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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

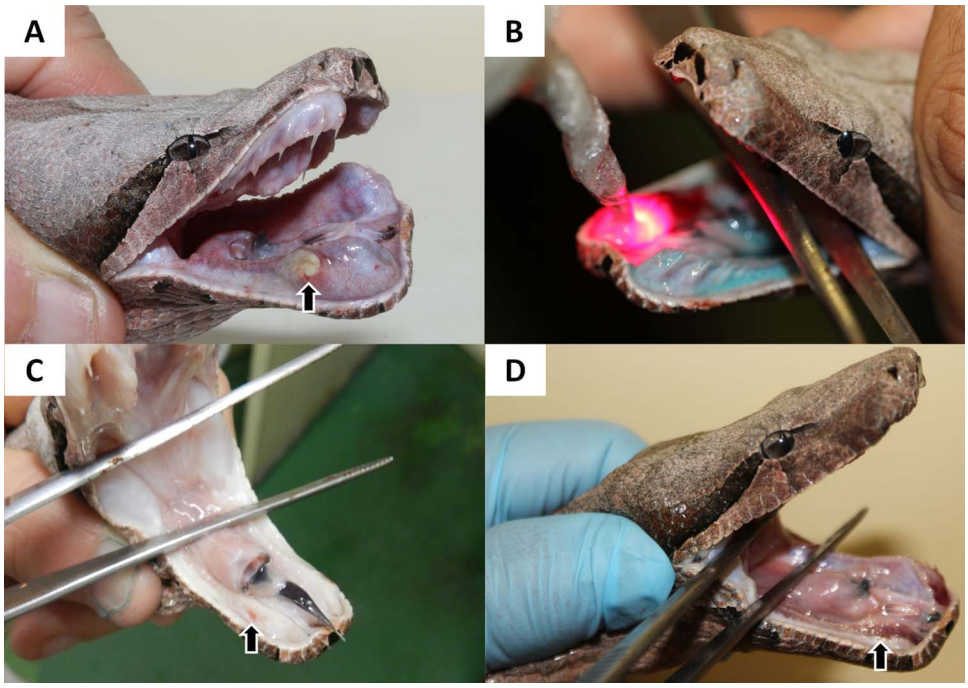

Fig. 1. Representative images of stomatitis in snakes treated by APDT. Initial lesion before treatment (A); application of the photosensitizer MB and irradiation with the diode red laser (B); Lesion aspect one week following APDT (C); significant reduction of the lesion at the end of treatment (3 months)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

consensus (ERIC) PCR analysis using primers ERIC1 and ERIC2 according protocol described by Versalovic et al. [7]. Fingerprint patterns were analyzed by a comprehensive pairwise comparison of band sizes, using the Dice coefficient through BioNumerics 7.6 software (Applied Maths, Sint- Martens-Latem, Belgium).

2.5. Determination of resistance genotype

Strains isolated from the same snake that showed 100% of genetic similarity in ERIC-PCR analysis, but different patterns of resistance phenotype before and after APDT, had the resistance genotype determined by PCR. The presence of antimicrobial resistance genes of beta-lactams (bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CMY}), aminoglycosides (*aadA, aadB, aphA, strAB*), tetracycline (*tetA, tetB, tetC*), sulfas (*sul1, sul2, sul3*), sulfa/trimetropim (*dfrA* variants), phenicols (*catB*, *cmlA, floR*), and quinolones (*qnrA, qnrB, qnrC, qnrD, qnrS, oqxAB, aac(6′)-Ib-cr, qepA*) was carried out as previously described [8–10].

2.6. Plasmid analyses

Plasmid profiles were obtained by extraction and purification using Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) and visualization after electrophoresis in 0.7% agarose gels subjected to 80 V for 5 h stained with SYBR Safe DNA gel stain (Invitrogen, São Paulo, Brazil). Plasmids of known sizes were used as molecular size markers.

3. Results

All animals improved and showed reduction of inflammatory signs and caseous material following the first week post-APDT (Fig. 1C). However, despite the clinical improvement, we observed signs of reinfection (petechiae and/or caseous material) on the first month. No regurgitation was observed after APDT procedure. Furthermore, the disease did not progress and no complications of the animals' clinical condition were observed. After 3 months, no caseous material was seen and significant reduction of petechiae was observed in case 1 (Fig. 1D). In cases 2 and 3 we observed notable reduction of caseous material and petechiae.

Bacterial identification revealed that *Pseudomonas aeruginosa* and *Escherichia coli* were present in all snakes. *Klebsiella pneumoniae* and *Morganella morganii* were also identified. For case 1, *K. pneumoniae*, *P. aeruginosa,* and *E. coli* were resistant to 10, 13, and 2 antibiotics, respectively (Fig. 2). Following APDT we noticed that resistance profile changed, i.e., *K. pneumoniae*, *P. aeruginosa*, and *E. coli* were resistant to 3, 10 and 0 antibiotics, respectively (Fig. 2).

DNA fingerprint analysis shown that *P. aeruginosa* and *E. coli* were replaced for other clones of the same species, band patterns of ERIC-PCR are showed in Fig. 2. However, the two *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 (phenicols, sulfa/trimetropim, tetracycline, and beta-lactams) (Fig. 2). Posterior analysis of resistance genotype showed the lack of three genes that encode resistance to sulfonamides, trimetropim and tetracycline (*sul1*, *dfrA12*, and *tetA*). Since the loss of resistance genes occurs most often due to a loss of plasmids, further analysis was conducted to verify the lack of plasmids. However, both *K. pneumoniae* isolates shown harbor a 100 kilobase (a hundred thousand base pairs) plasmid.

In case 2, interestingly, we observed that the oral microbiota was completely replaced following APDT. For case 3, we also observed a change in the resistance profile and microbiota after APDT. In fact, strains isolated after the treatment were different clones of the same species.

4. Discussion

It is well known that stomatitis in snakes is an immune-related disorder that primarily affects captive animals. Thus, it is important to highlight that ill snakes could be a serious problem for venom extraction, and, consequently, in snake antivenin production. Moreover, as it is a secondary infection, pet snakes can also be affected [1].

Few studies report Gram-negative bacteria as most commonly isolated from infectious stomatitis, mainly including *Pseudomonas* sp., *Aeromonas hydrophila*, *E. coli*, *Salmonella* sp., *Providencia* sp., *Klebsiella* sp., *Morganella* sp. and *Proteus* sp. [1]. In our study, all microorganisms isolated from the samples were Gram-negative bacteria, which reiterate the role of those pathogens in the disease etiology [1].

In case 1, we observed the same bacteria before and after treatment (*K. pneumoniae*, *P. aeruginosa,* and *E. coli*), indicating that those microorganisms remained in the oral cavity causing reinfection. Although DNA fingerprint analyses showed that only *K. pneumoniae* were the same clone, the other species were replaced by other strains. In case 3, we observed the presence of *P. aeruginosa* and *E. coli* before and after treatment period. On the other hand, in case 2 we noticed that *E. coli* and *M. morganii* were substituted by *P. aeruginosa*. We hypothesize that APDT favored microbiota alteration related to intrinsic Gram-negative bacteria colonization, since irradiation was only performed on the lesion.

Remarkably, both isolates of *K. pneumoniae* of case 1 were classified as a clone with 100% of genetic similarity, but showed different patterns of genotypic and phenotypic resistance. Given that, we suggest that APDT may have influenced the loss of *sul1*, *dfrA12*, and *tetA* genes. Regarding the genes that matched in both strains, *strAB* confers resistance to streptomycin, and bla_{SHV} and *oqxAB* confer resistance to beta-lactams and low level of resistance to fluoroquinolones, respectively. These two last genes are largely distributed in *K. pneumoniae* specie on chromosomal location. In *Enterobacteriaceae*, the genes that were lost can be found in chromosome or plasmids, but are commonly found in large conjugative plasmids [11,12]. We suppose that the lack of resistance phenotype to beta-lactams AMC, CLO, FOX and AZT after APDT could be related to overproduction of chromosomal *ampC* or alterations in expressions of outer-membrane porins in the strain isolated before APDT [13]. Plasmid analyses showed that the two isolates have a 100 Kb plasmid. However, the technique used allows visualization of plasmids up to 120 Kb, and *K. pneumoniae* can harbor plasmids larger than 250 Kb [14]. Loss of a plasmid is called “cure” and many methods are described to perform *in vitro* plasmid curing of bacteria. These methods are based on chemical and physical stress, like exposure to phenothiazine tricyclic compounds, ethidium bromide, acridine orange, or growth in high temperatures (45 °C) [15,16]. Thus, APDT could be a new approach to promote antiplasmid effects.

Although the loss of phenotype is related to the loss of genes, it is unclear whether this is related to the loss of a plasmid or deletion of a chromosomal region. However, our results suggest that APDT outcome is closely linked to alterations in the DNA. In fact, recently Costa and colleagues showed that MB is able to generate DNA damage in a zebrafish model [17]. Thus, further studies are warranted to a better comprehension of sublethal MB-mediated APDT on molecular targets of *K. pneumoniae*.

APDT studies in veterinary medicine have shown promising results in mammals and birds [18–21]. In this study, we investigate for the first time the use of APDT to treat reptile infections (ectothermic animals), which emphasizes the novelty of this investigation. Our study showed that APDT was able to improve infectious stomatitis in snakes, besides changing the *K. pneumoniae* resistance profile. Following APDT, the bacterial strains were mostly susceptible to enrofloxacin, chloramphenicol, sulfamethoxazole, and tetracycline, which have different mechanisms of action on bacteria. For example, enrofloxacin restrains the DNA gyrase activity preventing DNA synthesis [22], while chloramphenicol is bacteriostatic and inhibits protein synthesis [23].

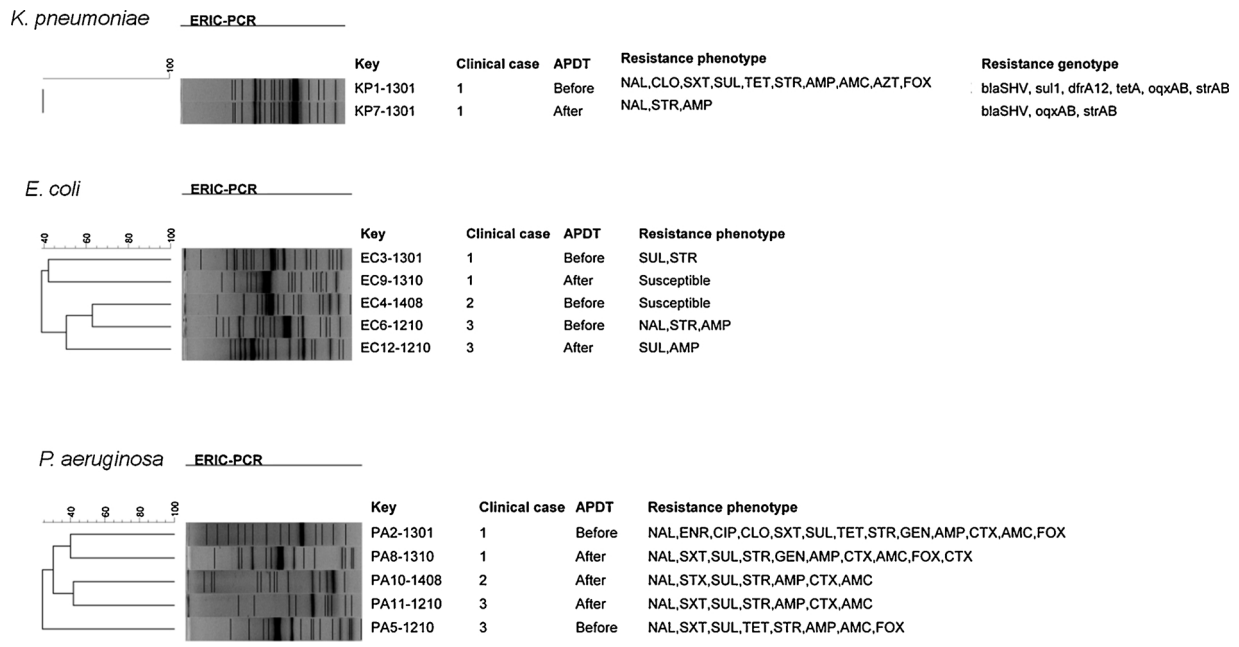

Fig. 2. ERIC-PCR dendrogram generated by BioNumerics software based on the Dice similarity index, indicating the genetic relatedness of two *K. pneumoniae*, five *E. coli* and five *P. aeruginosa* strains isolated from stomatitis lesions. The level of similarity (%) is shown on left. Resistance phenotype and genotype are also displayed on right.

Literature reports that APDT can damage the functional integrity of cell wall, DNA and membrane proteins [24].

In addition, the fact of a bacterial strain being reisolated with different phenotype and genotype resistance is a finding that may guide future research in the application of APDT. It is important to highlight that recent studies identified the plasmid-mediated colistin resistance mechanism (MCR-1) in humans and animals [25,26]. These new findings inspire the scientific community to pursue new antimicrobial approaches since colistin is considered the last-resort drug against Gram-negative bacteria. We will continue engaging with this investigation and encourage future studies to elucidate mechanisms behind APDT.

Conflict of interest

The authors declare that they have no conflict of interest.

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