https://dergipark.org.tr/tr/pub/ataunivbd
2020; 15(2): 106-112

Research Article/Araştırma Makalesi
DOI: 10.17094/ataunivbd.656701

Atatürk Üniversitesi Veteriner Bilimleri Dergisi
Atatürk University Journal of Veterinary Sciences

Determination of Seroprevalence of Infectious Bovine Keratoconjunctivitis Disease by ELISA and AGID*

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Geliş Tarihi/Received	Kabul Tarihi/Accepted	Yayın Tarihi/Published
07.12.2019	15.04.2020	27.10.2020

Bu makaleye atıfta bulunmak için/To cite this article:
Ataseven L: Determination of Seroprevalence of Infectious Bovine Keratoconjuctivitis Disease by ELISA and AGID. Atatürk University J. Vet. Sci., 15(2): 106-112, 2020. DOI: 10.17094/ataunivbd.656701

Abstract: Infectious Bovine Keratoconjunctivitis (IBK) is a highly contagious disease of conjunctiva and cornea in cattle which causes significant economic losses in cattle breeding. The objective of this study is to determine serum antibodies against *M. bovis* and obtaining information about the seroprevalence of IBK by AGID test and ELISA. Serum samples, collected from 552 cattle in different herds in Turkey were examined for specific antibodies against *Moraxella bovis* (*M. bovis).* The seropositivity of IBK in cattle sera with clinical signs was determined as 26.1% and 5.8% by ELISA and AGID, respectively. Seropositivity in sera samples of healthy appeared cattle were detected as 26.3% and 5.6% by ELISA and AGID, respectively. According to the results, the highest seropositivity (36.5%) was detected in the animals aged 12-24 months. Similar seroprevalence detection rates were recorded in cattle with and without clinical signs. Clinical signs, spesifically, does not give knowledge about the exact percentage of the IBK. Therefore, besides isolation and identification of the agent, serological tests like ELISA, as a screening test, should be preferred to determine the presence and spread of IBK in the herd.

Keywords: Agar gel immunodiffusion, ELISA, *Moraxella bovis*, Seroprevalence, Turkey.

Sığırların Infeksiyöz Keratokonjuktivitis Hastalığının Seroprevalansının ELISA ve AGID Testi ile Belirlenmesi

Öz: Sığırların infeksiyöz keratokonjunktivitis hastalığı (IBK) sığır kornea ve konjunktivasının çok bulaşıcı bir infeksiyöz hastalığı olup sığır yetiştiriciliğinde önemli ekonomik kayıplara neden olmaktadır. Bu çalışmada, ELISA ve AGID ile sığır serumlarında *M. bovis*'e karşı oluşmuş serum antikorlarının belirlenmesi ve IBK'nın seroprevalansı ile ilgili bilgi edinilmesi amaçlandı. Araştırmada Türkiye'deki çeşitli çiftliklerdeki sığırlardan temin edilen toplam 552 kan serumu *Moraxella bovis (M. bovis)*'e karşı oluşmuş spesifik antikorlar yönünden teste tabi tutuldu. Klinik bulgu gösteren hayvanlara ait serumların %26.1' i ELISA, %5.8' i ise Agar jel immünodifüzyon testi ile pozitif tespit edildi. Sağlıklı görünen hayvanlara ait serumların ise %26.3'ü ELISA, %5.6'sı Agar jel immünodifüzyon testi ile pozitif olarak tespit edildi. Sonuçlara göre en yüksek seropozitiflik 12 ila 24 aylık sığırlarda (%36.5) saptandı. Klinik bulgu gösteren ve klinik bulgu göstermeyen sığırlarda seroprevalans oranlarının benzer olduğu tespit edildi. Keratokonjunktivit gibi klinik bulgular, spesifik olarak IBK'nın sürüdeki gerçek oranı hakkında bilgi vermemektedir. Bu nedenle etkenin izolasyon ve identifikasyonunun yapılmasının yanısıra hastalığın sürüdeki varlığının ve yaygınlığının tespiti için tarama amacıyla ELISA gibi serolojik bir test tercih edilebilir.

Anahtar Kelimeler: Agar jel immünodiffüzyon, ELISA, *Moraxella bovis,* Seroprevalans, Türkiye.

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*The paper is summarized from the same named PhD thesis of author.

INTRODUCTION

Infectious Bovine Keratoconjunctivitis (IBK) has been associated with ocular disease characterized by lacrimation, photophobia, conjunctivitis, keratitis, corneal opacity, and corneal ulceration in cattle and, the disease causes severe economic loss in cattle breeding worldwide as a highly contagious disease (1-6). *Moraxella bovis* (*M. bovis*) is considered as primary etiologic agent of IBK (3,5,7,8) which was characterized as the Gram-negative aerobic/microaerophilic rod-shaped coccobacillus (9). Many serotypes of *M. bovis* vary in pathogenicity and some other Moraxella species like *Moraxella bovoculi* (*M. bovoculi*) and *Moraxella ovis (M. ovis*) have also been isolated from the IBK cases. According to new investigations, the most significant bias is the failure to show a specific association between cases and organism colonization, so, *M. bovoculi* or other alike bacteria may be the secondary invading pathogen in IBK lesions (6,10-12).

In order to determine the antibodies against *M. bovis* in natural or experimentally infected cattle; techniques like plate and tube agglutination tests, passive hemagglutination, agar gel immunodiffusion (AGID), indirect immunofluorescent and enzyme-linked immunosorbent assay (ELISA) can be used (7, 13-17). Although the immunodiffusion test can detect the serum antibodies against *M. bovis*, ELISA detects the antibodies against *M. bovis* both in blood sera and lacrimal secret (15,17). That's why, ELISA remains an important tool for diagnostic purposes, particularly in determining seroconvert animals within the population on field investigations about IBK.

Several studies indicated that the infection is more prevalent in the world including the USA, Australia, Ethiopia, India, Britain, Scotland, Ireland, Japan, Hungary, Nigeria, and New Zealand etc. (3,18-23). Although there have been reports about the isolation of *M. bovis* in Turkey (24-26) serological studies about the disease are limited (24). The objective of the present study was to determine the anti-*M. bovis* antibodies by ELISA and AGID and obtain data about the seroprevalence of IBK. This is the first use of ELISA for the investigation of the disease in Turkey.

MATERIALS and METHODS

Serum Samples

A total of 552 blood sera obtained from 483 healthy-appearing and 69 symptomatic (epiphora, conjunctivitis, keratitis, opacity) cattle from 6 different regions were examined. Herds sampled and the number of samples were shown in Table 1. The current study was carried out in accordance with ethical principles.

Table 1. The source of test serum and distribution of serum numbers by age groups.
Tablo 1. Test serumlarının kaynağı ve serum sayılarının yaş gruplarına göre dağılımı.

	Clinical signs (+/-)		Ages				Total
	+	-	0-6 months	7-11 months	12-24 months	>24 months	
Bala AE*(Ankara)	24	115	77	24	7	31	139
Gelemen AE* (Samsun)	9	43	-	20	32	-	52
Karaköy AE* (Samsun)	24	218	46	76	47	73	242
Bolu-Düzce-Gerede	4	72	11	12	15	38	76
Bursa	6	18	10	1	6	7	24
Kars	2	17	-	-	19	-	19
Total	69	483	144	133	126	149	552

*A.E. : Agricultural Enterprise (Tarım işletmesi)

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA protocol to *M. bovis* specific antibodies was adapted from Bishop et al. (15). *Haemolytic M. bovis* strain (IBH63 Iowa Strain, NADC, Ames) was used as a test and immunization antigen. In order to assess the absorbance values as positive or negative, 10 negative and 2 positive control sera were included in each plate. Sera, which have 3 fold OD values than the mean OD values of negative control sera were accepted as positive. Positive control immunesera were prepared according to the method reported by Pugh et al. (27) ELISA antigen was prepared by a method reported by Bishop et al. (15) and, the chessboard method was used to determine the optimal dilutions of conjugate and antigen which used in the assay.

Agar Gel Immunodiffusion (AGID)

Immunization antigen, test antigen, and positive sera were prepared according to the method reported by Gil-Turnes and Araujo (16). For the standardization of AGID test, different percentages of Noble Agar and Purified Agar were examined as test media, and different dilutions of AGID antigens were tested. It was concluded to use the 1% of purified agar in veronal buffer solution as test media and ½ of antigen dilution was concluded to use as test antigen. The protein quantity of the test antigen was determined as 1167 µg/ml by the Lowry method. The titer of immunsera was determined by the use of this antigen. At each test media (plate) one positive and one negative control sera were included.

Statistical Analysis

According to *M. bovis* antibodies, statistics expressed in numbers and percentages. The Kappa (K) test was used to determine the correlation between the tests and the difference between the age groups was evaluated by the Chi-square test (28).

RESULTS

Of 552 sera, 145 (26.3%) were found positive with ELISA, and 31 (5.6%) were found to be positive by AGID. While the correlation between ELISA and AGID test was found as 0.28 by Kappa analyze, the difference between analyses was found significant (P<0.001). The seroprevalence results of the sampled sera are shown in Table 2.

Table 2. ELISA** and AGID*** test results of serum samples.
Tablo 2. Serum örneklerinin ELISA** ve AGID*** testi sonuçları.

Sampling Areas	Number of Sera samples (n)	ELISA positive sera % (n)	AGID positive sera % (n)
Bursa	24	62.5 (15)	16.6 (4)
Kars	19	36.8 (7)	0 (-)
Bolu-Düzce-Gerede	76	25 (19)	5.26 (4)
Karaköy* (Samsun)	242	19 (46)	2.89 (7)
Gelemen* (Samsun)	52	59.6 (31)	7.69 (4)
Bala* (Ankara)	139	19.4 (27)	8.6 (12)
Total	552	26.3** (145)	5.6** (31)

*A.E. : Agricultural Enterprise, **Difference was found significant by Kappa Analyse (P<0.01).
** Enzyme Linked Immunosorbent Assay *** Agar Gel Immunodiffusion

Sera from the cattle with clinical signs and without clinical signs were found 26.1% (18/69) and 26.3% (127/483) positive via ELISA respectively; and 5.8% (4/69) and 5.6% (27/127) of them were found seropositive by AGID.

According to the seropositivity, the difference between the animals with or without clinical signs was determined as statistically not significant (P>0.05). The results were summarized in Table 3.

Table 3. The ELISA** and AGID*** results of clinical sign positive and negative animals.
Tablo 3. Klinik belirti gösteren ve göstermeyen hayvanların ELISA** ve AGID*** sonuçları.

Clinical sign (+/-) (n)	ELISA antibody positive* % (n)	AGID antibody positive* % (n)
Clinical sign positive (69)	26.1[a] (18/69)	5.8[b] (4/69)
Clinical sign negative (483)	26.3[a] (127/483)	5.6[b] (27/483)

* The difference between the groups with the same letters was not significant (P>0.05) and the difference between the groups with different letters in the same row was significant (P<0.01). ** Enzyme Linked Immunosorbent Assay *** Agar Gel Immunodiffusion

According to the age groups ELISA positivity rates were as follows 18.05% (26/144) in 0-6 months, 29.3% (39/133) in 7-11 months, 36.5% (46/126) in 12-24 months, and 22.8% (34/149) were positive in elder animals. The difference between the age groups was found significant statistically (P<0.01).

DISCUSSION and CONCLUSION

In Turkey, Erdeğer (24**)** reported seropositivity against *M. bovis* as 6.3% (32/507) in sera delivered from the slaughterhouse of Meat and Fish Authority (Ankara), Karacabey Agricultural Enterprise (Bursa) and Veterinary Control Central Research Institute (originated from Sivas, Bala, Malatya, Urfa) by AGID. AGID results (5.6%) here were compatible with the results of Erdeğer.

In the current study seropositivity against *M. bovis* was determined as 26.3% by ELISA. This rate is higher than the AGID results. In fact, ELISA can detect even very low levels of antibodies against the agent (29). Additionally, experimental infection and vaccine studies have shown that precipitant antibodies became to be determined on the 28th day by AGID (27,30). The difference between the antibody determination rates is thought to be because of the higher sensitivity of ELISA than AGID and the ability of ELISA to detect the antibodies that occur in the early stages of infection.

Clinical sign positive animals were determined as positive (5.8%) by AGID. This rate was found to be 5.6% in healthy-looking animals. Antibody positivity in healthy-looking animals suggests that these animals were previously infected or that they were persisted following an acute infection. After an IBK epidemic, the infection can persist for up to 10 weeks. Animals that do not show clinical signs are reservoirs especially for a young and sensitive herd (7,31). Eyes, which are less affected, healed in two weeks, and the animals who have not lost their vision have recovered in four weeks. While the recovery in some animals lasting 4-6 weeks, chronic keratoconjunctivitis may be developed in others (20,32). The reason for the low rate of precipitant antibodies in animals with clinical signs was suggested that *M. bovis* infection was due to the fact that antibody titers were not yet formed at a detectable level. Because, serum precipitant antibodies in experimentally generated infections were began to be determined on 28-48 days (27,32). Different variants of *M. bovis* vary in pathogenicity and pink eye has multifactorial etiology (6,10).

In this study, the sonicated antigen was used in the ELISA technique, and in 26.3% of the sampled animals, *M. bovis* specific antibodies were detected. 26.1% of the animals with clinical findings and 26.3% of healthy-looking animals were found positive with ELISA. The antibody positivity in animals without corneal signs in three herds previously IBK detected was reported between 33% to 100% (32). It suggested that this result might be due to a non-symptomatic low-pathogenic *M. bovis* infection. In the current study, the presence of *M. bovis* antibodies in single blood serum sampled from each animal was investigated. There was not any data about seroprevalence determined by ELISA in Turkey, so it could not be possible to compare here. The positivity in healthy-looking animals was considered due to a previous infection with *M. bovis*, or due to a low-pathogenic strain, or because of a persistent infection. The absence of detectable anti-*M. bovis* antibodies in the presence of clinical symptoms suggested other agents except for *M. bovis* that could

cause the same symptoms. In addition, it may be possible that the detectable level of IgG response could not be established in these animals due to acute infection.

Although infection is seen in adult and elder cattle, young animals are more sensitive to IBK infection is more severe in young animals and higher infection rates are reported in animals younger than 2 years old (1,3,34). In addition, symptoms can be observed in calves of healthy-looking infected mothers (31). In a previous study described by Pugh et al. (33), all the vaccinated and non-vaccinated calves (1.7 and 4-4.5 months old) has infected after *M. bovis* challange. However, no significant difference was found between the prevalence of IBK in very young and relatively older calves. Dodt (34), classified animals as cattle, young oxen, and calves in his prevalence study in Australia in northeastern Queensland. In his study, 50.11% of Shorthorn cattle, 18.53% of the young ox, and 30.46% of 6 months old calves were found IBK positive. On the other hand, positivity percentages of Brahman hybrids cattle, young oxen, and calves were 7.47%, 7.07%, and 5.18% respectively. In this study, the ages were grouped as 0-6, 7-11, 12-24 months, and <2 years. The highest percentage of ELISA positivity was 36.5% in 12-24 months of animals. The difference between these age groups was statistically significant. The presence of infection in young animals (12-24 months and 7-11 months, respectively) was more consistent with other studies. However, when the seropositivity rates of cattle older than 2 years of age and calves with 0-6 months of age (22.8% and 18.05%, respectively) were compared, the difference between rates were not found statistically significant but the relative difference between them was similar as described by Dodt (34).

In conclusion, serological data about IBK were obtained by ELISA in Turkey for the first time. Especially animals those who have persistent IBK infection are a reservoir for the young and sensitive herds. Therefore, it is very important to determine the presence of this very contagious disease (IBK) in the herd. By ELISA, we detect the prevalence of about 26% and by AGID it was about 5.6-5.8%. Serological tests like ELISA can be preferred to determine the presence and situation of IBK in the sensible herds. Also, by this study, the seroprevalence of *M. bovis* was determined by ELISA in Turkey for the first time. Infection is prevalent in cattle with and without clinical signs. According to the same seropositivity rates in these animals, it is concluded that clinical signs do not give knowledge about the exact situation of IBK in the herd. Therefore, besides isolation and identification of the agent, serological monitoring is necessary. As ELISA detects the antibodies against *M. bovis* in higher percentages, it can be preferred for this aim.

Conflict of interest

The author declares no conflict of interest.

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