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Conjunctival bacterial and fungal flora in clinically normal sheep

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ABSTRACT

Objectives: The aim was to identify conjunctival bacterial and fungal flora in clinically normal sheep.

Design: Prospective study.

Setting: Tuscany.

Participants: 100 eyes from 50 adult Massese female sheep were examined. The sheep included in the study were considered free of anterior ophthalmic abnormalities.

Primary and secondary outcome measures: Bacteria were identified by morphological assessment, Gram staining, biochemical tests. Identification of filamentous fungi was achieved at the genus level, and *Aspergillus* species were identified based on keys provided by other authors. Yeast colonies were highlighted, but not identified.

Results: Positive cultures were obtained from 100/100 eyes for bacteria, and from 86/100 eyes for fungi. A total of 14 types of bacteria and 5 types of fungi were isolated. Yeasts were isolated from 13/100 eyes. The most frequent fungal isolates were saprophytic fungi.

Conclusions: Conjunctival bacterial and fungal flora of clinically normal eyes were reported in sheep. The positivity obtained for conjunctival bacteria was higher compared to findings in the literature by other authors in the same species (100 per cent v 40 per cent), while our results were in line with a recent work performed on mouflons (*Ovis Musimon*) with a 100 per cent positivity for bacterial conjunctival fornix. In our survey, Gram-positive species were prevalent, as reported by other authors in different species. Few data are available in the literature regarding conjunctival fungal flora in healthy small ruminants. The prevalence of conjunctival fungal flora in this study was higher than findings reported in mouflons (86 per cent v 45 per cent). Differences in fungal prevalence may be due to different methods of managing herds, though further studies are required to verify this hypothesis. The similarities in bacterial and fungal isolates between sheep and mouflons suggest a genera pattern of conjunctival colonisation by bacteria and fungi.

INTRODUCTION

Bacterial and/or fungal flora of the normal conjunctival fornix has been described in many animal species (Wood and others 1965, Urban and others 1972, Whitley and others 1983, Zenoble and others 1983, Samuelson and others 1984, Moore and others 1988, Gionfriddo and others 1992, Gerding and others 1993, Davidson and others 1994, Dubay and others 2000, Cooper and others 2001, Silvanose and others 2001, Barbasso and others 2002, Petruzzi and others 2002, Pinard and others 2002, Tuntivanich and others 2002, Andrew and others 2003, Rosa and others 2003, Barsotti and others 2006, Nardoni and others 2007, Sgorbini and others 2008, 2010, Cousquer and others 2010, Taddei and others 2010, Johns and others 2011). Many factors, such as environment, age, geography, habitat and husbandry have been reported to influence the composition of conjunctival flora (Andrew and others 2003, Rosa and others 2003, Barsotti and others 2006, Sgorbini and others 2010, Johns and others 2011). Understanding the normal conjunctival flora is important in terms of possible implications in keratoconjunctivitis. Reports describing the normal conjunctival flora of sheep are scarce and old (Baker and others 1965, Spradbrow 1968, Hopkins 1973, Baas and others 1977, Araghi-Sooreh and Hatami-Lorzini 2012). In clinically normal sheep, 60 per cent of eye swabs have been found to be negative for bacterial growth (Ramsey 1999). The most commonly isolated bacteria were similar to *Branhamella* (*Neisseria*) *ovis* and were recovered in small numbers. Other frequently isolated organisms were *Micrococcus* species and *Streptococcus* species. Less commonly isolated bacteria were included in the genera *Corynebacterium*, *Achromobacter*, *Bacillus*, *Neisseria* (other than *N ovis*), *Staphylococcus*, *Pseudomonas*, *Moraxella* and *Escherichia*, (Baker and others 1965, Spradbrow 1968, Ramsey 1999, Waldridge and Colitz 2002). The fungi most frequently isolated from sheep and goats conjunctival fornix are *Aspergillus* species and *Mucor* species (Hopkins 1973, Baas and others 1977,

Waldridge and Colitz 2002). The aim of this work was to identify conjunctival bacterial and fungal flora in clinically normal sheep.

MATERIALS AND METHODS

A total of 100 eyes from 50 sheep were examined in August 2010. Approval to conduct this study was obtained from the Ethics Committee on Animal Experimentation of the University of Pisa (DL 116/92). All sheep were female, aged more than one year, and they all underwent similar management conditions. All the animals were considered healthy on the basis of clinical examination and free from anterior ophthalmic abnormalities, as determined by an ophthalmic examination performed after the conjunctival sampling.

The eye and periocular region were examined in normal light for gross abnormalities. Menace response, palpebral and corneal reflex tests were also performed. A Schirmer tear test was conducted on each eye using a commercial test strip (Dina strip Schirmer-Plus; GECIS sarl, Neung sur Beuvron, France). Tear production was recorded in millimetres wetting after 60 seconds. These procedures were performed in daylight or artificial light. The adnexa and anterior segment of both eyes were examined with a binocular magnifying loupe, a transilluminator 3.5 V (Heine, Berlin, Germany), and a portable slit-lamp biomicroscope (SL-14, Kowa Company, Tokyo, Japan). Pupillary light reflexes were also evaluated in a dark stable. Intraocular pressure was assessed by applanation tonometry (Tonopen-XL, Mentor, Norwell, Massachusetts, USA) after topical application of oxibuprocaine chlorhydrate 0.4 per cent (Benoxinato chlorhydrate INTES; ALFA INTES Industria Terapeutica Splendore S.r.l., Naples, Italy). Fluorescein staining was also performed. Sheep were not sedated for the ophthalmic examination or for sample collection, but only restrained manually.

Ocular specimens were obtained by retropulsing each eye through the closed upper eyelid and running a sterile swab (Cultiplast, LP Italiana S.p.A., Italy) along the surface of the ventral conjunctival fornix. The procedure was repeated twice in order to obtain specimens for bacterial and mycotic flora. Swabs collected for mycotic cultures were maintained in 2 ml of sterile saline solution with gentamicin (50 µl/ml), whereas sterile swabs for bacterial cultures were placed in Stuart transport media. Samples were collected before ophthalmic examination without topical anaesthesia. Special care was taken to ensure that the swab did not come into contact with the vibrissae, eyelids, or eyelashes. All the samples obtained from eyes with signs of anterior segment abnormalities were excluded from the study. All the samples were collected during the morning milking and maintained at 4°C for at least 12 hours.

Bacteriological samples

On reaching the microbiology laboratory, the conjunctival swabs were placed aseptically into a sterile tube containing 1 ml of physiological solution (Oxoid, Milan, Italy) and vortexed for 30 seconds. Subsequently, 100 µl aliquots were spread onto a Columbia agar plate containing 5 per cent sheep blood, which was incubated at 37°C for 24–48 hours. The same procedures were repeated for incubation in anaerobic conditions (Anagen Oxoid, Milan Italy). Colonies were counted by hand on both plates, using an illuminated colony counter when large numbers of colonies were present. The number of colonies on each plate was converted to number of bacteria per 1 ml of physiological solution (equal to the number of bacteria per eye) with the equation used in the literature (Ferguson and others 2003). Additionally, each conjunctival swab was inoculated in Tripticase Soya broth, incubated at 37°C for six hours and then streaked on whole media. The culture media used were: Columbia sheep blood agar, with and without Streptococcus Selective Supplement, Mannitol Salt agar, MacConkey agar (Oxoid, Milan, Italy). Plates were incubated at 37°C and examined for growth at 24, 48 and 72 hours. Representative colonies of bacteria were subcultivated onto Columbia blood agar plates and identified by morphological assessment, Gram staining, biochemical tests and, where necessary, using additional identification kits (Remel – Oxoid, Milan, Italy).

Mycological samples

Samples were plated onto Sabouraud dextrose agar (SDA, Oxoid, Milan, Italy) and malt extract agar (MEA, Oxoid, Milan, Italy), incubated at 25°C and examined daily from day 4 postincubation (p.i.), over a 21-day period to identify slow-growing organisms (Rosa and others 2003). Identification of colonies of filamentous fungi was achieved at the genus level on the basis of macroscopic and microscopic features of colonies, as described in the literature (Barnett and Hunter 1998). *Aspergillus* species were identified based on keys provided by other authors (Rapper and Fennell 1965). Yeast colonies were highlighted but not identified. Each test was carried out in duplicate to confirm the results. Colony forming units (CFU) were visually counted by the same operator. The total number of fungi and bacteria isolates per eye expressed in percentages (prevalence) in 100 normal eyes from 50 sheep was calculated.

RESULTS

Results regarding bacterial and fungal prevalence and CFUs are reported in Table 1. Positive cultures were obtained from 100/100 (100 per cent) eyes for bacteria and from 86/100 (86 per cent) eyes for fungi. A total of 14 species of bacteria were isolated: 10 types of Gram-positive bacteria and four Gram-negative bacteria. The Gram-positive bacteria were: five rods (*Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus licheniformis* and *Corynebacterium* species) and five cocci (*Enterococcus* species, coagulase-negative staphylococci, *Streptococcus* γ-hemolytic, *Staphylococcus aureus* and

TABLE 1: Bacteria and fungi isolated from 100 eyes of 50 Massese sheep, their prevalence and CFU

GRAM+bacteria	Prevalence (%) (n=100)	CFU/ml (mean)
Bacillus subtilis	48	1×10^2
Enterococcus spp.	35	9×10^1
Bacillus cereus	33	1×10^2
Corynebacterium spp.	15	5×10^1
Bacillus thuringiensis	10	9×10^1
Bacillus licheniformis	10	11×10^1
Coagulase-negative staphylococci	9	15×10^1
Streptococcus γ-hemolytic	8	8×10^1
Staphylococcus aureus	6	12×10^1
Micrococcus spp.	2	11×10^1
GRAM−bacteria		**CFU/ml**
Escherichia coli	28	7×10^1
Alcaligenes faecalis	16	1×10^2
Streptobacillus spp.	7	7×10^1
Moraxella spp.	3	6×10^1
Fungi		**CFU**
Mucor spp.	49	5
Aspergillus spp.	31	67
Penicillium spp.	26	23
Alternaria spp.	16	1
Cladosporium spp.	6	61

CFU, Colony forming unit

Micrococcus species). The Gram-negative bacteria were: *Escherichia coli*, *Alcaligenes faecalis*, *Streptobacillus* species and *Moraxella* species. The most frequently isolated Gram-positive bacteria were *B. subtilis* (48/100 eyes; 48 per cent), *Enterococcus* species. (35/100 eyes; 35 per cent) and *B. cereus* (33/100 eyes; 33 per cent), while the most frequently isolated Gram-negative bacteria were *E coli* (28/100 eyes; 28 per cent) and *A faecalis* (16/100 eyes; 16 per cent). Seven/100 eyes (7 per cent) were positive for one bacterial genus, while 93/100 (93 per cent) eyes were positive for two or more bacteria.

A total of five species of fungi were isolated: *Mucor* species, *Aspergillus* spcies, *Penicillium* species, *Alternaria* species and *Cladosporium* species. Yeasts were isolated from 13/100 eyes (13 per cent). The most frequently fungi isolated were: *Mucor* species in 49/100 eyes (49 per cent), *Aspergillus* species in 31/100 eyes (31 per cent), and *Penicillium* species in 26/100 eyes (26 per cent). Aspergilli were identified as *Aspergillus niger* and *Aspergillus flavus/parasiticus*. A total of 40/100 eyes (40 per cent) were positive for one fungus genus, 30/100 eyes (30 per cent) were positive for two fungi genera, and 16/100 eyes (16 per cent) were positive for three fungi genera; 4/100 (4 per cent) eyes were positive only for yeasts. CFUs ranged between 1 and 100.

All the bacteria and fungi isolated are reported in Table 1, along with the frequency of isolation per eye, and the mean CFU/ml recorded per microorganism for bacteria and CFUs per eye for fungi.

CONCLUSIONS

We investigated conjunctival bacterial and fungal flora in clinically normal eyes in sheep. All the eyes from the examined animals were positive for at least one microorganism. Bacteria were isolated in all the eyes examined, while fungi were present in 86/100 eyes. The positivity obtained for conjunctival bacteria was higher compared to findings in the literature by other authors in the same species (100 per cent v 40 per cent) (Spradbrow 1968). On the other hand, our results were in line with a recent work performed on mouflons (*Ovis Musimon*) with 100 per cent positivity for bacterial conjunctival fornix (Petruzzi and others 2002). In our survey, Gram-positive species were prevalent, as reported for other species (Whitley and others 1983, Dubay and others 2000, Silvanose and others 2001, Pinard and others 2002, Andrew and others 2003, Cousquer and others 2010, Taddei and others 2010). The most frequent Gram-positive bacteria isolated (*Bacillus* species, *Enterococcus* species, *Corynebacterium* species, *Sthaphylococcus* species) were similar to bacteria isolated previously from mouflons (Petruzzi and others 2002). Regarding Gram-negative bacteria, the prevalence of *E coli* and *A faecalis* isolated in sheep was higher compared to other ruminants or herbivores (Gionfriddo and others 1992, Tuntivanich and others 2002, Andrew and others 2003, Taddei and others 2010, Johns and others 2011), while in mouflons, these bacteria were not isolated (Petruzzi and others 2002).

Fungi are considered part of the normal conjunctival mycoflora in many species (Samuelson and others 1984, Gionfriddo and others 1992, Tuntivanich and others 2002, Andrew and others 2003, Rosa and others 2003, Barsotti and others 2006, Nardoni and others 2007, Sgorbini and others 2008, 2010). Few data are available in the literature regarding conjunctival fungal flora in healthy small ruminants (Petruzzi and others 2002, Sgorbini and others 2010). The prevalence of conjunctival fungal flora in this study was higher than findings reported in mouflons (86 per cent v 45 per cent) (Petruzzi and others 2002). The most frequent fungal isolates in this study were saprophytic fungi, such as *Aspergillus* species, *Penicillium* species and *Mucoraceae*. These filamentous fungi are also the most frequent isolates in all the species examined by other authors, (Samuelson and others 1984, Gionfriddo and others 1992, Petruzzi and others 2002, Tuntivanich and others 2002, Andrew and others 2003, Barsotti and others 2006, Nardoni and others 2007, Sgorbini and others 2008, 2010, Johns and others 2011) but with a different prevalence. The prevalence of *Mucoraceae* found in this study was higher in sheep than has been found in the literature in other ruminants (Sgorbini and others 2010), while *Penicillium* species was lower than in mouflons

(Petruzzi and others 2002) and cows (Sgorbini and others 2010). The prevalence of *Aspergillus* species in sheep was higher compared to cows for some authors (Samuelson and others 1984), but similar compared to results reported in a recent study (Sgorbini and others 2010). Differences in fungal prevalence may be due to different methods of managing herds, though further studies are required to verify this hypothesis. The similarities in bacterial and fungal isolates between sheep and mouflons suggest a genera pattern of conjunctival colonisation by bacteria and fungi.

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