Investigation of *Mycoplasma* Species in Diseased and Healthy Calves and Heifers in Al-Najaf Province, Iraq

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Abstract. *Mycoplasma infections are among the most common causes of eye infection in cattle worldwide. The purpose of this work was to use a DNA-based method to investigate the presence of Mycoplasma spp. in the conjunctival sac of diseased and healthy calves and heifers. Between May 2018 and August 2019, a total of 116 eye swab samples were taken from seven industrial dairy farms in Al-Najaf province, where 85 samples were collected from diseased animals and 31 from healthy animals. Concerning the PCR test, 6.4% and 28.2% were respectively positive for healthy and diseased animals. Conjunctivitis, keratoconjunctivitis and corneal ulcers were reported in 52%, 31% and 17% of the diseased group, respectively. In conclusion, Mycoplasma spp. important bovine pathogen causing multiple eye lesions that can not be identifed due to their asymptomatic nature.*

Introduction

Corneal and conjunctival infections are amongst the most common eye diseases in livestock worldwide. A predisposing eye infection was considered to be eye irritation caused by exposure to sunlight, dust, pollen, seeds, and buds. Most cases of ocular lesions show corneal ulcers, corneal blindness, corneal oedema, light anxiety, blepharospasm, and abundance of tear excretion (Underwood et al., 2015, Brooks et al., 2017).

Mycoplasma is one of the main pathogens affecting a competitive animal health condition in most countries (Nicholas & Ayling, 2003). Mycoplasma exploits chronic diseases that affect the animal, leading to the suppression of the body's general immune status, providing an appropriate environment for infection with different *Mycoplasma* species. In cattle, various *Mycoplasma* species are considered to be a real cause of mastitis, respiratory tract infections, arthritis, genital tract infections, otitis, keratoconjunctivitis, and abortion (Maunsell & Donovan, 2009, Calcutt et al., 2018), which leads to massive economic losses in the sector of animals worldwide (Aebi et al., 2012, Matilda et al., 2018, Loria et al., 2018).

Moraxella bovis is indeed among the most common causes of eye diseases in cattle, but mycoplasma is no less harmful. Other pathogens like *Moraxella ovis* and *Chlamydia species* are also regarded as a common cause. Mycoplasma has many species, including *M. agalactiae, M. bovis*, *M. californicum, M. bovirhinis, M. alkalescens*, *M. mycoides*, *M. dispar*, *M. canadense*, *M. bovigenitalium, M. conjunctivae,* and *M. bovoculi* (Loria et al., 2018, Salih & Rosenbusch, 2001, Nicholas et al., 2000).

Mycoplasma alone can cause an ocular lesion in cattle, but a mixed infection with *Moraxella bovis* may increase the severity and pathogenicity of the disease (Alberti et al., 2006, Gould et al., 2013).

Mycoplasma is a self-reproducing microorganism that is recognized as the smallest one. They spread globally, either as free-living or as parasites of humans, animals and plants (Razin, 1992). Mycoplasmas are phenotypically distinct from other bacteria by their minute size and complete absence of a cell wall, as well as restricted metabolic ability (Razin et al., 1998). Using antibiotics such as penicillin that inhibit cell wall synthesis is futile in treating mycoplasma diseases because it lacks the cell wall (Klöckner et al., 2016, Naveed et al., 2020).

Because of restricted genetic potency of mycoplasmas, they need a complicated growth medium to consist of the medium enriched with peptone, yeast extract, animal serum and animal cell (Smith, 2012, Gerdtzen, 2017). There is a limited condition for mycoplasma growth, such as a specific host, a specific tissue and a specific organ, that reflects the precise nutritional character (Volokhov et al., 2011)

In order to control mycoplasma infections, a precise diagnosis must be made and suspected animals must be exluded from the herd because there is no effective vaccine against mycoplasma infections (Nicholas et al., 2008b, HA, 2013).

In addition to the shared antigenicity among pathogenic and non-pathogenic mycoplasmas, the costly and time-consuming culture of mycoplasmas make diagnosis difficult by routine technique (Pilo et al., 2007, Smith, 2012). The PCR test provides a more

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precise method of mycoplasma detection in both acute and chronic infections and offers a greater scope for detection than culture and serological methods (Rossetti et al., 2010, Touati et al., 2014).

The occurrence of mycoplasma in Iraqi cattle is unclear, and not all diagnostic laboratories do isolate mycoplasma generally. For this reason, a DNA-based approach was employed to identify the presence of *Mycoplasma* spp. in the conjunctival sac of diseased and healthy calves and heifers.

2. Materials and Methods

2.1. Sample collection

A total of 116 eye swab specimens were collected from 85 diseased and 31 healthy animals during the study period between May 2018 and August 2019. The samples were taken from calves and heifers (6-2 months) of Holstein Friesian breed from 7 industrial dairy farms in Al-Najaf province-Iraq and transported by a cold chain to the laboratory. Clinical symptoms of the affected animals were reported. Furthermore, clinical cases were divided into three categories according to complication severity: conjunctivitis as a single sign; conjunctivitis and keratitis without corneal ulcers; conjunctivitis and keratitis involving a visible corneal ulcer.

The eye swab samples were taken from the conjunctival sac using sterile cotton moistened with sterile normal saline and stored in sterile tubes containing 1.5 mL of saline phosphate buffer and an ice pack for transfer to the laboratory to be stored at –70°C.

2.2. Preparing of DNA samples

DNA was extracted from the eye swab samples according to the protocol (Sinagen DNPTM kit) (SinaClonBioScience-Tehran, Iran).

2.3. PCR test

A 280 bp fragment of a highly conserved 16s rRNA coding region of mycoplasma genome was amplified using *Mycoplasma* spp. PCR detection Kit primer (SinaClonBioScience Company, Tehran, Iran). For this purpose, a final volume of 25 µL, consisting of 20 µL PCR MIX, 0.2µL Taq DNA polymerase and 4.8 µL DNA sample, was prepared in a PCR tube on ice. The 20 µL PCR MIX used in the reaction includes, in addition to $MgCl_2$ and dNTPs, specific primers for the 16s rRNA coding region. The PCR tubes containing the reaction mixture were then transferred to the thermocycler device (Bio-Rad™ - MJ Mini thermal cycler, USA). The thermocycling protocol was as follows: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 15 sec, 52°C for 20 sec and 72°C for 35 sec, with a terminal step of 5 min at 72°C. After completion of the thermocycler step, the PCR product samples were transferred to electrophoresis (5 volts/cm) using GelRed® Prestain Plus 6X DNA loading dye (Biotium, USA) with 1.5% agarose/TBE gels. Then the UV illuminator gel documentary (Siemens, Germany) was used to visualize a 280 bp amplicon. Positive and negative controls were also applied along with the samples (McAuliffe, Ellis, Ayling, & Nicholas, 2003; Tenk et al., 2006; Jain, Verma, & Pal, 2012).

Statistical analysis

Data obtained are expressed as mean ± SD. Using Excel and SPSS V.20 statistical applications, $P < 0.01$ values were considered statistically important.

Results

The PCR test of the current study indicated the presence of 280 bp fragments of 16s rRNA-specific coding gene of mycoplasma reflecting positive results, as shown in Fig. 1.

In general, 73.3% of the samples were taken from animals with different ocular lesions. The remainder of the 26.7% was collected without clinical evidence from healthy animals. Based on the findings of the polymerase chain reaction, overall positive samples were found to be 22.4% (26/116), 6.4% (2/31) in healthy animals, and 28.2% (24/85) in diseased animals (Fig. 2).

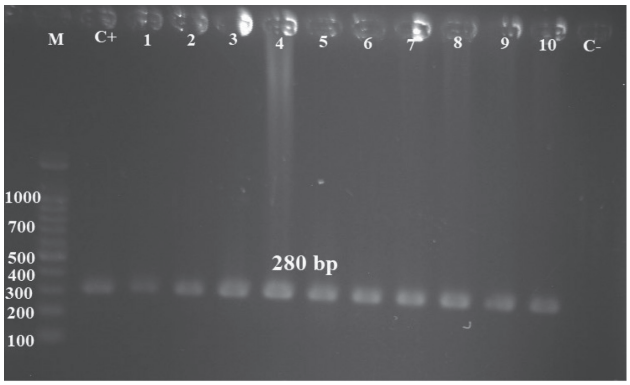

Fig. 1. Positive 280 bp bands of the Mycoplasma specified 16s rRNA gene coding region
Lane 1–10 shows eye swab samples. Lane C+ and Lane C– are both positive and negative controls. M: DNA ladder 100 bp.

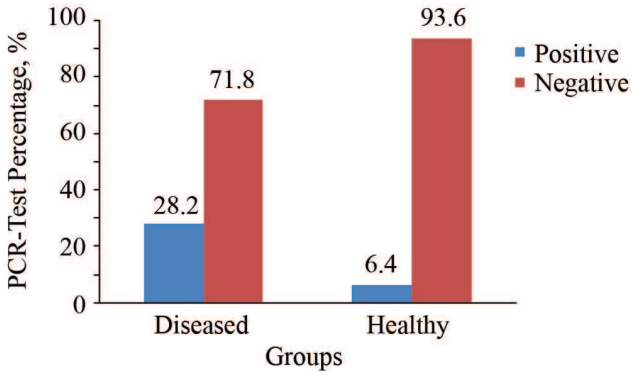

Fig. 2. Percentage of positive and negative results of the PRC test for both diseased and healthy animals

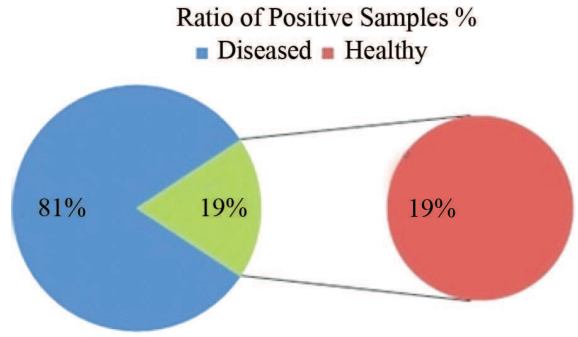

Fig. 3. The ratio of mycoplasm in healthy and diseased animals detected by PCR

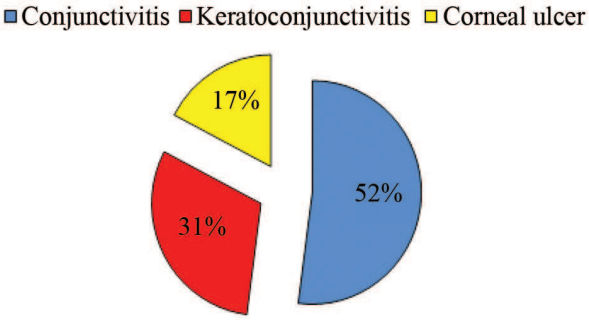

Fig. 4. Percentage of conjunctivitis, keratoconjunctivitis, and corneal ulcer in the positive samples of the diseased group

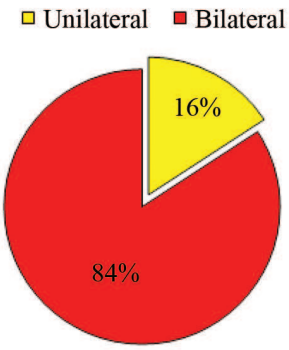

Fig. 5. Percentage of bilateral eye lesions

The ratio of identified microorganisms from diseased and healthy animals was 81% and 19%, respectively, revealing significant statistical differences (Fig. 3).

Conjunctivitis, keratoconjunctivitis and corneal ulcer were observed in 52%, 31% and 17% of the diseased animals, respectively, and 16% of the cases showed involvement of both eyes (bilateral infection) (Figs. 4 and 5).

Discussion

M. bovoculi and *M. conjunctivae* are the two most clinically important species to cause conjunctivitis and infectious bovine keratitis (IKC) (Romano et al., 2018, Tryland et al., 2017, Gupta et al., 2015).

In this study, a polymerase chain reaction was performed to determine the *Mycoplasma* species in the conjunctival sac of diseased and healthy calves and heifers.

In the current study, a 280 bp 16s rRNA fragment of *Mycoplasma* spp. was specifically detected. Besides, cross-amplification did not occur with other pathogens. The *Mycoplasma* spp. DNA sequence analysis (the data not shown) confirmed the positive result of the PCR test.

The total percentage of *Mycoplasma* spp. in eye swab samples was 22.4% (26/116). This percentage is lower than the proportion recorded by (Gupta et al., 2015) and (Raoofi et al., 2016) who reported a ratio of 37.5% and 56.7%, respectively. The difference can relate to the number of samples included in the study, the study season, the age of the animals studied and the area. In the diseased animals, the positive percentage of Mycoplasma identified was 28.2, while in healthy animals it was 6.4. We disagree with the results (Schöttker-Wegner et al., 1990) of 34.2% and 41.2%, respectively, in ill and healthy cattle, as well as the findings of Raoofi et al., (2016) who revealed 63.8% and 48.1% in infected and non-infected cattles. That can be attributed to the difference in both the total number of samples being examined and the season.

The present study shows that conjunctivitis, keratoconjunctivitis and corneal ulcers were found in 52%, 31% and 17% of the diseased group, respectively. Such findings are in line with those reported by (Sidal et al., 2007) who reported a ratio of 30.3%, 33.3%, and 36.4%, respectively. Infections of both eyes were detected in 16% of the cases, and this result is consistent with that of (Takele & Zerihun, 2000). Variation in virulence of different *Mycoplasma* spp., heat, dust, host immune status and likely concurrent infection with a bovine rhinotracheitis virus (bovine herpesvirus 1) or *Moraxella bovis* increases disease severity (Atkinson et al., 2008, Maunsell et al., 2011, Wilcock & Njaa, 2016).

Severe conjunctivitis with corneal opacity or ulceration is the most frequent and obvious sign of Mycoplasma ocular infection. Eyelid involvement with marked swelling is prominent. Conjunctivitis is prominent in many keratoconjunctivitis-producing infections (Schnee et al., 2015).

Corneal ulcers initially arise from the cytotoxic effect of the microorganism over 24 hours, resulting in corneal degradation (microscopically) without a sufficient inflammatory response (Nicholas et al., 2008a, Brooks, 2005). The corneal epithelium is eventually destroyed, followed by degradation of the keratocytes and degradation of the corneal stroma. An inflammatory response will then develop for several days leading to the development of large and deep corneal ulcers and oedema with stromal involvement, as well as corneal neovascularization (CNV) (Maggs, 2008).

Conclusion

The study found that the percentage of *Mycoplasma* spp. in animals with eye lesions was greater than in healthy animals, but only showing its existence in healthy animals suggests the latent or asymptomatic nature. Hence, *Mycoplasma* spp. infection can contribute to the severity of disease-related lesions.

Conflicts of Interest

The authors guarantee that the substances discussed at some stage in this manuscript are not affiliated with any business or entity having a financial interest or non-financial interest.

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