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A Study on Mycoplasmal and Viral Infections in Bovine Keratoconjunctivitis

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Running Title: Mycoplasmal and Viral Infections in IBK

Abstract

Background: Infectious bovine keratoconjunctivitis (IBK or "pink eye") is the most common infectious ocular disease of cattle throughout the world. 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 detection of *Mycoplasma* sp., bovine Herpesvirus 1 (BHV-1) and bovine viral diarrhea viruses (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. were detected in 63.6% and 47.2% of IBK affected and healthy eyes. BHV-1 were detected in 59.1% and 36.1% of affected and healthy eyes, respectively. BVDV were 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).

Conclusions: Based on the results of this study, BHV-1 may play a role as 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 Keratoconjunctivits, *Mycoplasma* sp.

Introduction

Infectious bovine keratoconjunctivitis (IBK or "pink eye") is the most common ocular disease of cattle occurring in their populations throughout the world. It is a highly contagious disease that spreads rapidly within a herd. The spread is by direct contact, nasal and ocular discharges, and by 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, Doan, & O'Connor, 2021). The progression of the clinical signs has been divided in to several stages. Blepharospasm, photophobia, conjunctivitis and a lot of watery discharge are the earliest signs of the disease evident in the stage 1. Corneal opacification, corneal vascularization and cloudiness of the discharges are characteristics of the stage 2. Corneal ulceration may develop at stage 3 (Sargison, Hutner, West, & Gwozdz, 1996).

Mixed infections are often established and act in concert or successively to result in pinkeye (Levisohn, Garazi, Gerchman, & Brenner, 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*, *Chlamydophila*, *Mycoplasma* and *Ureaplasma* sp., and bovine Herpesvirus (BHV-1) have been implicated (Alexander, 2010; Betbeze et al., 2020). *Mor. bovis* is the only pathogen to have reproduced IBK-like ocular lesions in various experimental models, including in gnotobiotic calves, indicating that it is capable of inducing disease in the absence of other potential pathogens. For other members of the genus, primarily *Moraxella bovoculi*, direct causality appears less clear, as reproduction of the disease following experimental infection with pure culture has not yet been achieved. (Loy, Hille, Maier, & Clawson, 2021).

Mycoplasma species may cause conjunctivitis of cattle, either alone or in conjunction with *Moraxella bovis* and therefore may increase the severity and may have a role in pathogenesis of the disease (Betbeze et al., 2020). In an outbreak in young calves, *Mycoplasma bovoculi* and *Mycoplasma bovis* were the only agents isolated from the affected eyes, However, *Moraxella bovis*, as the principal agent, was not isolated (Levisohn *et al*., 2004). Recently, the investigators hypothesized that herds with higher *Mycoplasma bovoculi* prevalence are predisposed to acute outbreaks of IBK, possibly due to synergism with *Moraxella* sp. and studies show that *Mycoplasma bovoculi* may be an under-detected component of IBK, and PCR testing may reveal it is present at high levels in diagnostic submissions and during outbreaks. Other Mycoplasmas, including *Mycoplasma bovirhinis* and *Mycoplasma bovigenitalium* in a mixed infection with bovine herpesvirus-1 (BHV-1), have been reported associated with IBK. The

significance of these findings is unknown because the role of the individual agents was not evaluated and the findings stem from a series of case reports (Loy, Clothier, & Maier, 2021).

Ocular manifestations of BHV-1 infection may occur as an isolated clinical entity or with involvement of other body systems. Conjunctivitis is the most common ocular manifestation, but corneal vascularization and 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, Ardans, Mihalyi, & Guerra, 1988; Nayar & Saunders, 1975; Pugh, Hughes, & Packer, 1970). A potential mechanism to explain the association of IBR with IBK is that BoHV-1 causes immune depression which could predispose the host to superinfection with bacterial pathogens (Loy, Clothier, *et al*., 2021). However, in a recent study, BHV-1 was not associated with IBK (Zbrun, Zielinski, Piscitelli, Descarga, & Urbani, 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 cases of acute or chronic cases of BVD, though the significance of these observations is uncertain (Perdrizet, Rebhun, Dubovi, & Donis, 1987). Corneal opacity might be seen as a part of acute mucosal disease(Betbeze et al., 2020). Likewise BHV-1, considering the role of BVDV in immunodeficiency, the virus may be of importance in the pathogenesis of IBK.

To date, a limited number of recent studies have been carried out to investigate isolation of Mycoplasmal and viral agents and to clarify the role of these agents in the pathogenesis of IBK. The

objective of this study is 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.

Materials and Methods:

Sampling

Samples were obtained from 10 dairy farms reported outbreaks of IBK during our study which were located in provinces "Tehran" and "Alborz", Iran. The herd size on these farms varied from small scale with around 200 cows to large scale farms with around 4000 cows. Samples were collected from female individuals (aged greater than 3 months). Those affected By IBK did not receive any treatment before the time of sampling. Only one of the eyes of each affected or healthy individuals was sampled. Totally, 80 individuals were sampled; 44 of those showed signs consistent with IBK; 36 were healthy showing no signs of IBK.

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

Samples were taken by sterile cotton swabs from conjunctival sac. The swabs were transferred to the tube containing 1.5 ml of sterile PBS (Phosphate Buffered Saline), then taken to the laboratory and

stored in -70 ˚C freezer until the time of 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 obtained samples. Samples were thawed at room temperature (22 °C). DNA was extracted according to the manufacturer's instruction.

Mycoplasma sp. PCR detection kit (CinnaGen™ - Cat. No.: PR901649) was used for detection at the genus level. The primer set in this kit, allows detection of about 50 species of *Mycoplasma*. Detection requires at least 1-5 femtograms (fg) of *Mycoplasma* DNA or 2-5 *Mycoplasma* per sample. Briefly, 5 µl of extracted DNA solution was mixed with 20µl 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 sec. at 94, 15 sec. at 52 °C and 35 sec. 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 instruction, genome extraction was performed using CinnaPure Viral (CinnaGen™ - Cat. No.: PR921733) kit which is capable of simultaneous isolation of 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 dH2O was 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 dH2O 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 ml containing 3 µl of cDNA, 12.5 µl PCR Master Mix (Cinnagen), 1 µl of each primer and 7.5 µl dH2O. 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 carried out 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 the table 1.

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 dH2O. The reaction mix was heated in thermocycler for 40 cycles of 94 °C 1 min, 60 °C 1 min and 72 °C 1 min. For the second round, the mixture was subjected to 30 cycles of 94 °C 1 min, 60 °C 1 min and 72 °C 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 indicated 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 Statistics 20.0. The Chi-square test was used to investigate statistical differences between different variables.

Results

In this study, the association between the presence of Mycoplasmal and viral agents present in conjunctival sac and stages of clinical signs was investigated. 55% of sampled eyes had clinical signs consistent with IBK. The remaining 45% were healthy eyes showing no clinical signs at the time of 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 4 summarized the results obtained by Polymerase chain reaction (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.

Discussion

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

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 seems quite high in both clinical and nonclinical animals. It has been demonstrated experimentally that prior infection by ocular instillation with *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 the 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 (Gupta *et al*., 2015). 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 as well as in 41.2% of healthy eyes (Schottker-Wegner, Binder, & Kirchhoff, 1990). In our study, although Mycoplasmal agents were presented in 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 *Mycoplasma bovoculi* were assessed. Briefly, their results indicated that herds early in the course of 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, Heller, Schubert, & Sachse, 2015). However, according to the observations of this study, although not significant, the more progressed the stage of the clinical disease, the higher the percentages of eyes infected with *Mycoplasma* species.

Infection with infectious bovine rhinotracheitis virus (BHV-1) 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 were first infected with *Mor. bovis* and then with BHV-1 (Pugh et al., 1970). Afterwards, 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 (Nayar & Saunders, 1975). 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. (George *et al*., 1988) Nonetheless, in a recent study, BHV-1 exposure as indicated by positive titer was not found to predispose calves to developing IBK in a

herd where Moraxella 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.

In the present study, the probable role of BVDV as a risk factor for IBK was also evaluated. 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 on the basis of the detection of antibody against BVDV. The disease was first reported in 1970 in Iran by serum neutralization test and the estimated seroprevalence were varied between 16% to 69% (Khodakaram-Tafti, Mohammadi, & Farjani Kish, 2016; Mokhtari & mahzonieh, 2014). The BVDV seroprevalence 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 high exposure of animals and their eyes in the presented study. However, our results did not show any significant difference between affected and healthy eyes in the presence of BVDV in the conjunctival sac. As well, no significant difference was seen between the clinical stage of the disease and the presence of the virus.

As mentioned earlier, immune depression is one of the potential mechanisms that might be associated with increased susceptibility to IBK after exposure to and infection with viruses like BHV-1 and BVDV. Based on our results, statistical 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 it might be concluded that 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 factor for developing IBK and enhancement of severity of lesions. Although, based on our results, statistical significant difference was only seen when BHV-1 was presented.

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Conflict of interest statement

None of the authors of this paper have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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بررسی عفونت های مایکوپلاسمایی و ویروسی در کراتوکونژنکتیویت عفونی گاوان

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چکیده فارسی

زمینه مطالعه: کراتوکونژونکتیویت عفونی گاوان شایع ترین بیماری چشمی گاوها در سرتاسر جهان می باشد. افزون بر موراکسلا بویس بعنوان عامل اصلی بیماری، ویروس رینوتراکئیت عفونی گاو (هرپس ویروس تیپ 1 گاوی) و گونه های مایکوپلاسما به احتمال بعنوان فاکتورهای خطر کراتوکونژونکتیویت عفونی گاوان مطرح می باشند.

350 **هدف:** این مطالعه با هدف ارزیابی وجود ارتباط بین شناسایی گونه های مایکوپلاسما، هرپس ویروس تیپ 1 گاوی و ویروس اسهال ویروسی گاودر کیسه ی ملتحمه ی چشم و کراتوکونژونکتیویت عفونی گاوان انجام شد.

روش کار: واکنش زنجیره ای پلیمراز (PCR) برای شناسایی گونه های مایکوپلاسما، هرپس ویروس تیپ 1 گاوی و ویروس اسهال ویروسی گاو در نمونه های اخذ شده از چشم های مبتلا به کراتوکونژونکتیویت عفونی و چشم های سالم کار گرفته شد.

355 **نتایج:** براساس نتایج آزمون پی سی آر، گونه های مایکوپلاسما به ترتیب در 63/6 درصد از چشم های مبتلا و 47/2 درصد از چشم های سالم شناسایی شدند. هرپس ویروس تیپ 1 گاوی به ترتیب در59/1 درصد از چشم های مبتلا و 36/1 درصد از چشم های سالم مورد شناسایی قرار گرفت. ویروس اسهال ویروسی گاو نیز در 65/9 درصد از چشم های مبتلا و 58/3 درصد از چشم های سالم شناسایی شد. هرپس ویروس تیپ 1 گاوی بعنوان تنها علمل دارای ارتباط معنی دار

(P<0.05)با ضایعات بیماری مورد شناسایی قرار گرفت (جدا شده از 59/1 درصد از چشم های مبتلا و 36/1 درصد از چشم های سالم).

نتیجه گیری نهایی: بنابراین بر اساس نتایج این مطالعه، هرپس ویروس تیپ 1 گاوی ممکن است بعنوان فاکتور خطر در پاتوژنز کراتوکونژونکتیویت عفونی گاوان نقش داشته باشد و مکانیسم هایی بغیر از تضعیف ایمنی نیز در پاتوژنیسیته ی بیماری دخالت داشته باشد.

کلمات کلیدی: بیماری های چشمی، کراتوکونژونکتیویت عفونی گاوان، گونه های مایکوپلاسما، ویروس اسهال ویروسی گاو، هرپس ویروس تیپ 1 گاوی

Tables:

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Primer	Sequence	Position in the NADL	Final product size (in base pairs)
324	5'- ATGCCC(A/T)TAGTAGGACTAGCA-3'	108-128	171 bp (Hyndman et al, 1998)
326	5'- TCAACTCCATGTGCCATGTAC-3'	395-375	
Pesti3	5'-CCTGAGTACAGGA/GTAGTCGTCA-3'	176-196	
Pesti4	5'-GGCCTCTGCAGCACCCTATCA-3'	345-325	

Table 1. Sequence of primer sets used for detection of BVDV.

Primer	Sequence	Position in the BHV-gD	Final product size (in base pairs)
P3	5'-GCTGTGGGAAGCGGTACG-3'	351-368	425 bp (Takiuchi et al, 2005)
P4	5'-GTCGACTATGGCCTTGTGTGC-3'	817-796	
P5	5'-CCTGAGTACAGGA/GTAGTCGTCA-3'	394-422	

Table 2. Sequence of primer sets used for detection of BHV1 virus.

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	Mycoplasma 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%

Table 3. Frequency and percentage of positive and negative PCR results compared based on health status. * shows significant difference ($p<0.05$) between affected and healthy eyes.

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	Mycoplasma PCR (p=0.803)		BHV-1 Semi-Nested PCR (p=0.317)		BVDV Nested PCR (p=0.582)	
	+	-	+	-	+	-
Stage 1	8 57.1%	6 42.9%	6 42.9%	8 57.1%	9 64.3%	5 35.7%
Stage 2	9 64.3%	5 35.7%	9 64.3%	5 35.7%	8 57.1%	6 42.9%
Stage 3	11	5	11	5	12	4

	68.8%	31.2%	68.8%	31.2%	75%	25%

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

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