

Equine keratomycosis due to *Aspergillus flavus* in Argentina

Queratomycosis equina por *Aspergillus flavus* en Argentina

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ANALECTA VETERINARIA

Universidad Nacional de La Plata, Argentina

ISSN: 1514-2590

Periodicidad: Frecuencia

continua vol. 44, e081, 2024

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Recepción: 03 Octubre 2023

Revisado: 26 Febrero 2023

Aprobación: 28 Febrero 2023

<https://doi.org/10.24215/15142590e081>



Abstract: A 9-year-old gelding presented a corneal ulcer unresponsive to treatment in his right eye. The trauma was caused by vegetal material. The ophthalmologic exam revealed paraxial corneal opacity, whitish discharge, peripheral oedema, congestion of conjunctival vessels and corneal angiogenesis. Microscopic examination of smears revealed the presence of neutrophils and fungal hyphae. On day 3 of culture, several small white fluffy colonies that rapidly turned to pale green were detected. These colonies were isolated to study their micro and macromorphology. Microscopic examination revealed the presence of colourless, thick-walled, roughened conidiophores, ranging from 340 to 1650 μm in length, with globose to sub-globose vesicles, ranging from 22 to 48 μm in diameter. Vesicles had one or two series of phialides. Conidia were globose, olive-green with slightly rough, thin walls that ranged from 3 to 7 μm in diameter. Molecular identification was also performed. Both techniques confirmed the presence of *Aspergillus flavus* complex. Topical treatment with natamycin resulted in healing within 10 days. To our knowledge, this is the first case of equine keratomycosis reported in Argentina.

Keywords: Equine, keratomycosis, *Aspergillus flavus* complex, natamycin.

Resumen: Un equino castrado de 9 años presentó una úlcera corneal en el ojo derecho, sin respuesta al tratamiento. El trauma fue causado por material vegetal. El examen oftalmológico reveló opacidad corneal paraxial cubierta por una descarga blanquecina y edema periférico, asociado a congestión de vasos conjuntivales y angiogénesis corneal. El examen microscópico reveló la presencia de neutrófilos e hifas fúngicas. Al tercer día de cultivo se detectaron varias colonias blancas que se tornaron de color verde pálido. El examen microscópico de estos aislamientos reveló la presencia de conidióforos incoloros, rugosos y de paredes gruesas, de entre 340 y 1650 μm de longitud, con vesículas globosas a subglobosas, de entre 22 y 48 μm de diámetro. Las vesículas tenían una o dos series de fiálides. Los conidios eran globosos, de color verde oliva con paredes delgadas y ligeramente rugosas que oscilaban entre 3 y 7 μm de diámetro. También se realizó la identificación molecular. Ambas técnicas confirmaron la presencia del complejo *Aspergillus flavus*. El uso tópico de natamicina resultó efectivo en la resolución de la lesión. Hasta donde sabemos, este es el primer caso de queratomycosis equina reportado en Argentina.

Palabras clave: Equino, queratomycosis, complejo *Aspergillus flavus*, natamicina.

Introduction

Equines, as well as other species, can develop ulcerative and non-ulcerative keratitis, but horses are considered more susceptible to corneal infections due to the position of the ocular globe, their usual behaviour, environment, or the use of hay as food. Most often, corneal infection originates from the contamination of a traumatic ulcer, or the entry of microorganisms, from the environment or the ocular microflora, into the corneal stroma by traumatic micropuncture (Mustikka *et al.*, 2020). Non-ulcerative keratitis is associated with decreased ocular surface defences, either due to immunosuppression (animal-specific or pharmacological) and/or due to alterations in the tear film.

The microbiota of the ocular surface is of great importance since it collaborates with local immunity, but in turn, it can exercise opportunism and trigger or complicate ocular disorders. This microbiota is made up of Gram-positive and Gram-negative bacterial and fungal organisms. Most scientific papers mention that the most frequently isolated fungi are *Aspergillus* spp. and *Penicillium* spp., but these investigations were carried out only in warm and humid climate regions (Das *et al.*, 2022). This study aimed to report the first case of equine keratomycosis due to *Aspergillus flavus* in Argentina.

Materials and methods

According to the owner, a 9-year-old gelding suffered a trauma in the right eye caused by vegetal material. After clinical examination by a local veterinarian, a chronic infectious ulcerative keratitis was diagnosed and treated with topical tobramycin 0.3 % every 8 hours, topical atropine every 12 hours, and intravenous flunixin meglumine 1.1 mg/kg every 24 hours. The ulcer persisted, together with a whitish discharge after 15 days. Therefore, the veterinarian decided to consult with a specialist. At the time of ophthalmologic examination, the horse was alert and in good general condition. The menace response and the dazzle reflex were positive for both eyes. Pupillary light reflexes were not performed because the right eye was under the effect of atropine. The animal presented blepharospasm and epiphora.

Before the inspection, the palpebral branch of the auriculopalpebral nerve was blocked with 2 mL of lidocaine 2 % (Richmond, Buenos Aires, Argentina), over the zygomatic arch. Lidocaine was injected subcutaneously with a 25-gauge, 5/8-inch needle adjacent to the nerve.

To perform the ophthalmologic examination on the horse, diffuse and focal direct illumination with magnification was used at first, followed by biomicroscopy using a slit-lamp (KangHua, model 6M, Chongqing Kanghua Ruiming, China). Then, direct and indirect ophthalmoscopy were performed with a direct ophthalmoscope (Heine beta 200, Germany) and an indirect ophthalmoscope (Keeler All Pupil, UK) with a Volk +20-D lens. The exam revealed paraxial corneal opacity with whitish discharge and peripheral oedema, associated with congestion of conjunctival vessels and the presence of few superficial vessels in the dorsal area of the cornea. The iris was in mydriasis due to atropine treatment (Figure 1). No alterations were observed in the anterior chamber, lens, or fundus. The contralateral eye did not present lesions.

Proparacaine (Poencaina, Poen, Argentina) was administered locally, and after 10 minutes the sample was taken with a sterile swab and placed in a transport medium. Then, the corneal surface was scraped with a cytobrush (Medibrush XL, Argentina) and 3 smears were taken for subsequent

microscopic observation. Removal of the discharge made it possible to see the bottom of the ulcer with the whitish stroma surrounded by oedema. Staining with fluorescein (0.25 % sodium fluorescein, Poen, Argentina) was performed, which revealed an ulcer of 4 mm diameter. After ophthalmological examination, treatment with 0.3 % ciprofloxacin ophthalmic solution (Denver Farma, Argentina) (1 drop every 6 hours) and 1 % atropine (Alcon, Argentina) ophthalmic solution every 24 hours was indicated. Biological material and the 3 smears were sent to the mycology laboratory for analysis.



Figure 1. Conjunctival hyperemia, paraxial corneal opacity, covered by a whitish discharge with mild vascular reaction, and mydriasis (due to atropine treatment).

Differential diagnoses included bacterial or mycotic ulcerative keratitis, stromal abscess, as well as nonhealing (indolent) ulcers, self-trauma-induced ulcerations, corneal degeneration, and ulcerations secondary to adnexal abnormalities (Richter *et al.*, 2020).

Giemsa and Gram staining were performed on the corneal scraping of the affected eye. Clusters of epithelial cells with fungal septate hyphae and few neutrophilic granulocytes were observed (Figure 2). Based on these preliminary results, treatment was started, 24 hours later, with natamycin 5 % ophthalmic solution (Farmacia Magister, Argentina) (1 drop every 6 hours) together with intravenous flunixin meglumine 1.1 mg/kg every 24 hours. Treatment was directed against fungi, as well as against corneal and intraocular inflammation, responses following fungal replication and dead hyphae. Bacterial contamination was also controlled. After 10 days, the opacity area was negative to fluorescein staining. Treatment continued for 5 more days, for a total of 15 days.

Samples were cultured in 9 cm diameter Petri dishes with agar Sabouraud medium chloramphenicol (50 mg/l) at 37 °C and checked daily for 14 days.

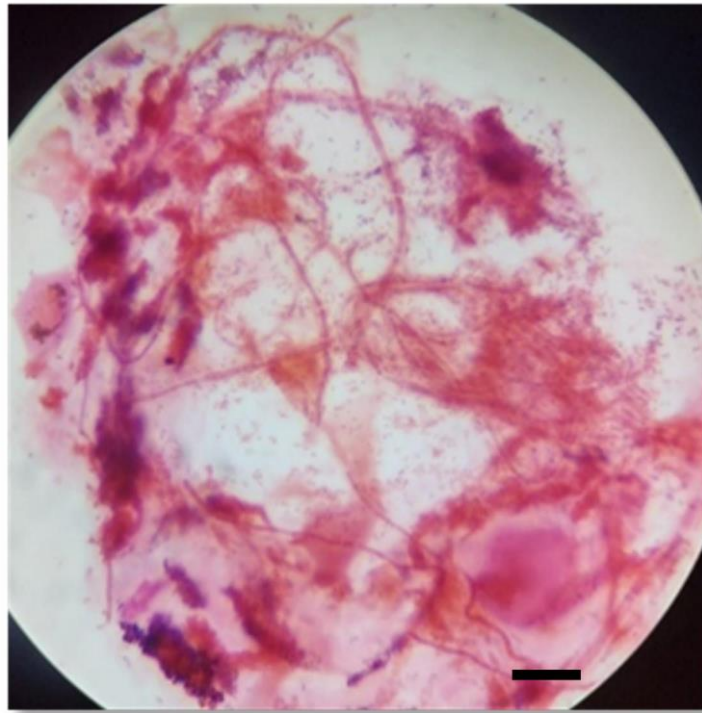


Figure 2. Clusters of epithelial cells with fungal septate hyphae and few neutrophilic granulocytes. Gram staining. Black bar = 20 μm .

Results

On day 3, several small white fluffy colonies that rapidly turned to pale green were detected. These colonies were isolated to study their micro and macromorphology. Microscopic examination revealed the presence of colourless, thick-walled, roughened conidiophores, ranging from 340 to 1650 μm in length, with 22 to 48 μm diameter globose to sub-globose vesicles. Vesicles had one or two series of phialides. Conidia were globose, ranging from 3 to 7 μm in diameter, olive-green, with slightly rough, thin walls. Macromorphology revealed fluffy olive-green colonies in Potato Dextrose Agar (Figure 3).

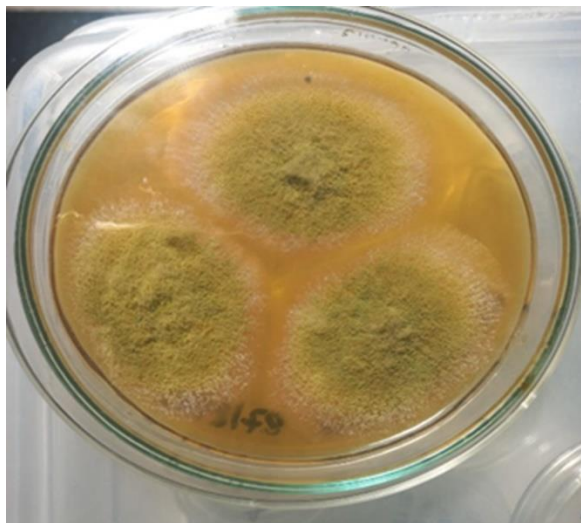


Figure 3. Macromorphology of *Aspergillus flavus* in Potato Dextrose Agar.

Molecular identification was carried out by PCR using primers that amplified the ITS1–5.8S–ITS2 region of ribosomal DNA (Ferrer *et al.*, 2001). The PCR product was sequenced, and the sequence obtained was compared against the GenBank database using BLAST software. A 99.33 % homology with the sequence OQ726517 of *Aspergillus flavus* was obtained which confirmed the definitive diagnosis of keratomycosis due to *Aspergillus flavus*.

Discussion and conclusions

There is a small number of published studies reporting the characterization of the equine ocular surface microbiota. These works were carried out in different geographical regions and climates of the world, which raises controversy regarding the results (Mustikka *et al.*, 2020). This is particularly evidenced in the characterization of the fungal flora because there is variability of the fungi isolated from the equine ocular surface and most of the investigations were performed in warm and humid climate regions. Works carried out on healthy animals suggest that the fungal flora remains stable (Tahoun *et al.*, 2020), while other authors conclude that it may vary according to age, sex, geographic location, climate and even race (Zak *et al.*, 2018).

The diagnosis of keratomycosis occurs frequently in horses in warm and humid areas. However, we reported this case in winter. Keratomycosis has been described in other species such as cattle and alpacas, among others (Voelter-Ratson *et al.*, 2013). In our case, the origin of the fungal contamination was likely trauma with vegetal material. However, it is known that this type of injury in horses is frequent due to the position of the eyeballs, exposure of the cornea, sudden reactions, and the surrounding environment (Plummer 2017). Brooks *et al.* (2013) demonstrated that fungi can penetrate the subepithelial layers without previous injury, due to changes in the tear film.

Several authors observed that most equine ocular keratomycosis occurred in warm and humid countries or regions and that the most frequently isolated aetiological agent was *Fusarium* spp., followed by *Aspergillus* spp, *Purpureocillium* spp., *Alternaria* spp., and *Scedosporium* spp. (Walther *et al.*, 2021). Moreover, Walther *et al.* (2021) reported that natamycin was effective against most fungi species but not against *Aspergillus flavus*; however, the ocular keratomycosis reported in this case responded effectively to treatment with this drug (Sherman *et al.*, 2018).

Natamycin is the treatment of choice for keratomycosis in most animal species. When used topically, it tends to accumulate within corneal ulcers, potentially increasing contact time. It is a commonly used drug for equine ocular fungal infections and is fungicidal *in vitro*, against many filamentous fungi isolated from horses with keratomycosis (Ledbetter *et al.*, 2017; Roberts *et al.*, 2020). It has been shown that it is possible to treat melting ulcers with natamycin combined with the cross-linking technique, with good results (Hellander-Edman *et al.*, 2013). Other authors report the use of systemic antifungals for the treatment of keratomycosis (Pearce *et al.*, 2009).

To the best of our knowledge, this is the first report of equine keratomycosis in Argentina, due to *Aspergillus flavus*. Furthermore, this is the southernmost equine keratomycosis reported worldwide to date. Even though natamycin has been reported as not effective against *Aspergillus flavus* infection, the patient responded effectively to this treatment. We consider that this diagnosis broadens the list of differential diagnoses for corneal infections.

Acknowledgements

The authors want to thank Solis Ailen for their help in laboratory work. This study was financed in part by the National University of La Plata, Argentina.

Conflict of Interest

There is no conflict of interest, including financial, personal, or other relationships, with other persons or organizations.

Declarations-Ethics approval

Examination and sample collection were performed under the consent of the farmer.

Author contribution

GLZ and HT conceived and designed the study, and critically revised the manuscript. GLZ, HT and FJR performed the experiment, analysed the data, and drafted the manuscript. MT and RDV helped in the implementation and execution of the study. MT, RDV and FJR performed and interpreted the laboratory analyses. All authors read and approved the final manuscript.

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