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Research Paper

Antimicrobial photodynamic therapy for infectious stomatitis in snakes: Clinical views and microbiological findings

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ABSTRACT

Background: Antimicrobial photodynamic therapy (APDT) has been broadly investigated as an alternative to treat localized infections, without leading to the selection of resistant microorganisms. Infectious stomatitis is a multifactorial disease frequently reported in captive snakes characterized by infection of the oral mucosa and surrounding tissues. In this study, we investigated methylene blue (MB)-mediated APDT to treat infectious stomatitis in snakes and verified the resistance phenotype and genotype before and after APDT.

Methods: Three Boid snakes presented petechiae, edema and caseous material in their oral cavities. MB (0.01%) was applied on the lesions and after 5 min they were irradiated using a red laser (λ = 660 nm), fluence of 280 J/cm^2, 8 J and 80 s per point, 100 mW, spot size 0.028 cm^2 and fluence rate of 3.5 W/cm^2. APDT was repeated once a week during 3 months. Samples of the lesions were collected to identify bacteria and antibiotic resistance profiles. To analyze the clonality of bacterial isolates before and after APDT, isolates were subjected to ERIC PCR analysis.

Results: Snakes presented clinical improvement such as reduction of inflammatory signs and caseous material. *Pseudomonas aeruginosa* and *Escherichia coli* were present in all snakes; *Klebsiella pneumoniae* and *Morganella morganii* were also identified in some animals. We also observed that the oral microbiota was completely replaced following APDT. However, *K. pneumoniae* isolates before and after APDT were a single clone with 100% of genetic similarity that lost resistance phenotype for seven antibiotics of four classes.

Conclusions: These results show that APDT can be used to treat infectious stomatitis in snakes.

1. Introduction

Infectious stomatitis or “mouth rot” is one of the most commonly diagnosed diseases in captive reptiles. It is characterized by the infection of the oral mucosa and surrounding tissues. Nutrition deficiencies, stress, poor oral hygiene, and oral trauma are considered primary and predisposing factors for the occurrence of this disease [1,2].

Gram-positive bacteria are predominant in oral cavity of healthy reptiles; in contrast, Gram-negative bacteria, fungi and viruses are commonly isolated in oral cavity of ill reptiles. It is believed that changes in oral microbiota are directly related to immunosuppression due to stress developed during captivity adaptation [1].

The first symptoms of stomatitis are petechiae in the oral cavity, inappetence, caseous material along the dental arcade, excessive production of saliva and regurgitation. In severe cases, stomatitis may induce progressive weight loss concomitant to other diseases such as pneumonia and chronic proliferative lesions, probably increasing the risks of osteomyelitis or septicemia development [1,2].

Usually, infectious stomatitis treatment is based on administration of broad-spectrum antibiotics (e.g., carbenicillin, ceftazidime, chloramphenicol, enrofloxacin or gentamicin). However, antibiotic treatment is considered long and often frustrating since immunosuppression development seems to be the main factor impairing clinical recovery [1,2].

In this context, antimicrobial photodynamic therapy (APDT) has emerged as a feasible alternative to treat localized infections. Its action is based in the association of a photosensitizer (PS), light and oxygen. This interaction culminates in a sequence of photophysical and

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Table 1
Biometric data of the snakes.

	Clinical case	1	2	3
Animals	Specie	*Boa constrictor constrictor*	*Boa constrictor amarali*	*Boa constrictor constrictor*
	Body mass (kg)	1.33	1.14	1.03
	Snout-vent length (cm)	124	126	100
	Total length (cm)	138	142	120
	Gender	Male	Female	Female

photochemical processes that result in reactive oxygen species generation to promote oxidative stress of cellular components leading to microorganism inactivation. Furthermore, due to its action on multiple targets, the development of resistant microorganisms seems unlikely [3].

In 2005, a review encouraged the use of APDT for infectious stomatitis in snakes [4]. More than ten years later with the scientific evidences provided by literature, our group is investigating the ability of methylene blue (MB)-mediated APDT to treat infectious stomatitis in captive snakes. In addition, to gain a deeper understanding we identified microorganisms associated to this disease and analyzed bacterial resistance phenotype and genotype before and after APDT.

2. Materials and methods

2.1. Animals

Three captive adult Boid snakes (Table 1) from the Laboratory of Herpetology of Butantan Institute (São Paulo, Brazil) presented repeated regurgitation after feeding. Their oral cavities were examined and revealed petechiae, edema and focal caseous material, which are considered common symptoms of stomatitis (Fig. 1A).

The snakes were housed individually in cages containing corrugated cardboard and a vessel of fresh water (*ad libitum*) inside a room with artificial lighting and climate controlled. Laboratory mice (*Mus musculus*) were offered monthly for feeding.

2.2. Bacterial identification and antibiotic resistance profiles

In all cases, after clinical diagnosis, a sample of the lesions was collected through a sterile swab and cultivated in brain–heart infusion broth, blood agar, and MacConkey agar. Strains were isolated and identified by colony morphology, biochemical tests, and Vitek 2 system, and were stored at −80 °C. Antimicrobial susceptibility was determined by disk diffusion test according to international standards [5,6]. Isolates were tested against a set of antimicrobial agents: ampicillin (AMP) (10 μg), cefoxitin (FOX) (30 μg), ceftazidime (CAZ) (30 μg), cefotaxime (CTX) (30 μg), cefepime (CPM) (30 μg), imipenem (IPM) (10 μg), amoxicillin + clavulanic acid (AMC) (20/10 μg), aztreonam (AZT) (30 μg), streptomycin (STR) (10 μg), gentamycin (GEN) (10 μg), amikacin (AMI) (30 μg), tetracycline (TET) (30 μg), chloramphenicol (CLO) (30 μg), ciprofloxacin (CIP) (5 μg), enrofloxacin (ENR) (5 μg), nalidixic acid (NAL) (30 μg), sulfonamides (SUL) (300 μg), sulfamethoxazole + trimethoprim (SXT) (23,75/1,25 μg). After 3 months, new samples of the lesions were collected to identify the microorganisms at the end of the treatment.

2.3. Treatment procedure

The same APDT procedure was carried out in all cases. To perform APDT, we kept the snake's mouth opened with an anatomical tweezer, removed all caseous material with a brush and applied about 1 mL of MB aqueous solution at a concentration of 0.01% (Sigma-Aldrich; St. Louis, MO, USA) directly on the lesions through a syringe. After 5 min, we irradiated punctually the lesions with a diode laser emitting a wavelength of 660 nm, fluence of 280 J/cm^2, 8 J and 80 s per point, 100 mW, spot size 0.028 cm^2 and fluence rate of 3.5 W/cm^2 (Therapy XT, DMC®, São Carlos, SP, Brazil) (Fig. 1B). The treatment was repeated once a week during 3 months and the clinical evaluation was based on the reduction of injured area, reduction of inflammatory signs and presence of reinfection during experimental time.

2.4. DNA fingerprint analysis (ERIC-PCR)

To analyze the clonality of bacterial isolates before and after APDT, isolates were subjected to enterobacterial repetitive intergenic

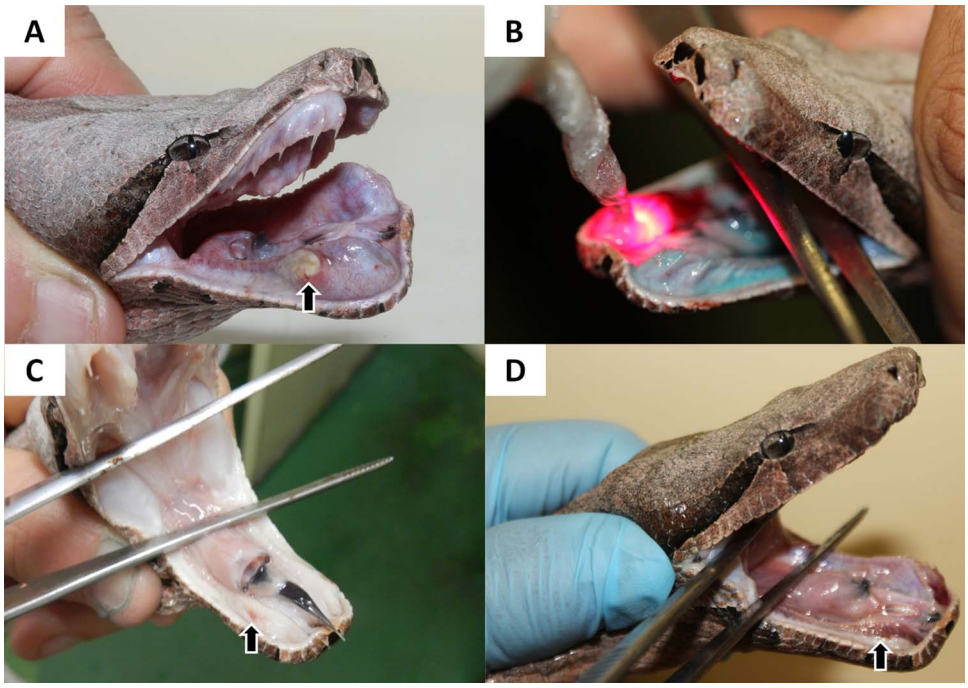

Fig. 1. Representative images of stomatitis in snakes treated by APDT. Initial lesion before treatment (A); application of the photosensitizer MB and irradiation with the diode red laser (B); Lesion aspect one week following APDT (C); significant reduction of the lesion at the end of treatment (3 months) (D). Arrows point to lesion.