Conjunctival bacterial and fungal flora in healthy donkeys in Central Italy

F. LAUS*, V. FAILLACE*, A.R. ATTILI, A. SPATERNA, B. TESEI, V. CUTERI
School of Biosciences and Veterinary Medicine, University of Camerino
*These authors contribute equally to the work

SUMMARY
Introduction - The normal eye microflora is composed of several species of fungi and bacteria. If the ocular defense barriers become weak, they can act as pathogens and cause infections. Therefore, characterization of conjunctival normal flora is essential in making diagnosis and treating eye infections. Bacterial and fungal flora of the normal eye has been reported for different mammals but few studies concerning donkeys are available. Aim - To evaluate the bacterial and fungal flora of healthy eyes of donkeys (Equus asinus) reared in three different Areas in Central Italy. Materials and methods - One hundred-fourteen mixed breed donkeys (93 females, 21 males) housed in Marche, Umbria and Lazio Region were included in the study and sampled on the ventral conjunctival fornix. Age ranged between 4 months and 16 years (mean: 7.3 years, SD ± 8.6). Animals were divided into three categories: foals: ≤ 1 year, n = 35; young: 1 < age ≤ 3 years, n = 9; and adult: ≥ 3 years, n = 70. Results and discussion - Twenty-one different bacteria genus and thirteen fungi/yeasts were isolated. The emergent Kocuria spp. was isolated in 61 cases. None significant effect of gender on bacterial and fungal isolation was observed. Significantly lower bacterial load was recorded in foals than adult donkeys. In relation to the Areas, differences were observed both for bacterial and fungal mean loads. Conclusion - The area in which donkeys are reared seems to be a significant factor influencing the conjunctival bacterial and fungal flora loads. The emerging human pathogen bacteria Kocuria spp. was isolated for the first time in donkeys. In the present study, new important information to facilitate the diagnosis of eye disease in an emergent species like donkeys are provided.

KEY WORDS
Bacteria, conjunctiva, donkey, fungi, Kocuria.

INTRODUCTION
Equids have relatively large, prominent eyes, which are susceptible to be damaged by straw or dirt from the environment resulting in more frequently observed ocular infections than in other domestic animals. Animal and human eye microflora is normally composed by several species of fungi and bacteria that remain in a balance with immunitary system of the host. The resident microbial flora contributes in preventing overgrowth of pathogenic bacteria but if the ocular defense barriers become weak, they may act as pathogens and cause infections. If a corneal abrasion occurs, these non-pathogenic microorganisms may infiltrate the corneal stroma and result in keratitis or in infected corneal ulceration usually difficult to treat. Therefore, characterization of conjunctival normal flora may be useful in treating eye infections. Bacterial and fungal flora of the normal eye has been reported for different mammals but few studies concerning donkeys are available. It is known that microbial population of the horses’ ocular surface is influenced by factors such as gender, geographic location and housing, so findings from individual studies may not be applicable to animals reared in other Areas of the world. Furthermore, equine corneal diseases can be affected by seasonal factors such as temperature and humidity. The aim of this study was to evaluate the bacterial and fungal flora of healthy eyes of mixed breed donkeys (Equus asinus) living in the Marche, Umbria and Lazio Regions (Central Italy).

MATERIALS AND METHODS
Animals
Between April 2014 and October 2014, 114 mixed breed donkeys (93 females, 21 males) housed in Central Italy (Fig. 1) were included in the study. Age ranged between 4 months and 16 years (mean: 7.3 years, SD ± 8.6). Animals were divided into three age categories: foals: ≤ 1 year, n = 35; young: 1 < age ≤ 3 years, n = 9; and adults: ≥ 3 years, n = 70. For each animal physical and ophthalmic examinations were performed. Only animals with none history or clinical signs related to eye disorders were included in the study.

Areas of sampling
Geographical distribution of sampled animals is reported in Figure 1.
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room temperature and then vortexed for 30 seconds. An ali-
saline solution (Oxoid, Milan, Italy), left for 5 minutes at
aseptically putted into a sterile tube containing 1 mL of 0.9%
for bacteriological examination, one conjunctival swab was
imens from each eye were obtained. Special care was taken
to ensure that the swabs did not come into contact with the
vibrissae, eyelids or eyelashes. Samples were placed in a coo-
the surface of the ventral conjunctival fornix, two ocular spe-
gh the closed upper eyelid and running a sterile swab along
by retropulsing the eye through
nerve blocks or topical anesthetics. By retropulsing the eye throu-
no food integration, and used for onotherapy and trekking.
Area 3. Rignano Flamino (42°12'00''N, 12°29'00''E), Lazio
Area 3. Rignano Flamino (42°12'00''N, 12°29'00''E), Lazio
Region. Animals (n = 23) were reared at pasture, ea-
ling grass with no food integration, and used for onotherapy and trekking.

Area 1. Abbadia di Fiastra (43°13'17''N, 13°24'19''E), Marche
Region. Animals in this farm (n = 55) were reared in large
paddocks with graze access and hay available ad libitum.

Milking was performed once a day with automatic machine.

Area 2. Castelluccio di Norcia (42°49'44''N, 13°12'21''E), Umbria Region. Animals (n = 23) were reared at pasture, eat-
ing grass with no food integration, and used for onotherapy and trekking.

Area 3. Rignano Flamino (42°12'00''N, 12°29'00''E), Lazio Region. Animals in this farm (n = 36) were reared with a semi-intensive system, housed in small paddock to allow separation between different category based on age and month of lactation. Milking was performed twice a day with automatic machine.

The temperature in all Areas during the sampling period ranged from 9.6 °C to 18.2 °C, humidity ranged from 72.1%, and 84.2%, wind ranged from 3 km/h to 17 km/h (Source: http://clima.meteoam.it, Military Aviation website).

Sampling
Conjunctival samples were obtained without sedation, nerve blocks or topical anesthetics. By retropulsing the eye through the closed upper eyelid and running a sterile swab along the surface of the ventral conjunctival fornix, two ocular specimens from each eye were obtained. Special care was taken to ensure that the swabs did not come into contact with the vibrissae, eyelids or eyelashes. Samples were placed in a cooled box and delivered to the laboratory within 2-4 hrs.

Laboratory analysis
For bacteriological examination, one conjunctival swab was aseptically putted into a sterile tube containing 1 mL of 0.9% saline solution (Oxoid, Milan, Italy), left for 5 minutes at room temperature and then vortexed for 30 seconds. An ali-
quot, 100 µL, was spread onto Columbia agar plate contain-
ing 5% sheep blood, with and without Streptococcus sup-
plement, Mannitol Salt agar, Mac Conkey agar, Pseudomonas
Cetrimide agar and Burkholderia cepacia selective agar
(Oxoid, Milan, Italy). Plates were incubated at 37 °C for 24-
72 hrs. in aerobic conditions. Gram-positive bacteria were identified using Gram staining, catalase and coagulase test, colony morphology, and biochemical gallery (Remel RapID, Oxoid, Milan, Italy). Gram-negative bacteria were identified by selective media agar, Gram staining, oxidase test, and biochemical gallery (Remel Rapid ID, Oxoid, Milan, Italy).

Samples for mycological investigation were maintained in 1 mL of sterile saline solution with 50 µL/mL gentamicin and stored at 4 °C for 24 hrs. An aliquot, 100 µL, was spread on Sabouraud dextrose agar (SDA, Oxoid, Milan, Italy), incubated at 25 °C and examined daily over a 21-day period.

Aspergillus species were identified following Rapper and Fen-
el’s keys (1965) while the identification of other filamentous fungi was achieved to the genus level.19 Yeast colonies
were identified by macro- and micro-morphologic charac-
tistics and on the basis of physiologic characteristics, such as presence of capsule by India Ink testing, urease production at 25 °C, and the germ tube test. On all isolates the Carbohydrate assimilation test was performed.

On each plate, the number of colony forming unit (CFU), was converted into number of bacteria/fungi per 1 mL of saline solution (equal to the number of flora per eye) using the equation by Ferguson et al. (2003).20

Statistical analysis
Effects of age, gender, and environment, on bacterial and fun-
gal frequency isolation were determined using χ2 test and an independent-samples student’s t-test. A multivariate analysis was performed considering three variables: the age, the gen-
der, and the environment. P-values less than 0.05 were con-
sidered significant. To calculate the sample size the Cannon and Roe’s (1982) formula was used, considering a population of about 1,000 donkeys (source: http://statistiche.izs.it), an estimated prevalence of infection of 2.5%, and a degree of confidence of 95%.21 Data were analysed using a statistical software program (STATA, version 13; STATA Corporation, College Station, Texas, USA).

RESULTS
Twenty-one different bacteria genus (Table 1) and thirteen fungi/yeasts were found (Table 2). All eyes were positive for at least one microorganism, except for the 5.9% of eyes of donkeys reared in Area 2. A total of 0.9% (n = 6) and 13.8% (n = 50) of eyes resulted negative for bacteria and fungi/yeasts, respectively.

Significant differences for each microorganism and fungi/yeasts per Area are reported in Tables 1 and 2. None significant effect of gender and age on bacterial and fungal isolation frequency (P > 0.05) was observed. Male (21136.8 ± 98439.2) and female animals (31553.5 ± 120329.1; t = -0.9204, P = 0.36) had similar bacterial and fungal mean loads (Fig. 2), while foals revealed a significan-
tly lower bacterial load (7924.8 ± 18132.2) than adult donkeys (41281.7 ± 138165.7; t = -3.3985, P < 0.001) (Fig. 3). In relation to the Area, differences were observed both for

Figure 1 - Areas of sampling and geographical distribution of animals.
Table 1 - Bacterial eye prevalence (%) and CFU/eye mean (±SD).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Frequency (N)</th>
<th>Prevalence (%)</th>
<th>CFU/eye (x10^2) - Mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Area 1</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>91</td>
<td>13.9</td>
<td>0.6* (± 1.6)</td>
</tr>
<tr>
<td><em>S. intermedius group</em></td>
<td>3</td>
<td>0.4</td>
<td>0.05 (± 0.001)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>46</td>
<td>7.0</td>
<td>0.2* (± 0.4)</td>
</tr>
<tr>
<td><em>S. hyicus</em></td>
<td>22</td>
<td>3.4</td>
<td>0.5* (± 0.7)</td>
</tr>
<tr>
<td>Kocuria rosea</td>
<td>21</td>
<td>3.2</td>
<td>0.1* (± 0.08)</td>
</tr>
<tr>
<td><em>K. kristinae</em></td>
<td>40</td>
<td>6.1</td>
<td>–</td>
</tr>
<tr>
<td>Micrococcus spp.</td>
<td>1</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp. γ</td>
<td>3</td>
<td>0.4</td>
<td>0.05 (± 0.001)</td>
</tr>
<tr>
<td><em>S. constellatus</em></td>
<td>22</td>
<td>3.3</td>
<td>–</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>43</td>
<td>6.5</td>
<td>–</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>67</td>
<td>10.1</td>
<td>0.09* (± 0.07)</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>96</td>
<td>14.5</td>
<td>0.08* (± 0.04)</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>63</td>
<td>9.5</td>
<td>0.1* (± 0.1)</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>4</td>
<td>0.6</td>
<td>0.1 (± 0.001)</td>
</tr>
<tr>
<td>Pantoea agglomerans</td>
<td>1</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>6</td>
<td>0.9</td>
<td>–</td>
</tr>
<tr>
<td>Leminorella grimponti</td>
<td>1</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>2</td>
<td>0.3</td>
<td>–</td>
</tr>
<tr>
<td>Negative eyes</td>
<td>6</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>610</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Legend: * P < 0.05

Table 2 - Fungal eye prevalence (%) and CFU/eye mean (±SD).

<table>
<thead>
<tr>
<th>Fungi and Yeast</th>
<th>Frequency (N)</th>
<th>Prevalence (%)</th>
<th>CFU/eye - Mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Area 1</td>
</tr>
<tr>
<td><em>Absidia</em> spp.</td>
<td>8</td>
<td>2.2</td>
<td>10.0 ± 5.8</td>
</tr>
<tr>
<td>Acremonium spp.</td>
<td>6</td>
<td>1.7</td>
<td>10.0 ± 0.1</td>
</tr>
<tr>
<td><em>Alternaria</em> spp.</td>
<td>13</td>
<td>3.6</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>23</td>
<td>6.4</td>
<td>6.1 ± 2.2*</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>44</td>
<td>12.3</td>
<td>10.0 ± 5.5</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>5</td>
<td>1.4</td>
<td>5.0 ± 0.1*</td>
</tr>
<tr>
<td><em>A. ochraceus</em></td>
<td>22</td>
<td>6.2</td>
<td>8.3 ± 4.4</td>
</tr>
<tr>
<td><em>A. penicilliodes</em></td>
<td>15</td>
<td>4.2</td>
<td>9.6 ± 5.2</td>
</tr>
<tr>
<td><em>A. terreus</em></td>
<td>16</td>
<td>4.5</td>
<td>5.0 ± 0.1*</td>
</tr>
<tr>
<td>Mucor spp.</td>
<td>49</td>
<td>13.7</td>
<td>6.1 ± 2.1</td>
</tr>
<tr>
<td>Penicillium spp.</td>
<td>23</td>
<td>6.4</td>
<td>6.7 ± 2.5</td>
</tr>
<tr>
<td><em>Pichia anomala</em></td>
<td>63</td>
<td>17.6</td>
<td>23.4 ± 32.3*</td>
</tr>
<tr>
<td>Rhodotorula spp.</td>
<td>20</td>
<td>5.6</td>
<td>12.5 ± 6.2</td>
</tr>
<tr>
<td>Negative eyes</td>
<td>50</td>
<td>13.8</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>357</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Legend: * P < 0.05
bacterial and fungal mean loads between Areas 1-2 (bacteria \( t = -5.758, P < 10^{-4} \); fungi \( t = -3.1638, P = 0.002 \)); Areas 1-3 (bacteria \( t = -8.45, P < 10^{-4} \); fungi \( t = -4.8116, P < 10^{-4} \)); and between Areas 2-3 only for bacteria mean load: \( t = -2.2528, P = 0.02 \) (Fig. 4).

Analysing the data by Area, in the Lazio and Umbria Region the higher bacterial and fungal mean loads were recorded; in particular, in the Area 1, significant differences were recorded for bacteria (CFU/eye) between foals (16.1 ± 27.7) and adult donkeys (39.5 ± 106.7; \( t = -2.3304, P = 0.02 \)), and for fungal flora both between foals (15.2 ± 19.5) and adult animals (41295.1 ± 90383.3; \( t = -2.0760, P = 0.04 \)). In Area 3, the eyes of adult donkeys had more bacterial mean load (107513.0 ± 218993.0) than foals (25970.2 ± 25173.0; \( t = -2.8716, P = 0.004 \)), and slight differences were found for fungal mean load (26.1 ± 27.1 vs 143.8 ± 336.2; \( t = -2.0040, P = 0.048 \)).

In relation to the gender, only in the Area 1 female (33.4 ± 94.0) had a slightly significant greater bacterial mean load than male donkeys (12.5 ± 11.4; \( t = -1.9793, P = 0.048 \)). The statistical analysis carried out by age, confirmed that the higher bacterial loads were in the Area 3 both for foals (\( t = -11.2307, P > 10^{-4} \)) and adult donkeys (\( t = -7.1519, P > 10^{-4} \)); while in the Area 2 the donkeys showed the higher fungal loads with significant differences in foals (\( t = -3.8666, P = 0.0002 \)) and adults (\( t = -2.4509, P = 0.01 \)). The multivariate analysis confirmed that the Area in which donkeys were reared is the significant factor influencing the conjunctival bacterial and fungal flora mean loads (\( P < 10^{-4} \)).

DISCUSSION

Staphylococcus spp. strains were confirmed as the most prevalent opportunistic Gram-positive bacteria isolated from conjunctival swabs in animals.\(^{14,17,22}\) On the contrary, the isolation of Kocuria spp. was never recorded in previous studies. These catalase-positive and coagulase-negative, Gram-positive, coccoid bacteria are usually found as tetrads and irregular clusters. Bacteria of genus Kocuria belongs to the family Micrococccaeae, order Actinomycetales, class Actinobacteria and are frequently found as normal skin and oropharynx commensals in humans and other mammals.\(^{23,24,25}\) Kocuria spp. have been ascertained as responsible for several human infections, mostly in immunocompromised hosts and it is considered an emerging microbe since 5 of the 19 species in this genus are known to be opportunistic pathogen.\(^{25,26}\) The documented infections caused by Kocuria spp. in humans are limited but many cases might have been missed owing to their misidentification as Staphylococcus spp. due to the inadequate biochemical tests and to the automated identification systems.\(^{25,26,27}\)

If donkeys can be a potential source of infection for themselves or for other animals will require further investigations. In a previous study performed in donkeys, the prevalent bacteria isolated were Staphylococcus spp., and Enterobacter spp. among the Enterobacteriaceae family.\(^{14}\) The study was performed in South Italy, with a Mediterranean warm climate, in semi-intensive stables, and the Authors did not isolate bacteria such as Corynebacterium spp. or Bacillus spp., instead found in the present study. The findings reported by Foti et al. (2012) were more similar to those recorded in the Area 2 of our work.\(^{14}\) However, climate and housing of Area 2 were very different from that of the cited study and it is possible that different variables have influenced the results, such as seasonal sampling, not evaluated in this study.

Aspergillus spp., Macau spp. Penicillium spp., and the yeast Pichia anomala, were the more represented fungal flora, as reported in horses.\(^{12}\) Aspergillus spp., Cladosporium spp., Alternaria spp., Penicillium spp., Fusarium spp. and yeasts have
been predominantly isolated from conjunctiva and cornea of healthy horses.6,7,16,28 In the present study, Aspergillus spp. was isolated from 35% of healthy eyes, in accordance with surveys carried out on Amiata donkeys and in horses.1,4,12,29 Furthermore, a high percentage (17.6%) of isolations was observed for the yeast Pichia anomala, close to the 11% recorded by Nardoni et al. (2007), but much high if compared with that observed in horses.31,32 This high frequency of Pichia anomala could be a typical feature of donkeys and should be further investigated.

In this study the Authors have compared results with those of a previous one performed on horses living in the similar geographic Area, and with the same climate, showing that the donkeys’ conjunctiva is more colonized by fungi than those of horses, even if this difference is not significant.39

In the present study, no differences between male and female have been found, except for the Area 1 where a weak significant difference was observed. An explanation for this data cannot be provides and only an influence of the hormonal stress of pregnancy can be supposed.

The study revealed Gram-negative flora only in the Area 2 where young donkeys were positive for Shigella spp. and Klebsiella spp., while adult donkeys were positive to all the four Gram-negative isolated bacteria (Pantoea agglomerans, Shigella spp., Leminorella grimontii, and Klebsiella spp.). Foals from the three Areas had an exclusive Gram-positive flora. Moreover, a significant increasing trend of mean bacterial load with age has been found in each Area, with higher mean bacterial load in adult donkeys reared in the Area 3 and higher mean fungal load in adult donkeys reared in the Area 2.

The effect of gender and age on bacteria and fungi presence has not been evaluated in none of the previous studies performed on donkey but some variability regarding frequency and composition of conjunctival flora in relation to the age has been reported for horses.4,12,14,29,30 In a study, performed in UK on conjunctival bacteria and fungi, no differences were found in horses for age and gender, but animals with Gram-negative bacteria were older than horses with Gram-positive bacteria.19 On the contrary, Andrew et al. (2003) found young horses having an increased incidence of Gram-negative bacteria other than fungal isolations.4 Khosravi et al. (2014) did not find any differences for fungal isolation in relation to age and gender in Iranian horses.6 However, they found Caspian miniature horses having the highest mean CFU compared with other breeds and explained these findings with the closer contact with food because of their small size. Such statement concerning the size could account for differences found in donkeys and foals. According with the present study, surveys carried out in horses in Central Italy and in Brazil failed to find age differences on positivity for fungal isolation.16,28,29 Although the lack of difference between males and females can therefore now be proposed, the meaning of differences among age categories needs further investigation in domestic Equids.

Many studies have tried to analyse the factors affecting the normal equine conjunctival flora in horses. Andrew et al., (1998) state that geographic location, environmental and husbandry conditions influence the presence or absence of fungal flora, such as the mycotic burden.31 One study from Brazil showed an increasing incidence of fungi in stabled animals compared those that were reared outside, similar to the findings of another study performed in USA where a higher fungal prevalence was associated with dust, humidity and hygiene issues in the stables.7,16 In contrast to these reports, in a study conducted in Switzerland, differences between stabled and outdoor horses were not found, but the Authors consider that the prevalence of certain fungal species was influenced by the different type of housing and bedding.32 In the present paper, donkeys reared on pasture, resulted to have a higher burden of microflora confirming that environmental influence on ocular cultures has not yet been established and needs further investigations. However, seems to be ascertained that warmer season causes a higher fungal prevalence.17,29,32 Whiteley et al. (1983) suggested that the low percentage of conjunctival isolations obtained during cold conditions could be due to the less amount of dust, pollens and flies in comparison with warmer periods.55 Another study performed in the same Area but during the summer, gave a higher percentage of positivity.7 Differently, effect of season on conjunctival isolation was not found by Andrew et al. (2003).4

Sampling in this study was performed in different months during the year, but never in extremely cold or hot conditions. This could account for a preeminent influence of housing respect to the climate when differences for fungal and bacterial isolations among studied Areas were evaluated and could be supposed that differences found among the three Areas were due to the management (greater overcrowding in the Area 3, feeding with hay, more time spent in closed environments in Areas 1 and 3) rather than the climate.

CONCLUSIONS

Microorganisms never isolated before in donkey’s conjunctiva have been detected in this study, and others have been confirmed. Hypotheses for these differences are provided. The identification of new potentially pathogenic species should encourage research in this regard. The influences of subjective and environmental factors on conjunctival microflora have been partially studied in horse but the data are very scarce in donkeys. Since the comparison between horses and donkeys is never completely appropriate, further studies are needed to establish how the variables affect the conjunctival flora in donkeys and consequently the pathophysiology of diseases involving the eye structures.

References

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