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Effective treatment and decolonization of a dog infected with carbapenemase (VIM-2)-producing *Pseudomonas aeruginosa* using probiotic and photodynamic therapies

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Abstract

Background—Carbapenem-resistant bacterial infections are a critical problem in veterinary medicine with limited treatment options.

Objective—To describe effective probiotic and photodynamic therapy of a dog with gut colonization and ear infection caused by a hospital-associated lineage of carbapenemase (VIM-2)-producing *Pseudomonas aeruginosa*.

Animals—A 5-year-old Lhasa apso dog presented with otitis externa.

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Conflict of interest No conflicts of interests have been declared.

Methods—Unilateral otitis externa caused by carbapenem-resistant *P. aeruginosa* was treated with antimicrobial photodynamic therapy (aPDT) using methylene blue as photosensitizer [wavelength 660 nm, fluence 140 J/cm^2, 8 J and 80 s per point (six equidistant points), 100 mW, spot size 0.028 cm^2 and fluence rate 3.5 W/cm^2]. The isolated bacterial strain was also tested for susceptibility to *in vitro* aPDT where the survival fraction was quantified by colony forming unit counts after exposure to increasing light doses. For decolonization, probiotic supplements were orally administered (once daily) for 14 days. Effectiveness of probiotics and photodynamic therapy was evaluated by clinical and microbiological culture assays.

Results—Complete resolution of clinical signs was achieved by day 7 after aPDT. Samples collected immediately and after seven and 14 days following aPDT were negative for VIM-2-producing *P. aeruginosa*. Oral and rectal swabs collected on days seven, 14 and 21 after probiotic therapy, confirmed effective gastrointestinal decolonization.

Conclusions and clinical importance—Combined use of aPDT and probiotics could be a promising therapeutic strategy for treatment of superficial infections produced by carbapenem-resistant bacteria, while avoiding recurrent infection due to intestinal bacterial carriage of these multidrug-resistant pathogens.

Introduction

Following the global spread of carbapenemase-producing pathogens causing human clinical infections, carbapenem-resistant Gram-negative bacteria have now emerged as causes of animal infections worldwide.[1,2] In this regard, gut colonization has contributed to rapid dissemination and is considered to be a potential risk factor for infection.[1–3]

Probiotics such as *Lactobacillus* spp. and *Bifidobacterium* spp. have been investigated as candidates to reduce gut colonization by multidrug-resistant (MDR) pathogens. Probiotics can act as microbial barriers through competitive inhibition of pathogen binding sites and prevention of the production of virulence factors,[4] and have shown promising results in laboratory mice.[5] In addition, the use of prebiotics (nutritional ingredients) combined with probiotics could be a tool against MDR-related colonization, reestablishing the normal gut microbiota.[4]

Antimicrobial photodynamic therapy (aPDT) is an attractive approach to treating localized bacterial infections, including those produced by MDR pathogens. This technique combines a photosensitizer (PS), light and oxygen to locally generate high yields of reactive oxygen species (ROS) that inactivate microbial cells. Additionally, the non-targeted specificity of aPDT makes the development of aPDT-resistant bacteria unlikely, and ensures aPDT displays broad-spectrum bactericidal activity.[6]

A molecular epidemiological study reported the zooanthroponotic transmission of a high-risk hospital clone of VIM-2-producing *Pseudomonas aeruginosa* in a household setting, which was associated with the hospitalization of the pet owner.[7] In that report the pet dog became colonized in the gastrointestinal tract and suffered from severe otitis externa (OE) that was resistant to treatment with commercial antimicrobials. In this study, we report the

effectiveness of probiotics and photodynamic therapy in the treatment and decolonization of the pet dog.[7]

Case report

A 5-year-old Lhasa apso dog was presented with head shaking, pain on palpation of the right ear pinna, pruritus, purulent discharge and erythema of the ear for one month (Figure 1a). Veterinary medical records revealed unsuccessful treatment with enrofloxacin and no other history of otitis. Otoscopic examination was performed in both ears, where no foreign debris or abnormalities of the tympanic membranes were observed. Clinically, the infected ear presented with no evidence of structural changes (i.e. induration, mineralization or hyperplasia). No signs of infection in the left ear were observed.

After clinical diagnosis of unilateral OE, samples from the infected and healthy ears and oral and rectal cavities were collected using sterile swabs. Carbapenem-resistant *P. aeruginosa* isolates were recovered from the right ear, oral and rectal samples, and displayed identical MDR profiles.[7] After confirmation of metallo-β-lactamase (VIM-2)-producing *P. aeruginosa* from the infection site, gut and oral cavity,[7] two therapeutic strategies were adopted.

In vitro photodynamic inactivation—Firstly, to determine the "*in vitro*" susceptibility of *P. aeruginosa* strains to aPDT, the VIM-2-producing *P. aeruginosa* ICBDVIM-2 isolated from the infected dog[7] was compared to a *P. aeruginosa* reference strain (ATCC® 27853™). Overnight *P. aeruginosa* Mueller-Hinton (MH) broth cultures were harvested, washed twice in phosphate buffered saline (PBS) and then adjusted to a 0.5 McFarland scale and diluted 10x to reach a concentration of ~1×10^7 cell/mL. Methylene blue (MB, Sigma-Aldrich, St Louis, MO, USA) solution was used as a photosensitizer at a concentration of 100 μM prepared in PBS. Aliquots of 100 μL *P. aeruginosa* suspensions were suspended in 100 μL MB solution and transferred into wells of a 96-well sterile microplate, which were incubated for 5 minutes in the dark prior to light irradiation. Growth control groups were comprised of PBS only, a dark control MB solution without light irradiation, and a light control composed of cells suspended in PBS and irradiated with the higher fluence applied (25 J/cm^2). Light irradiation was performed using a red (660 nm) LED device with constant irradiance (100 mW/cm^2; LEDBox, BioLambda, São Paulo, Brazil), varying the time to achieve different levels of radiant exposure (5, 10, 15, 20 and 25 J/cm²). After treatment, bacterial suspensions were serially diluted and plated onto MH agar. Colonies were counted after 24 h incubation at 37 ºC. Each assay was performed in triplicate, in three independent experiments.

Interestingly, both *P. aeruginosa* strains were equally susceptible to aPDT (Figure 2). As expected, higher fluences gave higher inactivation rates and at 25 J/cm^2 zero colonies were obtained. Moreover, no bacterial inactivation was observed in the control groups (P>0.001).

In vivo probiotic and photodynamic therapies—For bacterial decolonization a *Saccharomyces cerevisiae* (9×10^9 CFU/kg)-based probiotic feed supplement (Organew, Vetnil, Louveira, São Paulo, Brazil), together with another probiotic compound (Probiótico Vetnil, Brazil) containing *S. cerevisiae* (3.33×10^5 CFU/g), *Lactobacillus acidophilus* (3.33×10^7 CFU/g), *Bifidobacterium bifidum* (3.33×10^7 CFU/g), *Enterococcus faecium* (1.66×10^7 CFU/g) and *L. plantarum* (1.66×10^7 CFU/g) were tested. In brief, 2.5 g/Organew and

2.0 g/Probiótico Vetnil were orally administered (once daily) for 14 days. No diet changes were performed during the treatment period. After probiotic treatment, oral and rectal swab samples were collected on days seven, 14 and 21. In this respect, no positive rectal and oral samples were obtained after seven days of probiotic treatment.

For the OE treatment, a single aPDT session was performed. The dog was anaesthetized and the ear was cleansed with sterile physiological solution to remove the purulent exudate. Then, 7 mL of MB aqueous solution at a final concentration of 100 μM (Sigma-Aldrich) was applied directly on the surface of the ear canal using a flexible catheter coupled to a syringe (Figure 1b). After 5 min, the MB solution was removed by aspiration. Following that, the entire ear canal was irradiated with a diode laser emitting at λ= 660 nm, fluence 140 J/cm^2, 8 J and 80 s per point, 100 mW, and fluence rate 3.5 W/cm^2 (Therapy XT, DMC®, Brazil) (Figure 1c). Since the laser tip was much smaller than the ear canal area, six points spaced apart by 5 mm were irradiated equally over the vertical and horizontal surfaces of the affected ear canal. The infected ear was cleaned daily with sterile physiological solution by the pet owner. New samples were collected immediately, seven and 14 days after aPDT.

Regarding gut colonization by VIM-2-producing *P. aeruginosa*, no positive rectal and oral samples were obtained after seven, 14 and 21 days following probiotic treatment. The clinical analysis of OE performed on the 7th day revealed reduction of purulent exudate and regression of signs of inflammation and pain. On the 14th day, no exudate and erythema were observed (Figure 1d). Additionally, the VIM-2-producing *P. aeruginosa* isolated prior to aPDT was not recovered immediately after treatment, nor at seven or 14 days post-treatment. The animal was followed up for three months and no recurrence of OE was observed during this period.

Discussion

Otitis externa is one of the most common disease processes reported in companion animals, and particularly in dogs. Although it is well documented that several agents could be involved in the pathogenesis of OE in dogs (i.e. bacteria, fungus, and more rarely, parasites), meticillin-resistant *Staphylococcus* spp. and MDR *P. aeruginosa* have played the paramount role as causative agents in those recurrent infections that appear to be resistant to all the usual treatments.[8–10] One important human pathogen, VIM-2-producing *P. aeruginosa,* was reported in dogs with otitis in Korea,[9] supporting the urgent need to develop effective alternative treatments for carbapenem-resistant bacteria in OE infections. VIM-2-producing *P. aeruginosa* represents a new challenge for clinicians, although this resistance pattern remains atypical in veterinary settings.[7,9]

It should be noted that for optimum outcomes, the presence of any sort of secretion in the infected site should be cleared before performing the aPDT protocol. So far, no potential side-effects related to MB-mediated aPDT have been reported for superficial cutaneous lesions.[6,11] Therefore, the use of aPDT and probiotics is a promising therapeutic strategy to be used in the treatment of superficial infections produced by MDR bacteria,[6,11] and for avoiding short-term recurrent infections due to intestinal carriage of such pathogens.[12]

In summary, this report describes a therapeutic strategy using probiotics and aPDT for the treatment of external ear canal infection and gut decolonization of a high-risk clone of VIM-2-producing *P. aeruginosa* in a dog. These findings support the results of previous studies that have shown lactobacilli-based probiotics to be effective against planktonic and biofilm modes of growth of MDR *P. aeruginosa* clinical isolates.[4]

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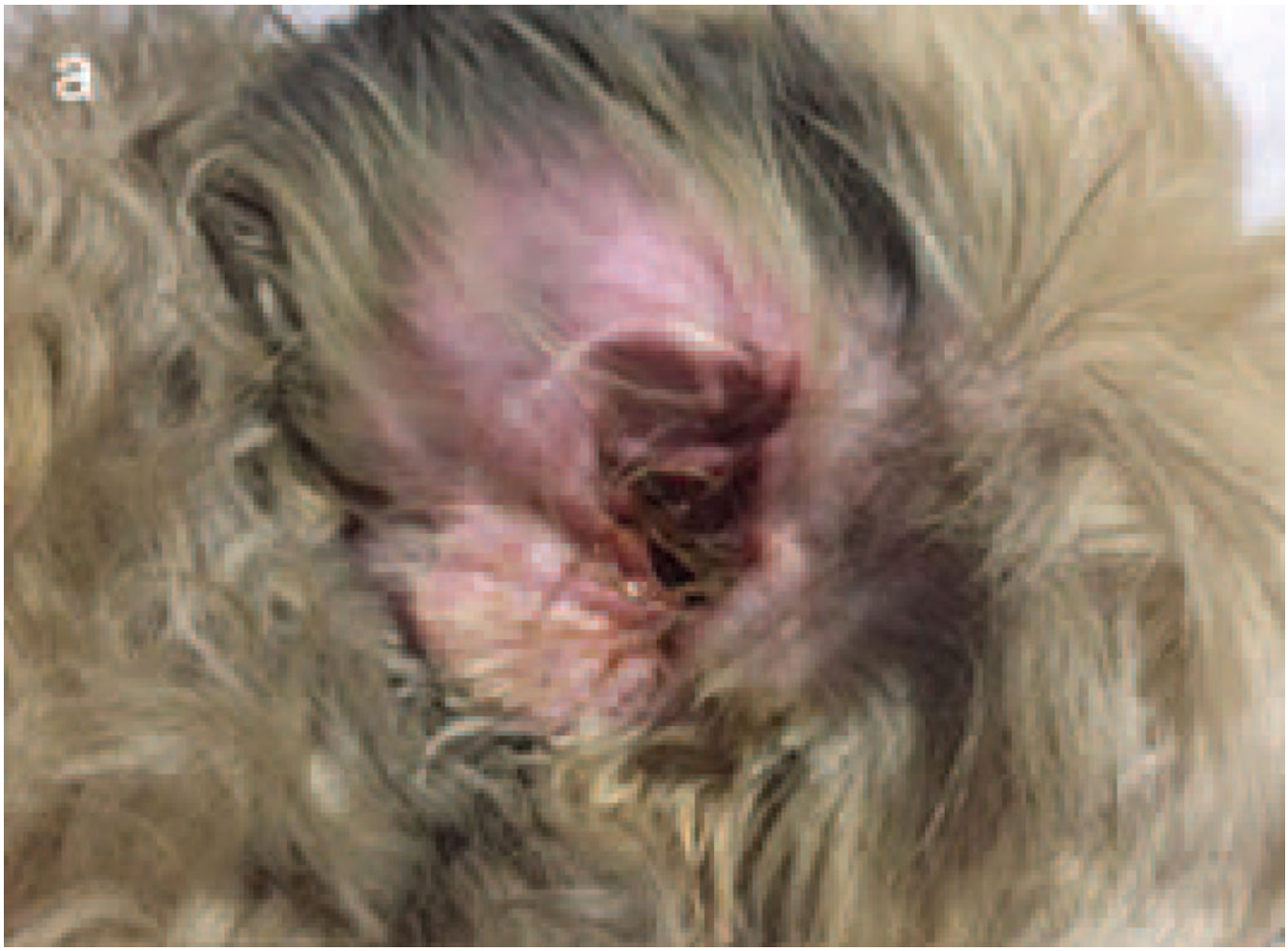

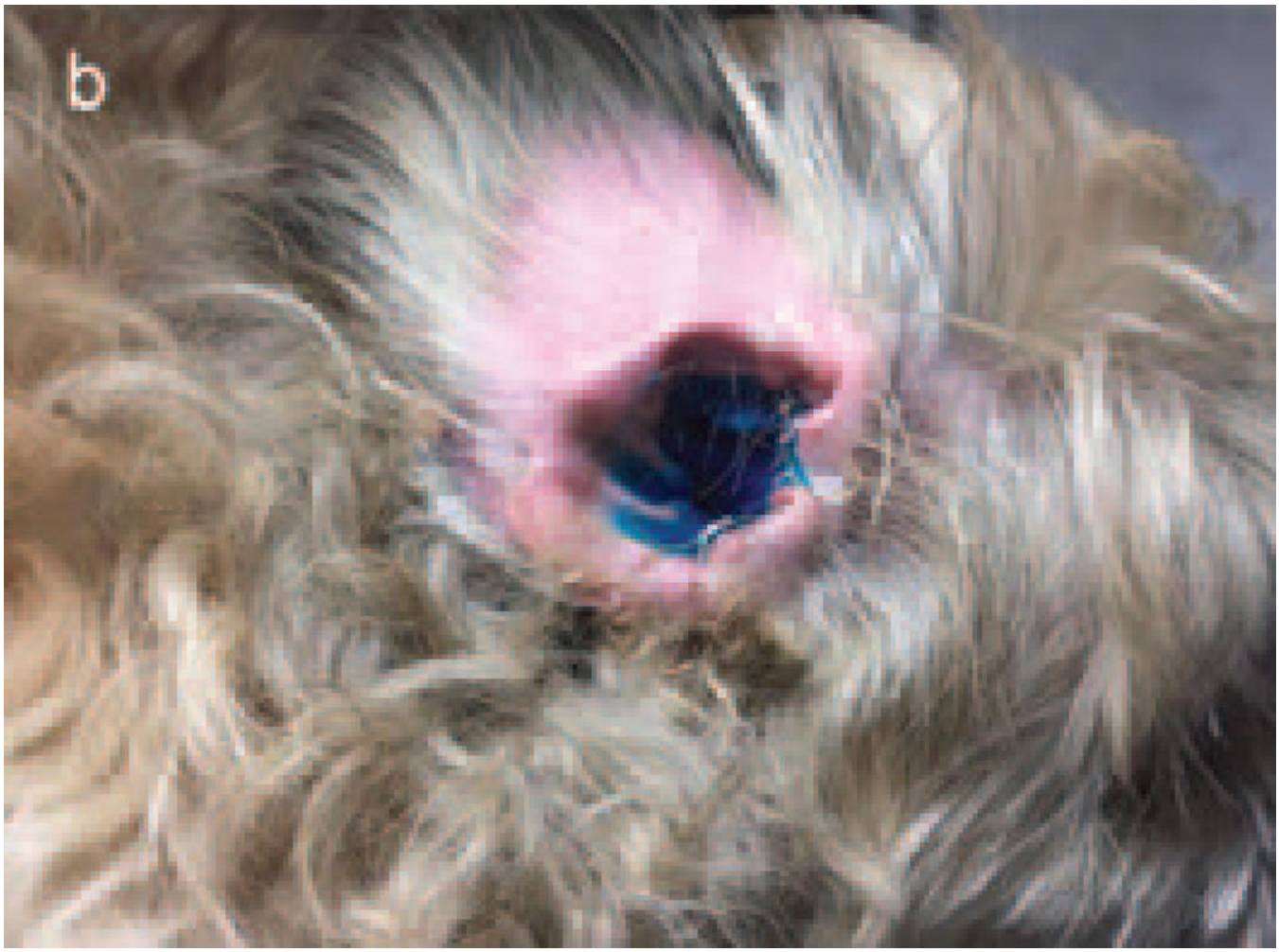

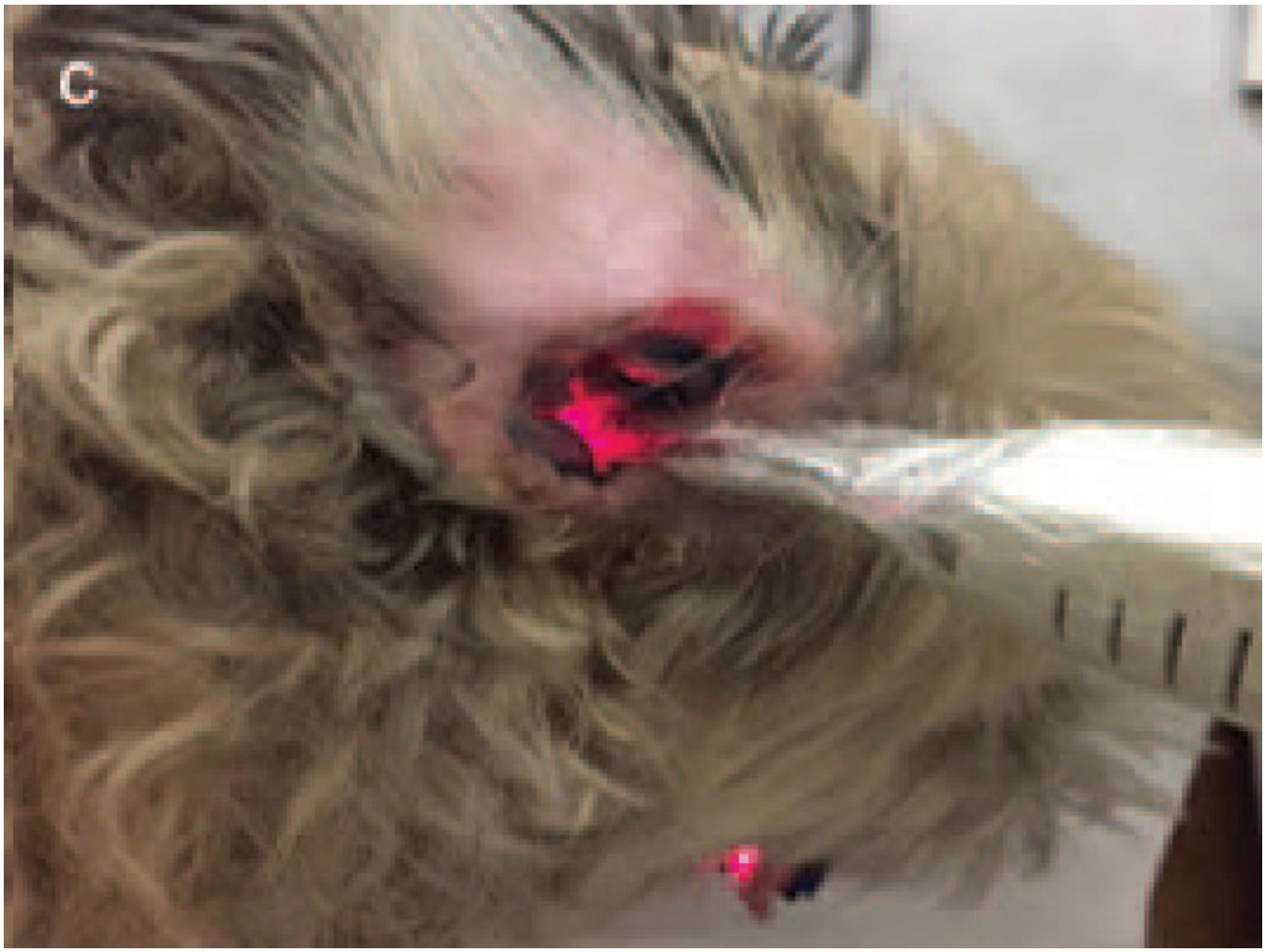

Figure 1. Representative images of otitis externa and aPDT in a dog infected with a VIM-2-producing *Pseudomonas aeruginosa*.

a) Otitis externa lesion presenting with erythema and purulent exudate.

b) Application of the photosensitizer MB previous aPDT.

c) aPDT irradiation with diode laser.

d) Absence of exudate and reversion of otitis externa 14 days after aPDT. The VIM-2-producing *P. aeruginosa* strain, isolated before aPDT, was not recovered immediately and at seven and 14 days after treatment.

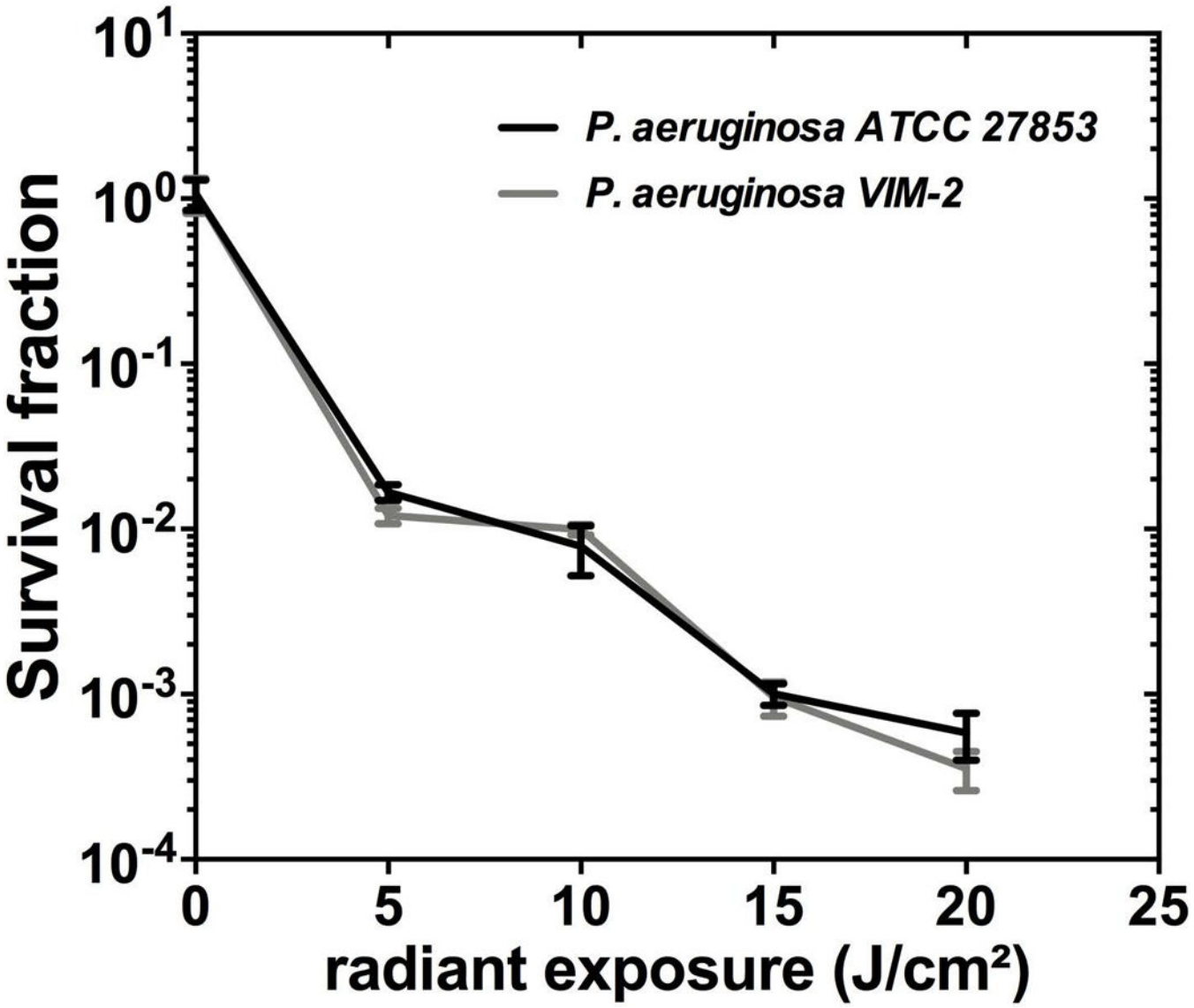

Figure 2. MB-mediated aPDT inactivation kinetics against *Pseudomonas aeruginosa* ATCC 27853 and VIM-2-producing *P. aeruginosa* ICBDVIM-2.[7]

One-way ANOVA with Bonferroni post hoc was used to compare each group in relation to its other strain counterpart and in to non-irradiated controls. All comparisons in relation to non-irradiated controls were significant; with *P* values lower than 0.001, while all comparisons in relation to strains at the same radiant exposure point were not significant.