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Inhibition potential of *Caryocar brasiliense* on methicillin-resistant *Staphylococcus pseudintermedius* isolated from the ocular surface of dogs with ophthalmopathies

[*Potencial de inibição do* Caryocar brasiliense *sobre* Staphylococcus pseudintermedius *resistente à meticilina isolado da superfície ocular de cães com oftalmopatias*]

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ABSTRACT

The indiscriminate use of antibiotics has contributed to the emergence of multiresistant bacteria. Faced with this, the search for antibiotics from plants has proven to be a promising alternative. The objective of this work was to isolate and identify *Staphylococcus sp*. resistant to methicillin of the ocular surface of dogs with ophthalmopathies and to evaluate its susceptibility to alcoholic extract of the bark and hexane extract of the pulp of *Caryocar brasiliense.* Biological material was collected from the ocular surface of 21 dogs presenting ophthalmopathies. We isolated 64 *S. pseudintermedius*, among these, 4 isolates were identified as methicillin-resistant *S. pseudintermedius* (MRSP). The alcoholic extract of *C. brasiliense* peel was able to inhibit the bacterial growth of MRSP isolates at a concentration of 2.2%. Thus, the extract from the *C. brasiliense* peel has antimicrobial potential and represents an alternative in the control of MRSP.

Keywords: herbal medicine, MRSP, ophthalmology, PCR, pequi

RESUMO

O uso indiscriminado de antibióticos tem contribuído para o surgimento de bactérias multirresistentes. Diante disso, a busca por antibióticos a partir de plantas tem se mostrado uma alternativa promissora. O objetivo deste trabalho foi isolar e identificar Staphylococcus *sp. resistente à meticilina da superfície ocular de cães com oftalmopatias e avaliar sua susceptibilidade ao extrato alcoólico da casca e ao extrato hexânico da polpa de* Caryocar brasiliense. *O material biológico foi coletado da superfície ocular de 21 cães com oftalmopatia. Isolaram-se 64* S. pseudintermedius; *entre esses, quatro isolados foram identificados como* S. pseudintermedius *resistente à meticilina (MRSP). O extrato alcoólico da casca de* C. brasiliense *foi capaz de inibir o crescimento bacteriano dos isolados de MRSP na concentração de 2,2%. Dessa forma, o extrato da casca de* C. brasiliense *possui potencial antimicrobiano e representa uma alternativa no controle de MRSP.*

Palavras-chave: fitoterapia, MRSP, oftalmologia, PCR, pequi

INTRODUCTION

Staphylococcus sp. methicillin-resistant (MRS) and methicillin-resistant *S. pseudintermedius* (MRSP) occur on the ocular surface of dogs and can cause infections and zoonotic potential (Bardiau *et al*., 2013; Mouney *et al*., 2013), highlighting a problem for treatments conventional and public health (Kang *et al*., 2014).

Caryocar brasiliense (*C. brasiliense*) is a native plant to the Brazilian Cerrado of economic, cultural, and nutritional importance, popularly known as *pequi*. Studies evaluated the antimicrobial activity of the ethanolic and aqueous extract of *C. brasiliense* leaves against *Staphylococcus sp*. strains isolated from sick cattle

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(Ribeiro *et al*., 2018) and *in vitro* (Pinho *et al*., 2012; Baptista *et al*., 2018), finding divergent results. In addition to the antimicrobial activity, the compilation of studies involving this plant species indicates its therapeutic potential, presenting antiparasitic, antioxidant, antitumor, healing, and antifungal action (Bruno Filho *et al*., 2021).

The objective of this work was to isolate and identify bacteria of the *Staphylococcus sp*. resistant to methicillin (MRS) of the ocular surface of dogs with ophthalmopathies and to evaluate their susceptibility to alcoholic extract of the peel and hexane extract of the pulp of *C. brasiliense*.

Ripe fruits of *C. brasiliense* in natura were purchased from a commercial establishment in the city of Jataí, Goiás. The husks were sliced to obtain smaller pieces and, consequently, a larger contact surface. The plant material was added to organic solvent according to its polarity, using 92.8% alcohol for the peel and hexane for the pulp. The different parts of *C. brasiliense* remained in percolation for seven days, and the volume of evaporated solvent was replaced during this period. Then, the solvents were separated in a rotoevaporator, and the extracts remained in a water bath until they reached a constant weight, confirming the total elimination of solvents from the medium. The extracts were kept at a temperature equivalent to -4 °C until use.

The study included the use of 21 dogs diagnosed with unilateral or bilateral ophthalmopathies, of different breeds, different ages, and both sexes, attended at the ophthalmology service of the Veterinary Hospital of the Federal University of Jataí (HV/UFJ), from July to September 2020. All patients were examined for signs of ophthalmic disease or general practice.

The eye examination was complete, standardized, and conducted in a timely manner. The Schirmer tear test (STT) (Schirmer test - Ophthalmos, São Paulo, Brazil), Fluorescein test (Fluoresceina strips - Ophtalmos, São Paulo, Brazil) were conducted under physical restraint. The upper and lower eyelids and the nictitating membrane, bulbar and palpebral conjunctivas, cornea, anterior chamber, iris, and lens were evaluated microscopically (Portable slit-lamp SL-14, Kowa). The posterior segment was evaluated by PanOptic Veterinary Ophthalmoscope (Welch Allyn PanOptic™). All ocular clinical manifestations were considered, the presence of some type of secretion and conjunctivitis, blepharitis, entropion, uveitis, corneal ulcer or opacity, for example.

All procedures adopted in this study were approved by the Ethics Committee in the Use of Animals of the Federal University of Jataí, Protocol number 013/2019. They also followed the care recommended by the ARVO (National Institutes of Health Publications Association for Research in Vision and Ophthalmology).

All samples were obtained, processed, and analyzed by the same investigator. The collections were carried out during the ophthalmic examination, after the performance of the Schirmer tear test, and before the instillation of 0.5% proxymetacaine eye drops (Anestalcon, Alcon, São Paulo, SP) to measure the intraocular pressure. With the help of a swab of cotton wool sterile, biological material was collected from the conjunctival sac of dogs with ophthalmopathies. The swabs were pressed directly and lightly into the inferior conjunctival sac of each eye, using rotational movements. Afterward, the specimens were placed in Stuart semi-solid transport medium and sent to the Laboratory of Veterinary Microbiology at UFJ. On the same day of collection, the material was inoculated into Mannitol Agar and kept in an oven at 37°C for 24 hours. Four colonies from each plate were picked to obtain pure cultures.

After growing the colonies on Mannitol Agar, Gram stain was performed. For biochemical identification of the isolates, the following tests were applied: Catalase test, coagulase, Voges-Proskauer test (VP), DNase, sensitivity to Bacitracin (300 UI) and Polymyxin (0.04 UI), and PYR test, according to methodology from the laboratory.

Staphylococcus sp. methicillin-resistant (MRS) were identified using cefoxitin disk 30µg, as described by CLSI/M100-S20 (Performance…, 2010). To identify methicillin-resistant *S. pseudintermedius*, an oxacillin 1µg disk was used. To perform the disk diffusion antibiogram, the following antibiotics were used: enrofloxacin 5µg, clindamycin 2µg, ampicillin 10µg, penicillin 1UI, imipenem 10µg, chloramphenicol 30µg, tetracycline 30µg, ciprofloxacin, neomycin 30µg, erythromycin 15µg, amikacin 30µg, cephalothin 30µg, ceftriaxone 30µg, cephalexin 30µg,

Tobramycin 10µg, Doxycycline 30µg, vancomycin 30µg, gentamicin 10µg and sulfazotrim 25µg. The isolates were placed in tubes containing 3mL of nutrient broth and incubated at 35 °C until they reach the 0.5 MacFarland standard. After incubation, cultures were seeded with the aid of sterile swabs, in plates containing Mueller-Hinton agar and, after approximately three minutes, the time necessary for the medium surface to dry, the discs containing the antimicrobial were added. The reading was carried out after 18h of incubation at 35 °C, by measuring the inhibition zones, using a millimeter ruler.

Minimum inhibitory concentration (MIC) was performed according to Hernández-Avilés *et al*. (2018). Serial dilutions were made from the extracts of *C. brasiliense* in phosphate-buffered saline (PBS), placed in Falcon microtubes, where the strains were inoculated. To dilute the pulp extract, polysorbate 80 (Tween 80) at a concentration of 10% was used as a demulcent and PBS (Perches *et al*., 2012). Afterward, the tubes remained in an oven at 37°C for 24 hours. A 10 µl aliquot of each tube was inoculated in Petri dishes containing Muller-Hinton agar, which remained 24 hours in an incubator at 37 °C, for colony counting. Bacterial suspension in PBS at standard concentration 1x106 CFU/mL and PBS solution as a negative control was used as a positive control. The evaluations were carried out in triplicate.

For PCR analysis, the extraction of bacterial genomic DNA, an elevation of the stock culture was cultured in 1mL of BHI broth for 12 hours at 35°C in a test tube, the entire volume was transferred to a 1.5 microtube mL which was centrifuged at 5000 rpm for 4min. The pellet was discarded, and the pellet was washed 3 times with 200µl of TE buffer. Subsequently, the pellet was resuspended in 100µl of TE buffer, the microtubes were heated to 95 °C in a water bath for 10 min and then centrifuged at 5000 rpm for 20 seconds. The supernatant (100 µL) was transferred to a 500 µL microtube and frozen at -20 °C and stored. The detection of the MecA gene through polymerase chain reaction (PCR) was performed according to Neves *et al*. (2007). The oligonucleotide primers used to detect a 533 fragment were: 5' AAA ATC GAT GGT AAA GGT TGG C 3' and 5' AGT TCT GCA GTA CCG GAT TTG C 3'. 96-well PCR plates will be used using 2 µL of previously diluted DNA (30ng); 2.5µL of 1x PCR buffer (50 mM KCl, 200 mM TRIS-HCl, pH 8.4); 2.5 U Taq DNA polymerase; 0.2 mM dNTP; 1.5mM MgCl2, 1 µg of each primer and sterile Milli Q water to complete the reaction volume in 20 µL. The samples were placed in a thermocycler with the following cycle: 2 min at 94 °C; 1 min at 94 °C; 2 min at 52 °C; 2 min at 72 °C; 39 cycles from step 2; 5 min at 72 °C and keeping the samples under refrigeration at 5 °C. All products, after the amplification process, were analyzed on agarose gel with 1.5% ethidium bromide and processed at 65 V for 1h 30min.

RESULTS AND DISCUSSION

There was bacterial growth in all collected samples. A total of 154 isolates were identified, of which 17 were discarded for being *Micrococcus sp.* whereas 137 isolates corresponded to the genus *Staphylococcus sp.* (Table 1). Despite composing the normal conjunctival microbiota of dogs, *Staphylococcus sp*. are opportunistic pathogens with infectious potential in cases where there are eye injuries (Oriá *et al*., 2013).

Table 1. Species and occurrence of isolates of the *Staphylococcus* genus

Species	Occurrence	%
S. pseudointermedius	64/137	46.7
S. schleiferi	17/137	12.4
S. hyicus	12/137	8.8
S. sp.	44/137	32.1

In 33,3% of the cases of ulcerative keratitis there was the presence of multiresistant *S. pseudintermedius*, corroborating the findings of Ekapoppahan *et al*. (2018). In addition to the multiresistant profile, more than 90% of isolated *Staphylococcus* corresponded to *Staphylococcus sp.* resistant to methicillin. Although the pathophysiology is still not well understood, there seems to be a proportional relationship between the extent of the ulcer and the presence of methicillin-resistant bacteria (Ekapoppahan *et al*., 2018).

Multi-resistance to different classes of antibiotics was found by 59.4% (38/64) of *the S. pseudintermedius* isolates. Of these, 4 isolates (25, 44, 50, and 53) corresponded to MRSP, being present in cases of entropion, ectropion, uveitis, and keratoconjunctivitis sicca (Table 2). The results corroborate the multidrug resistance profile. A frequency of *S. pseudintermedius* in 50% of the evaluated dogs was detected, where three corresponded to MRSP.

Table 2. Pathotypes and antimicrobial resistance profile of *Staphylococcus pseudintermedius* isolated from ophthalmopathies in dogs

		MRSP Search	antibiogram																		
Isolated	ophthalmopathy	Oxa	Pen	Tob	South	Cro	Cfl	Cfe	Neo	Cli	Dox	Im	Enr	Clo	Gen	Ami	Amp	Eri	Tet	Van	Resistance Profile*
1	entropion	s	R	R	R	s	s	s	R	s	s	s	R	s	R	s	s	s	R	s	MDR
2	corneal ulcer	s	R	S	I	s	s	s	R	s	s	s	s	s	I	s	s	s	R	s	MDR
3	Florida Spots	s	s	R	I	R	s	s	R	s	I	s	s	s	I	s	s	s	R	s	MDR
4	Florida Spots	s	R	S	s	s	s	s	s	s	s	s	s	s	s	s	I	s	R	s	SDR
5	KCS	s	R	S	s	s	s	s	I	s	I	s	R	s	s	s	I	s	R	R	MDR
6	corneal ulcer	s	s	S	s	s	s	s	I	s	s	s	s	s	I	s	s	s	R	s	SDR
7	KCS and Distichiasis	s	R	S	R	s	s	s	I	s	R	s	I	s	s	s	I	s	R	R	MDR
8	corneal ulcer	s	s	S	s	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	SDR
9	KCS	s	R	S	s	s	s	s	s	s	s	s	R	s	s	s	s	s	R	s	MDR
10	KCS	s	R	S	s	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	SDR
11	KCS	s	R	S	s	s	s	s	R	s	s	s	s	s	s	s	s	s	R	s	MDR
12	chalazion	s	s	S	s	s	s	s	I	s	s	s	s	s	s	s	s	s	R	s	SDR
13	Uveitis	s	s	S	s	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	SDR
14	KCS	s	R	S	I	s	s	s	s	s	s	s	s	s	s	s	s	s	R	R	SDR
15	corneal ulcer	s	s	S	I	s	s	s	s	s	s	s	s	s	I	s	s	s	R	s	SDR
16	KCS	s	R	S	s	s	s	s	s	s	s	s	R	s	s	s	s	s	R	s	MDR
17	chalazion	s	s	S	s	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	SDR
18	entropion	s	R	R	R	s	s	s	R	s	s	s	R	s	R	s	s	s	R	s	MDR
19	chalazion	s	s	S	s	s	s	s	R	s	s	s	s	s	s	s	s	s	R	R	SDR
20	corneal ulcer	s	s	I	I	s	s	s	R	s	s	s	s	s	R	s	s	s	R	s	SDR
21	Florida Spots	s	R	S	s	s	s	s	R	s	s	s	s	s	s	s	I	s	s	R	SDR
22	KCS, Trichiasis and Distichiasis	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	NDR
23	entropion	s	R	R	R	s	s	s	R	s	I	s	R	s	R	s	s	R	R	s	MDR
24	Uveitis	s	s	s	s	s	s	s	s	s	s	s	s	s	I	s	s	s	R	s	SDR
25	Entropion and Ectropion	R	R	s	R	s	s	s	R	R	R	I	s	s	s	s	I	R	R	R	MDR/MRSP
26	KCS, Trichiasis and Distichiasis	s	s	s	s	s	s	s	I	s	s	s	s	s	s	s	s	s	s	s	NDR
27	KCS and Distichiasis	s	R	s	R	s	s	s	I	s	s	s	R	s	s	s	s	s	R	s	MDR
28	entropion	s	R	I	R	s	s	s	R	I	I	s	R	s	R	s	s	R	R	R	MDR
29	KCS	s	R	s	s	s	s	s	s	s	I	s	R	s	s	s	s	s	R	R	MDR
30	entropion	s	R	s	I	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	SDR
31	trichiasis	s	R	s	R	s	s	s	s	s	s	s	R	s	s	s	s	s	R	s	MDR
32	KCS and Distichiasis	s	R	s	R	s	s	s	s	s	s	s	R	s	s	s	I	s	R	s	MDR
33	KCS	s	R	s	s	s	s	s	s	s	I	s	R	s	s	s	I	s	R	R	MDR
34	trichiasis	s	R	I	R	s	s	s	s	I	s	s	s	R	I	s	I	R	R	s	MDR
35	entropion	s	R	s	I	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	SDR
36	entropion	s	R	s	R	s	s	s	s	s	R	s	s	s	s	s	s	s	R	R	MDR
37	KCS and Distichiasis	s	R	s	R	s	s	s	s	s	s	s	R	s	s	s	I	s	R	s	MDR
38	entropion	s	R	s	R	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	MDR
39	entropion	s	R	R	R	s	s	s	s	I	s	s	R	s	R	s	s	R	R	s	MDR
40	KCS and Distichiasis	s	R	s	R	s	s	s	s	s	R	s	R	s	s	s	I	s	R	R	MDR
41	entropion	s	R	R	R	s	s	s	s	I	s	s	R	s	R	s	s	R	R	s	MDR
42	Entropion and Ectropion	s	R	s	R	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	MDR
43	entropion	s	R	s	R	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	MDR
44	KCS	R	R	s	s	s	s	s	s	s	s	s	R	s	s	s	s	s	R	s	MDR/MRSP
45	KCS, Trichiasis and Distichiasis	s	R	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	SDR
46	entropion	s	R	s	R	s	s	s	s	I	R	s	s	s	s	s	s	s	R	R	MDR
47	KCS	s	R	s	s	s	s	s	s	s	R	s	R	s	s	s	s	s	R	R	MDR
48	Uveitis	s	s	s	I	s	s	s	s	s	R	s	s	s	I	s	s	s	R	s	SDR
49	entropion	s	R	s	R	s	s	s	s	s	s	s	s	s	s	s	s	s	s	R	MDR
50	Uveitis	R	s	R	s	R	s	s	s	s	R	s	s	I	s	s	s	R	R	s	MDR/MRSP
51	entropion	s	R	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	R	SDR
52	chalazion	s	s	s	s	s	s	s	s	s	I	s	s	s	s	s	s	s	R	R	SDR
53	entropion	s	R	s	R	s	s	s	s	s	I	s	s	s	s	s	s	s	R	s	MDR
54	KCS, Trichiasis and Distichiasis	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	NDR
55	Uveitis	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	SDR
56	trichiasis	s	R	s	R	s	s	s	R	I	I	s	s	R	s	s	I	R	R	R	MDR
57	chalazion	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	SDR
58	trichiasis	s	R	s	R	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	SDR
59	Uveitis	s	s	s	s	s	s	s	s	s	I	s	s	s	s	s	s	s	R	s	SDR
60	KCS	s	R	s	s	s	s	s	s	s	I	s	R	s	s	s	s	s	R	s	MDR
61	entropion	s	R	s	R	s	s	s	s	s	s	s	s	s	s	s	s	S	R	s	MDR
62	KCS	s	R	s	s	s	s	s	I	s	s	s	R	s	s	s	s	s	R	s	MDR
63	entropion	R	R	s	R	s	s	s	s	s	s	s	s	s	s	s	s	s	R	s	MDR/MRSP
64	corneal ulcer	s	s	R	I	s	s	s	R	s	I	s	s	s	I	s	s	s	R	s	MDR

Caption: KCS (Keratoconjunctivitis Sicca) NDR (no drug resistance); SRD (single drug resistance); MDR (multi drug resistance); MRSP (methicillin resistant *Staphylococcus pseudintermedius*); Oxa (Oxacillin); Pen (Penicillin); Tob (Tobramycin); Sul (Sulfamethoxazole with Trimethoprim); Cro (Ceftriaxone); Cfl (Cephalothin); Cfe (Cephalexin); Neo (Neomycin); Cl (Clindamycin); Dox (Doxycycline); Imi (Imipenem); Enr (Enrofloxacin); Clo (Chloramphenicol); Gen (Gentamicin); Ami (Amikacin); Amp (Ampicillin); Eri (Erythromycin); Tet (Tetracycline); Van (Vancomycin).

Among the *S. pseudintermedius* isolates from animals diagnosed with keratoconjunctivitis sicca (KCS), 71.4% (15/21) were multiresistant, and of these, one was also identified as MRSP (isolate 44). The indiscriminate use of antibiotics linked to tear film deficiency leads to the emergence of multiresistant isolates (Pereira *et al*., 2019).

S. pseudintermedius is a bacterium capable of causing topical and systemic infections. The increasing prevalence of MRSP isolates is a matter of concern both for animals and in the context of public health. It is hypothesized that commensal *S. pseudintermedius* involved in infectious processes may acquire genetic characteristics responsible for the development of methicillin resistance (Lynch and Helbig, 2021). The longitudinal transmission of the etiological agent is still not well understood, however, the detection of MRSP in humans, veterinary professionals, and veterinary hospitals has been frequent (Marques *et al*., 2013; Fleber *et al*., 2018).

Due to its intrinsic resistance character, the MRSP isolates were subjected to the action of *Caryocar brasiliense* extracts. The pequi pulp extract was not able to inhibit bacterial growth at any of the concentrations used, so it was found that concentrations lower than 25% of the hexane extract of the fruit are not effective in controlling methicillin-resistant *S. pseudintermedius*. On the other hand, the peel extract was able to inhibit bacterial growth at a concentration equal to or greater than 2.2%, with 27.5mg/mL being defined as the minimum inhibitory concentration of this compound. The pequi peel corresponds to more than 70% of the fruit's weight and is generally not used. As it is not used in human food, studies regarding its toxicity are still scarce (Arruda *et al*., 2012). Almeida *et al*. (2010) determined the LD50 of the hydroalcoholic extract of peel bran as equivalent to 0.31mg/mL when administering the solution intraperitoneally in rats. However, other routes must be evaluated as well as different preparations of the extract.

The peel of *C. brasiliense* has gallic acid, tannins, and phenols in its composition. Such metabolites have recognized antibacterial action (Rocha *et al*., 2015). Pinho *et al*. (2012) showed that peel bran extract did not show efficient bactericidal action against *S. aureus* and Escherichia coli. The biochemical composition of plant matter can change as a result of seasonality and environmental conditions where it was cultivated and still have an influence on the final concentration of active principles as a result of its form of extraction. Such factors justify the opposite findings to the present work.

Although there are no studies using *C. brasiliense* on the ocular surface, the use of herbal medicines in ophthalmology is promising. Solutions based on *Copaifera multijuga*, *Citrus limon*, *Ottonia martiana*, and *Aloe vera* (in vitro) have already been evaluated in the treatment of ophthalmopathies and presented encouraging results, showing that plant compounds have the potential to be used in ophthalmic therapies (Curto *et al*., 2014; Dias *et al*., 2017 Lisbão *et al*., 2012; Perches *et al*., 2012).

Although the isolates were phenotypically resistant to methicillin, the polymerase chain reaction analysis found the absence of the MecA gene in the DNA of the MRSP isolates. It is possible that the resistance of these isolates comes from other pathways, as this characteristic is regulated by a complex gene system. According to Gagetti *et al*. (2019) other genes, such as the *blaZ* gene, are also responsible for resistance to β-lactams, and mutations in different genes can result in resistance to other classes of antibiotics. McCartney *et al*. (2015) point out that horizontal gene transfer is common among the population of *Staphylococcus pseudintermedius*.

CONCLUSIONS

The presence of methicillin-resistant *Staphylococcus pseudintermedius* was identified on the ocular surface of dogs with ophthalmopathies. The extract from the pequi peel has antimicrobial potential and represents a viable alternative for MRSP control. The assessment of susceptibility to plant compounds with antimicrobial action is of great significance for the development of new therapies against the resistance of these infectious agents. Preclinical studies should be carried out to assess its toxicity on the ocular surface.

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