 20.9 %, and *Coxiella burnetii* at 1.6 %. They did not find any positivity for *C. abortus* in the study. In addition, a multi-ethiologic situation in terms of bacterial agents was not reported in the positive results obtained in the study. In this case, the presence of *C. abortus* and *Campylobacter* spp. in a single aborted fetus was demonstrated simultaneously with a multidisciplinary approach.

Among the studies conducted worldwide, Ramo et al. (2022) in Spain reported both *Campylobacter* spp. and *C. abortus* positivity in 10 cases in a study conducted with qPCR in sheep and goat abortion materials. In addition, IHC staining results that were consistent with experimental and field studies investigating *Chlamydial* antigen localization were obtained (Livingstone et al. 2017).

In line with our studies and our report, domestic animals should not be ignored because they are not constantly exposed to a single abortion agent and because different etiological agents may play a role together and cause multiple etiological abortions. In an abortion study previously published (Deniz and Oruc 2023), *Brucella* factors were shown to accompany *Chlamydia* infection. In this regard, multi-etiological abortion cases due to *Chlamydia* should also be investigated, as should other multi-etiological miscarriages. Moreover, a multidisciplinary approach in which multiple factors are taken into consideration instead of a single etiological factor in abortion cases is necessary. Recognizing this and making a complete diagnosis will enable more accurate and better protection measures to be taken for animal health.

In our study, *Campylobacter* spp. was diagnosed by bacteriologic isolation, and *C. abortus* was diagnosed using qPCR and IHC methods. Therefore, it would be more beneficial to test many factors in abortion cases with multidisciplinary diagnostic methods.

In the studies by Tuzcu et al. (2010), Aydin et al. (2020), and Karakurt et al. (2020), multifocal necrotic hepatitis with a characteristic target board appearance in the liver was detected histopathologically in *Campylobacter* spp. positive abortions, whereas typical liver lesions were not observed in this case. It is thought that the reason why typical liver lesions were

not observed in this case may be due to the fact that the amount of bacteria was not at a level that caused typical lesions in the liver.

Many bacterial, viral, and parasitic agents found in Türkiye were investigated in the study, such as *Coxiella burnetii* and *Leptospira* spp. *Mycoplasma* spp. *C. pecorum, C. psittaci*, and *sheep pox virus* were not evaluated as abortion agents. Simultaneous screening for these abortion agents in the identification of multi-ethiologic abortion cases will help to identify abortion agents and control multi-ethiologic abortion.

CONCLUSION

In conclusion, *Campylobacter* spp. and *C. abortus* were detected in sheep offspring in Türkiye in this study, and it was found that similar abortion patterns should be considered in abortion control. It is known that *Brucella* species and *C. abortus* are the most common abortion pathogens in small ruminants in Türkiye. In abortion cases, *Brucella* is usually evaluated first, and in case of negativity, other agents are evaluated one by one according to their prevalence in sheep. In the case of positivity for any of these agents, a separate evaluation for other bacterial, viral, and parasitic agents is not usually performed. In this study, we tried to emphasize the importance of performing a multidisciplinary evaluation for bacterial, viral, and parasitic agents simultaneously in every abortion sample that comes to laboratories for diagnostic purposes. The knowledge of the existence of abortions with multifactorial aetiology will contribute to the diversification of the measures (preventive vaccination, treatment, etc.) to be taken in the fight against abortion factors. In addition, it was concluded that these infectious agents should be taken seriously and studied individually with a broader range of diagnostic methods rather than relying on limited methods of laboratory diagnosis.

Conflict of Interest: The authors declare that there are no conflicts of interest.

Authors' Contributions: Case review, evaluation of findings, images, manuscript writing and submission of the manuscript were performed by ID. The discussion section was organized and contributed to the discussion by EO.

Ethical Approval: "This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules." (Ref No: 701825, Date: 02/2024).

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