

## Original Article

## A Study on Mycoplasmal and Viral Infections in Bovine Keratoconjunctivitis

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## ABSTRACT

**Background:** Infectious bovine keratoconjunctivitis (IBK or “pink eye”) is the most common infectious ocular disease in cattle worldwide. In addition to *Moraxella bovis* as the principal causative agent, infectious bovine rhinotracheitis virus (BHV-1) and *Mycoplasma* species probably act as risk factors for IBK.

**Objectives:** This study aimed to evaluate the association between the detection of *Mycoplasma* sp., bovine herpesvirus 1 (BHV-1), and bovine viral diarrhea virus (BVDV) in the conjunctival sac of the eye and IBK.

**Methods:** Polymerase chain reaction (PCR) was employed to detect *Mycoplasma* sp., BHV-1, and BVDV in samples collected from IBK-affected and healthy eyes.

**Results:** Based on the PCR results, *Mycoplasma* sp. was detected in 63.6% and 47.2% of IBK-affected and healthy eyes, respectively. BHV-1 was detected in 59.1% and 36.1% of affected and healthy eyes, respectively. BVDV was detected in 65.9% and 58.3% of affected and healthy eyes, respectively. BHV-1 was the only agent significantly ( $P < 0.05$ ) associated with IBK lesions (isolated from 59.1% of affected vs 36.1% of healthy eyes).

**Conclusion:** Based on the study results, BHV-1 may be a risk factor in the pathogenesis of infectious bovine keratoconjunctivitis, and mechanisms other than immune depression might be involved in its pathogenicity.

**Keywords:** BHV-1, BVDV, Infectious bovine keratoconjunctivitis, *Mycoplasma* sp

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## 1. Introduction

**I**nfectious bovine keratoconjunctivitis (IBK or “pink eye”) is the most common ocular disease of cattle worldwide. It is a highly contagious disease that spreads rapidly within a herd. The spread is by direct contact, nasal/ocular discharges, and mechanical vectors. IBK is most common in calves, typically affecting one eye, although both eyes may be affected. Irritation of the eye caused by intense sunlight, dust, pollen, or grass seeds is a major predisposing factor to infection. Corneal ulcers, corneal edema, photophobia, blepharospasm, and lacrimation are the main clinical manifestations of IBK (Angelos, 2010; Kneipp, 2021; Maier et al., 2021). The progression of the clinical signs has been divided into several stages. Blepharospasm, photophobia, conjunctivitis, and a lot of watery discharge are the earliest signs of the disease in stage 1. Corneal opacification, corneal vascularization, and cloudiness of the discharges are characteristics of stage 2. Corneal ulceration may develop at stage 3 (Sargison et al., 1996).

Mixed infections are often established and act concomitantly or successively, resulting in pink eye (Levisohn et al., 2004). *Moraxella bovis* is the most common bacterial species associated with the disease and appears well-suited to cause IBK. Other agents, such as *Moraxella ovis*, *Moraxella bovoculi*, *Chlamydomphila*, *Mycoplasma*, and *Ureaplasma* sp., and bovine herpesvirus (BHV-1) have been implicated too (Alexander, 2010; Betzebe et al., 2020). *Mor. bovis* is the only pathogen to have reproduced IBK-like ocular lesions in various experimental models, including gnotobiotic calves, indicating its capability to induce disease without other potential pathogens. For other members of the genus, primarily *Mor. bovoculi*, direct causality appears less clear, as reproduction of the disease following experimental infection with pure culture has not yet been achieved (Loy et al., 2021b).

*Mycoplasma* species may cause conjunctivitis in cattle, either alone or in conjunction with *Mor. bovis*, and therefore may increase the disease’s severity or may have a role in the pathogenesis of the disease (Betzebe et al., 2020). In an outbreak in young calves, *Mycoplasma bovoculi* and *Mycoplasma bovis* were the only agents isolated from the affected eyes. However, *Mor. bovis* was not isolated as the principal agent (Levisohn et al., 2004). Recently, the investigators hypothesized that herds with higher *Myc. bovoculi* prevalence are predisposed to acute outbreaks of IBK, possibly due to syn-

ergism with *Moraxella* sp., and studies show that *Myc. bovoculi* may be an under-detected component of IBK, and polymerase chain reaction (PCR) testing may reveal its presence at high levels in diagnostic submissions and during outbreaks. Other *Mycoplasma* species, including *Mycoplasma bovirhinis* and *Mycoplasma bovigenitalium* in a mixed infection with bovine herpesvirus-1 (BHV-1), have been reported to be associated with IBK. The significance of these findings is unknown because the role of the individual agents was not evaluated, and the results were inferred from a series of case reports (Loy et al., 2021a).

Ocular manifestations of BHV-1 infection may occur as an isolated clinical entity or involving other body systems. Conjunctivitis is the most common ocular manifestation, but corneal vascularization, perilimbal edema, and opacification also occur in severe cases. Various studies have considered infectious bovine rhinotracheitis virus (BHV-1) as a risk factor for IBK (George et al., 1988; Nayar & Saunders, 1975; Pugh et al., 1970). A potential mechanism to explain the association of IBK with BHV-1 is that BoHV-1 causes immune depression, which may predispose the host to superinfection with bacterial pathogens (Loy et al., 2021a). However, in a recent study, BHV-1 was not found to be associated with IBK (Zbrun et al., 2011). Ocular abnormalities in congenitally affected calves are the main ocular manifestations of bovine viral diarrhea virus (BVDV) infection. Ocular discharges have been reported in acute or chronic cases of BVD, though the significance of these observations is uncertain (Perdrizet et al., 1987). Corneal opacity might be seen as a part of acute mucosal disease (Betzebe et al., 2020). Similar to BHV-1, considering the role of BVDV in immunodeficiency, the virus may be important in the pathogenesis of IBK.

To date, a limited number of studies have been conducted to investigate the isolation of Mycoplasmal and viral agents and clarify their role in the pathogenesis of IBK. This study aims to evaluate the association of *Mycoplasma* species, BHV-1, and BVD viruses with infectious bovine keratoconjunctivitis (IBK) in industrial dairy farms around Tehran Province, Iran.

## 2. Materials and Methods

### Study sampling

Samples were obtained from 10 dairy farms that reported IBK outbreaks during our study in Tehran and Alborz Provinces, Iran. The herd size on these farms varied from small scale with around 200 cows, to large-scale farms, with about

4000 cows. Samples were collected from female cows (aged older than 3 months). Those affected by IBK did not receive any treatment before sampling. Only one of the eyes of each affected or healthy individual was sampled. A total of 80 individuals were sampled; 44 showed signs consistent with IBK, and 36 were healthy, showing no signs of IBK.

At the time of sampling, affected eyes were categorized based on the clinical disease stage. Conjunctivitis is characteristic of stage 1, stages 2 and 3 are characterized by corneal opacification and corneal ulceration, respectively.

Samples were taken from the conjunctival sac with sterile cotton swabs. The swabs were transferred to the tube containing 1.5 mL of sterile PBS (phosphate buffered saline), then taken to the laboratory and stored at -70°C until performing PCR assay to detect *Mycoplasma* species, BVD, and BHV-1 viruses.

***Mycoplasma* sp. DNA extraction and polymerase chain reaction**

CinnaGen DNP™ kit (CinnaGen™-Cat. No.: DN8115C) was used to extract *Mycoplasma* sp. DNA from the obtained samples. Samples were thawed at room temperature (22°C). DNA was extracted according to the manufacturer’s instructions.

*Mycoplasma* sp. PCR detection kit (CinnaGen™-Cat. No.: PR901649) was used for detecting the genus level. The primer set in this kit allows the detection of about 50 species of *Mycoplasma*. Detection requires 1-5 femtograms (fg) of *Mycoplasma* DNA or 2-5 *Mycoplasma* per sample. Briefly, 5 µL of extracted DNA solution was mixed with 20 µL ready-to-use PCR mix. All PCR assays were performed with the preheated thermocycler (Bio-Rad™-MJ Mini thermal cycler) using an initial denaturation step of 2 min at 94°C, followed by 35 cycles with 15 s at 94°C, 15 s at 52°C, 35 s at 72°C, and a final extension step of 5 min at 72°C. Finally, 10 µL of PCR products were visualized on 1.5% agarose gel after

staining with ethidium bromide. The presence of 280 bp (base pairs) fragments indicates positive test results.

**Viral genome extraction and polymerase chain reaction**

**Genome extraction**

Samples were thawed at room temperature (22°C). According to the manufacturer’s instructions, genome extraction was performed using a CinnaPure Viral (CinnaGen™-Cat. No.: PR921733) kit capable of simultaneously isolating viral DNA and RNA.

**cDNA synthesis for BVDV**

For cDNA synthesis, 8 µL of RNA, 1 µL of random hexamer (Cinnagen), and 3.5 µL of dH<sub>2</sub>O were denatured at 65°C for 5 min and cooled on ice. The following was added to each reaction tube, 1 µL of M-MuLV reverse transcriptase (Cinnagen), 2 µL 10X reaction buffer, 2 µL dNTP (Cinnagen), 0.5 µL ribonuclease inhibitor (Vivantis), and 2 µL dH<sub>2</sub>O to give a final reaction volume of 20 µL, which was then incubated for 60 minutes and 10 minutes at 42°C and 70°C, respectively.

**Nested RT-PCR for BVDV**

In the first round of nested PCR, amplification of cDNA was carried out in a total volume of 25 µL containing 3 µL of cDNA, 12.5 µL PCR Master Mix (Cinnagen), 1 µL of each primer, and 7.5 µL dH<sub>2</sub>O. Using the thermocycler, the reaction mix was subjected to 35 cycles of 95°C 1 min, 53°C 1 min, and 72°C 1 min. For the second round, 30 cycles were conducted with denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and elongation at 72°C for 1 min.

The primer sets’ sequences, position in the NADL strain of the BVDV genome, and the expected size of amplified products are indicated in Table 1.

**Table 1.** Sequence of primer sets used for detecting BVDV

Primer	Sequence	Position in the NADL	Final Product Size (in Base Pairs)
324	5'-ATGCCC(A/T)TAGTAGGACTAGCA-3'	108-128	171 bp
326	5'-TCAACTCCATGTGCCATGTAC-3'	395-375	
Pesti3	5'-CCTGAGTACAGGA/GTAGTCGTCA-3'	176-196	
Pesti4	5'-GGCCTCTGCAGCACCCATCA-3'	345-325	

**Table 2.** Sequence of primer sets used for detecting BHV1 virus

Primer	Sequence	Position in the BHV-gD	Final Product Size (in Base Pairs)
P3	5'-GCTGTGGGAAGCGGTACG-3'	351-368	
P4	5'-GTCGACTATGGCCTTGTGTGC-3'	817-796	425 bp
P5	5'-CCTGAGTACAGGA/GTAGTCGTCA-3'	394-422	

### Semi-nested PCR for BHV-1

For the first round of amplification, the PCR reaction was performed in a solution containing 3 µL of extracted DNA, 12.5 µL PCR Master Mix (CinnaGen), 1 µL of each primer, and 7.5 µL dH<sub>2</sub>O. The reaction mix was heated in a thermocycler for 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For the second round, the mixture was subjected to 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

The first and second round primer sets were designed to amplify a fragment of 468 bp and 425 bp of BHV-1 gD (glycoprotein D gene). The sequences are listed in Table 2.

### Agarose gel electrophoresis

Aliquots of 10 µL from PCR products were submitted to electrophoresis in a 1.5% agarose gel for approximately 45 min. The gel was stained with ethidium bromide and visualized under UV light.

### Statistical analysis

Statistical evaluation of obtained data was performed using SPSS software, version 20. The chi-square test was used to investigate statistical differences between different variables.

### 3. Results

This study investigated the association between Mycoplasma and viral agents present in the conjunctival sac and stages of clinical signs. About 55% of sampled eyes had clinical signs consistent with IBK. The remaining 45% were healthy eyes showing no clinical signs at sampling.

Among the affected eyes, 31.8% (14 out of 44), 31.8% (14 out of 44), and 36.4% (16 out of 44) had signs consistent with stages 1, 2, and 3, respectively.

Tables 3 and Table 4 summarize the results obtained by PCR.

BHV-1 virus prevalence was significantly different ( $P < 0.05$ ) between healthy and infected eyes.

Among positive PCR results, 62.2% of eyes infected by *Mycoplasma* sp., 66.7% by BHV-1, and 58% by BVDV were affected by various stages of IBK.

### 4. Discussion

Even though *M. bovis* is the primary causative agent, the roles of other species of *Moraxella* (*Mor. ovis* and *Mor. bovoculi*), *Mycoplasma* sp. (mainly *Myc. bovoculi*), and BHV-1 are not well understood. BHV-1 virus and *Mycoplasma* sp. are probably the most important associated agents besides *Mor. bovis* and likely act as risk factors for IBK.

**Table 3.** Frequency and percentage of positive and negative PCR results compared based on health status

Eyes	No. (%)					
	<i>Mycoplasma</i> PCR (P=0.141)		BHV-1 Semi-nested PCR (P=0.041)*		BVDV Nested PCR (P=0.486)	
	+	-	+	-	+	-
Affected	28(63.6)	16(36.4)	26(59.1)	18(40.9)	29(65.9)	15(34.1)
Healthy	17(47.2)	19(52.8)	13(36.1)	23(63.9)	21(58.3)	15(41.7)
Total	45(56.3)	35(43.7)	39(48.8)	41(51.2)	50(62.5)	30(37.5)

\*Significant difference ( $P < 0.05$ ) between the affected and healthy eyes.

**Table 4.** Frequency and percentage of positive and negative PCR results compared based on the clinical stage of the disease

Stages	No. (%)					
	<i>Mycoplasma</i> PCR (P=0.803)		BHV-1 Semi-nested PCR (P=0.317)		BVDV Nested PCR (P=0.582)	
	+	-	+	-	+	-
1	8(57.1)	6(42.9)	6(42.9)	8(57.1)	9(64.3)	5(35.7)
2	9(64.3)	5(35.7)	9(64.3)	5(35.7)	8(57.1)	6(42.9)
3	11(68.8)	5(31.2)	11(68.8)	5(31.2)	12(75)	4(25)

Either alone or in conjunction with *Mor. Bovis*, *Mycoplasma* sp. may have a role in the pathogenesis of IBK and the severity of related lesions (Betbeze et al., 2020; Pugh et al., 1970). Surveys indicate that the prevalence of *Myc. bovoculi* in cattle eyes is quite high in both clinical and nonclinical animals. It has been experimentally demonstrated that prior infection by ocular instillation of *Myc. bovoculi* enhances and prolongs colonization of *Mor. bovis* and *Mor. ovis*. The observation that *Myc. bovoculi* and *Moraxella* sp. may be isolated in co-infection also suggests that *Myc. bovoculi* may play a role in the development of IBK syndrome (Levisohn et al., 2004). In the study by Gupta et al. (2015) aiming to determine the role of *Mycoplasma* sp. in infectious keratoconjunctivitis of ruminants, 37.5% of bovine samples evaluated by PCR assay were *Mycoplasma* positive. In the study by Schöttker-Wegner et al. (1990), no correlation between clinical findings and isolation of Mycoplasmas could be demonstrated. Mycoplasmas were found in 43.2% of eyes with clinical symptoms of IBK and in 41.2% of healthy eyes (Schöttker-Wegner et al., 1990). Although Mycoplasmal agents were presented in a higher percentage of affected eyes, no significant difference was found between affected and healthy eyes. In the study by Schnee et al. (2015), the prevalence of infection with *Mor. ovis*, *Mor. bovoculi*, and *Myc. bovoculi* were assessed. Briefly, their results indicated that herds early in IBK had a higher prevalence of *Myc. bovoculi* detected by PCR than those recovering from or without clinical IBK. Thus, the investigators concluded that herds with high *Myc. Bovoculi* prevalence are more predisposed to outbreaks of IBK (Schnee et al., 2015). However, according to the observations of this study, although not significant, with the higher stage of the clinical disease, the higher the percentage of eyes infected with *Mycoplasma* species.

Bovine rhinotracheitis virus (BHV-1) infection has been considered a risk factor for developing clinical cases of IBK. The virus is reported to be highly prevalent in

Iran. The seroprevalence of BHV-1 in Iran were 31.9% and 72.2% in two recent studies published in 2015 and 2020, respectively (Nikbakht et al., 2015; Noaman & Nabinejad, 2020). In an experimental study, Pugh et al. (1970) elucidated the role of BHV-1 as a risk factor for IBK. First, by inoculating calves with the virus, all animals developed viral conjunctivitis. After inoculation with *Mor. bovis*, 70% of animals developed IBK lesions. Second, another group of calves was first infected with *Mor. bovis* and then with BHV-1. Afterward, only 50% of the animals developed IBK lesions. In the experiment by Nayar and Saunders (1975) in calves, simultaneous inoculation of eyes with *Mor. bovis* and BHV-1 increased the severity of lesions compared to those calves inoculated merely by *Mor. bovis*. George et al. (1988) evaluated the effects of a modified-live infectious bovine rhinotracheitis virus vaccine on experimentally induced infectious bovine keratoconjunctivitis. The findings indicated that calves vaccinated against BHV-1 with modified live virus vaccines were more susceptible to infection with *Mor. bovis* compared to unvaccinated animals. Nonetheless, in a recent study, BHV-1 exposure, as indicated by positive titer, was not found to predispose calves to develop IBK in a herd where *Mor. bovis* was present (Zbrun et al., 2011). In contrast to the findings of Zbrun et al., our results showed a significant difference in the presence of BHV-1 in the conjunctival sac of affected and healthy eyes. Based on our results, although not significantly different, the higher the stage of the disease, the higher the percentage of eyes infected with BHV-1. In addition, considering our anecdotal and unpublished observation, vaccination with a conventional combined inactivated vaccine of IBR, BVD, and PI3 after the herd outbreak we sampled in one of the farms resulted in a much milder outbreak of IBK in the following year. Accordingly, exposure to BHV-1 could be associated with IBK.

The present study evaluated the probable role of BVDV as a risk factor for IBK. BVDV-associated immunodeficiency may be of importance in the pathogenesis and susceptibility of cattle to IBK. The prevalence of BVD in Iran has been mainly reported based on antibody detection against BVDV. The disease was first reported in 1970 in Iran by serum neutralization test, and the estimated seroprevalence varied from 16% to 69% (Khodakaram-Tafti et al., 2016; Mokhtari & Mahzoonieh, 2014). The BVDV seroprevalence rates were 64.4% and 52.8% in two more recent studies published in 2015 and 2020, respectively (Nikbakht et al., 2015; Noaman & Nabinejad, 2020). The result of these studies could justify the high exposure of animals and their eyes in the presented research. However, our results showed no significant difference between affected and healthy eyes in the presence of BVDV in the conjunctival sac. Also, no significant difference was seen between the disease's clinical stage and the virus's presence.

As mentioned earlier, immune depression is a potential mechanism associated with increased susceptibility to IBK after exposure to and infection with viruses like BHV-1 and BVDV. Based on our results, a statistically significant difference was only seen when BHV-1 was presented, and the presence of BVDV in the conjunctival sac did not differ significantly between healthy and affected eyes. Therefore, mechanisms other than immune depression might be involved and should be considered when pathogenicity and association of BHV-1 with IBK outbreaks are investigated.

In conclusion, *Mycoplasma* sp. and BHV-1 virus could act as risk factors for developing IBK and enhancement of severity of lesions. Although, based on our results, a statistically significant difference was only seen when BHV-1 was presented.

## Ethical Considerations

### Compliance with ethical guidelines

All procedures were conducted according to the animal care guideline of the Research Committee of the Faculty of Veterinary Medicine, University of Tehran.

### Funding

The paper was extracted from the PhD thesis of Parham Mottaghian, approved by Department of Internal Medicine, University of Tehran.

## Authors' contributions

Conceptualization and supervision: Afshin Raofi; Methodology: Omid Madadgar and Iradj Ashrafi Tamai; Investigation, writing original draft, review & editing: Parham Mottaghian; Data collection: Parham Mottaghian and Arya Badiçi; Data analysis: Parham Mottaghian; Funding acquisition and resources: Afshin Raofi and Parham Mottaghian.

## Conflict of interest

The authors declared no conflict of interest.

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## مقاله پژوهشی

## بررسی عفونت‌های مایکوپلاسمایی و ویروسی در کراتوکونژنکتیویت عفونی گاوان

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## چکیده



**زمینه مطالعه:** کراتوکونژنکتیویت عفونی گاوان، شایع‌ترین بیماری چشمی گاوها در سرتاسر جهان است. افزون بر موراکسلا بویس به‌عنوان عامل اصلی بیماری، ویروس رینوتراکثیت عفونی گاو (هرپس ویروس تیپ ۱ گاوی) و گونه‌های مایکوپلازما احتمالاً به‌عنوان فاکتورهای خطر کراتوکونژنکتیویت عفونی گاوان مطرح می‌باشند.

**هدف:** این مطالعه با هدف ارزیابی وجود ارتباط بین شناسایی گونه‌های مایکوپلازما، هرپس ویروس تیپ ۱ گاوی و ویروس اسهال ویروسی گاو در کیسه ملتحمه چشم و کراتوکونژنکتیویت عفونی گاوان انجام شد.

**روش کار:** واکنش زنجیره‌ای پلیمرز برای شناسایی گونه‌های مایکوپلازما، هرپس ویروس تیپ ۱ گاوی و ویروس اسهال ویروسی گاو در نمونه‌های دریافت‌شده از چشم‌های مبتلا به کراتوکونژنکتیویت عفونی و چشم‌های سالم به کار گرفته شد.

**نتایج:** براساس نتایج آزمون پی‌سی‌آر، گونه‌های مایکوپلازما به‌ترتیب در ۶۳/۶ درصد از چشم‌های مبتلا و ۴۷/۲ درصد از چشم‌های سالم شناسایی شدند. هرپس ویروس تیپ ۱ گاوی به‌ترتیب در ۵۹/۱ درصد از چشم‌های مبتلا و ۳۶/۱ درصد از چشم‌های سالم و ویروس اسهال ویروسی گاو نیز در ۶۵/۹ درصد از چشم‌های مبتلا و ۵۸/۳ درصد از چشم‌های سالم شناسایی شدند. هرپس ویروس تیپ ۱ گاوی به‌عنوان تنها عامل دارای ارتباط معنادار ( $P < 0/05$ ) با ضایعات بیماری مورد شناسایی قرار گرفت (جدادشده از ۵۹/۱ درصد از چشم‌های مبتلا و ۳۶/۱ درصد از چشم‌های سالم).

**نتیجه‌گیری نهایی:** براساس نتایج این مطالعه، هرپس ویروس تیپ ۱ گاوی ممکن است به‌عنوان فاکتور خطر در پاتوژنز کراتوکونژنکتیویت عفونی گاوان نقش داشته باشد و مکانیسم‌هایی به غیر از تضعیف ایمنی نیز در پاتوژنیسیته بیماری دخالت داشته باشد.

**کلیدواژه‌ها:** بیماری‌های چشمی، کراتوکونژنکتیویت عفونی گاوان، گونه‌های مایکوپلازما، ویروس اسهال ویروسی گاو، هرپس ویروس تیپ ۱ گاوی

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