

Scientific Report

Molecular diagnosis of *Mycoplasma conjunctivae* in an outbreak of infectious keratoconjunctivitis in sheep

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Summary

Infectious keratoconjunctivitis (IKC) is a painful, highly contagious ocular disease in sheep and goats. This study was carried out for identification and characterization of causative agent of ocular disease in a sheep flock consisting of 300 ewes in Mashhad, Iran. Several ocular swabs were taken from affected animals. The samples were pooled and processed in a laboratory for isolation of suspicious agent. Following inoculation of the pooled sample in PPLO broth and agar, turbidity and growth of colonies were observed in them, respectively. Sequencing of the 1013 bp PCR product of 16S rDNA gene revealed that the causative agent of the outbreak has 100% sequence identity to *Mycoplasma conjunctivae*. Based on our knowledge this is the first documented report of isolation and molecular characterization of *M. conjunctivae* in Iran.

Key words: Infectious keratoconjunctivitis, Sheep, PCR

Introduction

Infectious keratoconjunctivitis (IKC), also known as contagious ophthalmia or pink eye, is an ocular disease in sheep and goats which is caused by *Mycoplasma conjunctivae* (Aitken Obe, 2007). *Mycoplasma conjunctivae* was first reported from an Australian sheep suffering from an ocular disease. This agent belongs to the *Mycoplasma neurolyticum* cluster of the *hominis* group, and is closely related to *Mycoplasma ovipneumoniae* (Giangaspero *et al.*, 2010).

Purchasing and entering of animals such as a ram with mild or inapparent infections to a clean flock, is the main route of transmission of IKC between and within flocks. Wind in winter, and transportation of the animals are involved in the spread of disease. Greater intensity of sunlight and flies around the head and eyes can be effective in severity of the disease in summer (Akerstedt and Hofshagen, 2004; Aitken Obe, 2007; Scott, 2010).

Cost of care and treatment, pregnancy toxemia in heavily multigravid pregnant ewes secondary to inability of feeding due to blindness, are the major economic losses of the disease (Aitken, 2007; Scott, 2010).

Depending on the injury to the cornea and occurrence of unilateral or bilateral lesions, the blindness may be temporary/partial or persistent/absolute (Scott, 2010). When the cornea is involved, the disease may progress toward corneal ulceration. In severe cases, in addition to anterior uveitis, ulceration of cornea may progress to rupture of the anterior chamber, but the latter status is uncommon (Whitley and Albert, 1984; Scott, 2010). Two of the most important differential diagnoses include presence of foreign bodies within the conjunctival sac

and entropions. Response to treatment is a good indicator for diagnosis of IKC (Scott, 2010).

Materials and Methods

In summer 2012, a sheep flock consisting of 300 ewes was visited in Mashhad, Iran. Ocular symptoms included eye inflammation, lacrimation, corneal opacity, and varying degree of blindness, mucoid to purulent ocular discharge, tear staining of the face, photophobia, blepharospasm either unilaterally or bilaterally in about 10 percent of the flock were observed in affected sheep. Mucopurulent nasal discharge was also seen in some involved animals. On close examination, conjunctivitis, with hyperaemic conjunctivae and injected sclera vessels were seen (Fig. 1a). Corneal opacities and keratitis were detected in a few head of the affected sheep, but there was no corneal ulcer in animals (Figs. 1b-d). On clinical examination, mild fever (40°C), decreasing ruminal movements and strength, and pulmonary harsh sounds were found. Blood samples were taken for complete blood count and determination of total protein and fibrinogen. Ocular swabs were collected from six severely affected animals and submitted to microbiology lab immediately. The samples were pooled and cultured for *Mycoplasma* spp. in PPLO broth and agar as described by Baas *et al.* (1977) and Bradbury (1998). DNA was extracted by using boiling method and the extracts were stored at -20°C till further use. Using genus specific primers, the polymerase chain reaction was performed; GPF; 5-GCT GGC TGT GTG CCT AAT ACA-3 and MGSO; 5-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3 (Lierz *et al.*, 2007; Ongor *et al.*, 2011)



Fig. 1: (a) Injected sclera vessels and tear staining of the face in the sheep with IKC. (b, c and d) Keratoconjunctivitis in the sheep with IKC

for amplifying 16S rDNA gene. The PCR were performed in a TC 512 Temperature Cycling System (Techne, UK) in a reaction volume of 50 μ l containing: 25 μ l of Taq DNA polymerase 2 x master mix red containing; 2 mM $MgCl_2$, Tris-HCl pH = 8.5, $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.2% tween 20, 0.4 mM dNTPs, 0.2 units/ μ l ampliqon Taq DNA polymerase inert red dye and stabilizer (Ampliqon, Denmark), 2 μ l (10 pmol/ μ l) of each primer (Bioneer, South Korea) and 25 ng template DNA and DDW up to 50 μ l final reaction PCR volume. The amplified products were detected by staining with ethidium bromide (0.5 mg/ml) after electrophoresis at 80 V for 2 h (7 V/cm) in 1.5% agarose gels. The PCR product was gel purified using the Invisorb Spin DNA Extraction Kit (Bioneer, South Korea) and sequenced using the same forward and reverse primers by a commercial DNA sequencing service (Bioneer, South Korea). The sequence of the product was compared to the 16S rRNA gene sequences of *Mycoplasma* spp. deposit in GeneBank using BlastN (NCBI, USA).

Results

In hematological examination, leukocytosis, neutrophilia, eosinophilia and monocytosis were observed, but the other parameters were within normal range. No common pathogenic bacteria or purulent agents were

isolated. Chlamydia was not detected in conjunctival smears. Sequence was used to perform individual nucleotide-nucleotide searches with the BLASTn algorithm at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>) and was 100% identical to type strain HRC/581. The sequence was submitted to GeneBank and the ATRJR100MCIR isolate was given the accession number: KC633276.

The affected sheep were treated with oxytetracycline 20%, IM (20 mg/kg) twice, with interval of 72 h, and flunixin meglumin, IM (2.2 mg/kg) three times, with interval of 24 h. In addition to the listed drugs, tylosin 20%, IM (17 mg/kg), was administrated three times for sheep with pulmonary involvement. Because relapses are common in treated and naturally recovered animals, it was advised to isolate the affected animals and a single dose of oxytetracycline 20% was injected to all the flock sheep at risk of the disease as prophylactic treatment (Aitken Obe, 2007). The control of flies was also recommended. At follow up two weeks later, recovery was reported.

Discussion

Definite diagnosis of IKC is based on the isolation of the causative agent in culture, immunologic identification, or by using molecular techniques such as PCR. *Mycoplasma* are highly fastidious, they typically

take weeks to culture and also their culture needs enriched and selective media, so it is laborious and not always successful (McAuliffe *et al.*, 2005). For diagnosis and epidemiological studies, detection of specific antibodies against *M. conjunctivae* by an indirect ELISA test is used. One of the major problems about the serological detection of mycoplasmal infection in ruminants is the high antigenic similarity between related *Mycoplasma* species (Belloy *et al.*, 2001; McAuliffe *et al.*, 2005). Various molecular techniques for the detection of *M. conjunctivae* are described and PCR is a rapid, sensitive, and specific test (Aitken Obe, 2007; Giangaspero *et al.*, 2010; Scott, 2010). However, under field conditions, other microorganisms, such as *Branhamella (Moraxella) ovis*, *Chlamydophila* spp, *E. coli*, *Staphylococcus aureus*, *Pseudomonas*, *Listeria monocytogenes* and other *Mycoplasma* species, especially respiratory pathogens are frequently isolated concomitantly, it seems that the main or primary pathogen is *Mycoplasma conjunctivae* and the other microorganisms exacerbate the disease as secondary agents (Akerstedt and Hofshagen, 2004; Aitken Obe, 2007). All ages of sheep may be affected, but in adult animals, the clinical signs are more common and severe (Scott, 2010).

Outbreak of IKC occurs at all seasons of the year, but according to some records, it is more obvious in winter because of housing and close contact during feeding and mating (Aitken Obe, 2007; Scott, 2010). In many cases, the disease is self-limiting, but by using some systemic and topical antimicrobial drugs, the recovery process is accelerated. It should not be forgotten that *Mycoplasma conjunctivae* infection is transmissible to humans, so great care should be taken in the handling of affected animals (Akerstedt and Hofshagen, 2004).

Since outbreaks of infectious keratoconjunctivitis in small ruminants flocks occur occasionally in the Razavi Khorasan province, to develop methods for controlling the disease, epidemiologic, immunologic, and ethologic studies are required in the area on the wild and domestic ruminants. While ovine infectious keratoconjunctivitis has been described throughout the world, this is, to our knowledge, the first time *M. conjunctivae* was isolated in Iran in association with the disease. Subtyping of the isolate will be the aim of future investigations.

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