Photodiagnosis and Photodynamic Therapy 13 (2016) 276–281

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

In vitro photoinactivation of bovine mastitis related pathogens

Fábio Parra Sellera[a,*], Caetano Padial Sabino[b], Martha Simões Ribeiro[b], Ronaldo Gomes Gargano[a], Nilson Roberti Benites[c], Priscilla Anne Melville[c], Fabio Celidonio Pogliani[a]

[a] Department of Internal Medicine, School of Veterinary Medicine and Animal Science, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87 – Cidade Universitária, 05508-270, São Paulo, SP, Brazil
[b] Center for Lasers and Applications, Av. Lineu Prestes, 2242 – Cidade Universitária, 05508-000, São Paulo,SP, Brazil
[c] Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Science, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87– Cidade Universitária, 05508-270, São Paulo SP, Brazil

ARTICLE INFO

Article history:
Received 13 July 2015
Received in revised form 18 August 2015
Accepted 19 August 2015
Available online 24 August 2015

Keywords:
Antimicrobial
Dairy cows
Photodynamic inactivation
Treatment
Veterinary medicine

ABSTRACT

Background: Bovine mastitis is considered the most important disease of worldwide dairy industry. Treatment of this disease is based on the application intramammary antibiotic, which favors an increase in the number of resistant bacteria in the last decade. Photodynamic inactivation (PDI) has been investigated in different areas of Health Sciences, and has shown great potential for inactivating different pathogens, without any selection of resistant microorganisms. The objective of this study was to investigate the efficacy of PDI in the inactivation of pathogens associated with bovine mastitis.
Methods: We tested the effectiveness of PDI against antibiotic resistant strains, isolated from bovine mastitis, from the following species: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, and the alga *Prototheca zopfii*. Nine experimental groups were evaluated: control, no treatment; light only, irradiation of a red light-emitting diode (λ = 662 (20) nm) for 180 s; exposure to 50 μM methylene blue alone for 5 min; and PDI for 5, 10, 30, 60, 120 and 180 s.
Results: S. dysgalactiae, *S. aureus*, and *C. bovis* were inactivated after 30 s of irradiation, whereas *S. agalactiae* was inactivated after 120 s and *P. zopfii* at 180 s of irradiation.
Conclusion: These results show that PDI can be an interesting tool for inactivating pathogens for bovine mastitis.

1. Introduction

Although various factors are involved in the development of mastitis, microorganism-mediated infection is considered to be the leading cause. In addition to reproductive problems and podal infections, this disease is thought to be the main contributor of reductions in milk productivity and quality [1].

The occurrence of this disease involves the interaction between different factors, including those intrinsic to the animal as well as human and environmental aspects. Factors linked to the animal aspects are those related with an impaired immune system. Altered neutrophil function, impaired antibody response and inflammatory cytokines production are usually found at the transition period [2,3] and in the presence of immunosuppressive infectious diseases [4]. The most susceptible animals can yet develop infectious diseases such as mastitis and metritis [5,6]. Bacteria are among the main causative agents of bovine mastitis; however, fungi, yeasts, viruses, and algae have also been associated with this disease [7,8].

Given its economic importance, treatment strategies for bovine mastitis have long been investigated. Despite the development of various active substances for mastitis treatment, there is still no consensus on the optimal therapeutic approach. In general, the correct maintenance of the animal homeostasis, adoption of hygienic sanitary measures and administration of local intramammary antibiotics are respectively the procedures most commonly used for the prophylaxis and treatment of this disease [1,7,9].

In the last few decades, there has been increased public concern related to the inspection of organs and the persistence of drug residues in milk products, which has motivated a series of studies currently under investigation [10–12]. In many countries, there was

* Corresponding author at: Av. Prof. Dr. Orlando Marques de Paiva, 87 – Cidade Universitária - 05508-270, São Paulo/SP – Brazil. Fax: +55 11 3091 1330.
E-mail addresses: fsellera@usp.br, fabio_coy@hotmail.com (F.P. Sellera), caetanosabino@gmail.com (C.P. Sabino), marthasr@usp.br (M.S. Ribeiro), gargano21@gmail.com (R.G. Gargano), benites@usp.br (N.R. Benites), melville@usp.br (P.A. Melville), fabiocp@usp.br (F.C. Pogliani).

http://dx.doi.org/10.1016/j.pdpdt.2015.08.007
1572-1000/

a reduction in the use of antimicrobial substances due to concern and public demand. However, the continued or indiscriminate use of antimicrobial agents for the treatment of bovine mastitis in some countries has favored a process of selection of the microorganisms, resulting in the development of strains resistant to the active compounds [13–15]. Therefore, identification of an efficient treatment for mastitis through interventions that do not lead to the appearance of biological residues in products of animal origin and do not promote the selection of drug-resistant microbial populations is urgently required.

Photodynamic inactivation (PDI) is a therapeutic procedure that has been investigated for the treatment of microbial diseases. The antimicrobial properties from PDI combine a harmless photoactivatable dye with visible light and molecular oxygen. Its mechanism of action is based on the excitation of photosensitive (*i.e.*, light-sensitive) substances by light at proper wavelengths, resulting in the formation of reactive oxygen species, which in turn damage subcellular structures that are fundamental to the survival of microorganisms, and so far no resistant microorganisms have been reported [16–18]. Factors that will determine the selective death of host cells or microbial inactivation are directly related to the affinity of photosensitive molecules used as well as to the light and chemical dosimetry applied [16].

Phenothiazinium dyes such as methylene blue (MB) have been broadly investigated as photosensitizer (PS) for both gram-positive and negative bacteria demonstrating effective eradication [19]. In fact, much of the use of MB as a lead compound has been due to its low toxicity in the human patient [20]. Another advantage of this cationic PS is its affinity for targeting microorganisms rather than host cells. Therefore, short-period irradiation treatments with MB tend to cause minimal damage to host cells but yet present effective antimicrobial activity [21].

In this study, we aimed investigate the ability of PDI to inactivate antibiotic-resistant bacterial strains of *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Corynebacterium bovis*, and a strain of the alga *Prototheca zopfii* isolated from cattle with clinical mastitis.

2. Materials and methods

The study was approved by the animal care and use committee of the School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil (reference number 2,246,120,214).

2.1. Microorganisms

Due to the importance of the prevalence of clinical or subclinical mastitis in Brazil [22–24], wild-type strains of *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, and *C. bovis* were chosen. The alga *P. zopfii* (wild-type strain) was used in this experiment because of its clinical importance, not only in Brazil but worldwide. The occurrence of mastitis caused by the alga has been described in several countries with no responsive treatment [25,26].

The strains were wild-type isolated from the milk of cows with clinical mastitis at different farms in the State of São Paulo, Brazil. Milk samples were collected aseptically in sterile tubes, they were transported to the lab under refrigeration (4 °C), where they were subjected to microbiological examination. From each milk sample collected, an aliquot of 10 mL was placed in Petri dishes with 5% sheep blood agar. The dishes were incubated, in anaerobic condition, at 37 °C, and the reading was performed after 24 and 48 h. The isolated bacteria (*S. aureus, S. agalactiae, S. dysgalactiae* and *C. bovis*) were identified according Murray [27]. The RapID system was used to confirm the result. The same laboratory protocol used for the isolation of the bacteria, as described above, was used for the isolation of *P. zopfii*, but these were identified according to their morphological, macroscopic and microscopic characteristics, besides determination of assimilation of different substrates according to Pore [28].

Bacteria were subject to *in vitro* susceptibility tests with paper discs containing the following antimicrobial drugs: amikacin (30 μg), ampicillin (10 μg), cephalexin (30 μg), cephalothin (30 μg), ceftiofur (30 μg), clindamycin (2 μg), chloramphenicol (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), oxacillin (1 μg), penicillin (10 IU), sulfamethoxazole + trimethoprim (25 μg), tetracycline (30 μg), and vancomycin (30 μg). The tests were carried out on Mueller–Hinton agar plates with the addition of 5% sheep blood in the cases of *Streptococcus* spp. and *C. bovis*. The disc diffusion method was performed according to the technique described by Bauer et al. [29]. Tests were evaluated based on the measured halo diameter, which reflects the degree of inhibition on microorganism growth. The concentrations used and interpretation criteria (inhibition zone diameter) adopted were chosen based on recommendations of the Clinical and Laboratory Standards Institute [30].

2.2. Light source and photosensitizer

To assay PDI, a red light-emitting diode (LED probe) was used ($\lambda = 662 \pm 20$ nm), with an optical power of 320 mW, beam diameter of 2 cm, irradiance of 100 mW/cm^2. The LED probe was fixed on a holder 1 mm apart from the surface of a microwell plate, which kept the beam area at 3.14 cm^2 and coincided to a single well area from the 24-well microwell plate. According to the literature, irradiance of 100 *m*W/cm^2 does not produce any photothermal effect since 300 mW/cm^2 induces an increased of 0.5 °C on solution after 2 min of irradiation using red light and MB [31]. Therefore, there was no need to control the temperature to prevent heating effects.

The PS employed for this study was MB (Sigma–Aldrich; St. Louis, MO, USA), which was diluted in sterile PBS (phosphate buffer solution, pH 7.4) to a working concentration of 50 μM.

2.3. Study design

S. aureus, S. agalactiae, and *S. dysgalactiae* were cultivated in brain-heart infusion (BHI) agar, and *P. zopfii* in Sabouraud agar overnight while *C. bovis* was grown in blood BHI agar for 2–3 days. They were then suspended in sterile PBS and homogenized, either with a vortex agitator (bacteria) or through repeated pipetting (*P. zopfii*).

The concentration of the inoculum was determined by assessing the turbidity of the suspension with a spectrophotometer. The absorbance of the PBS suspension containing the microorganisms was adjusted to 0.9 (1) at $\lambda = 600$ nm, which corresponds to a concentration of approximately 10^8 cfu/mL for bacteria and 10^5 for *P. zopfii* [32]. For PDI assays, bacterial suspensions were diluted to a working concentration of 10^7 cfu/mL and *P. zopfii* concentration was the same as obtained in the spectrophotometer (10^5 cfu/mL).

The experimental groups were compared to their respective control groups (C), which did not receive any light or PS treatment. Among the light-only (L) groups, suspensions of microorganisms were irradiated in the absence of MB for 180 s, resulting in a total of 18 J/cm^2 of delivered energy density. To MB groups, we added MB solution to microbial suspension inside the tubes and then they were placed in the dark during the entire experimental procedure time to assess the toxicity of the PS alone. For the PDI groups, MB was added to the suspensions and, after 5 min of pre-irradiation time, they were irradiated for 5 s, 10 s, 30 s, 60 s, 120 s or 180 s (energy densities of 0.5 J/cm^2, 1 J /cm^2, 3 J/cm^2, 6 J/cm^2, 12 J/cm^2 and 18 J/cm^2, respectively).

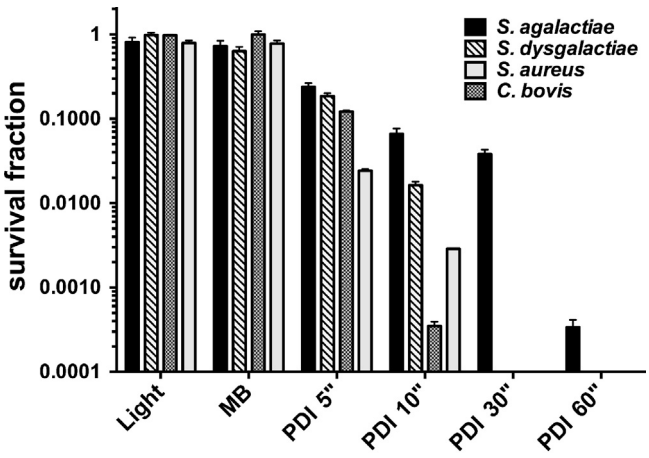

Fig. 1. Photodynamic inactivation of *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Staphylococcus aureus* and *Corynebacterium bovis*. Bacterial strains were incubated with 50 μM methylene blue (MB) for 5 min and irradiated with a red LED (100 mW/cm^2). Data are the means of nine independent experiments and the bars are the SD.

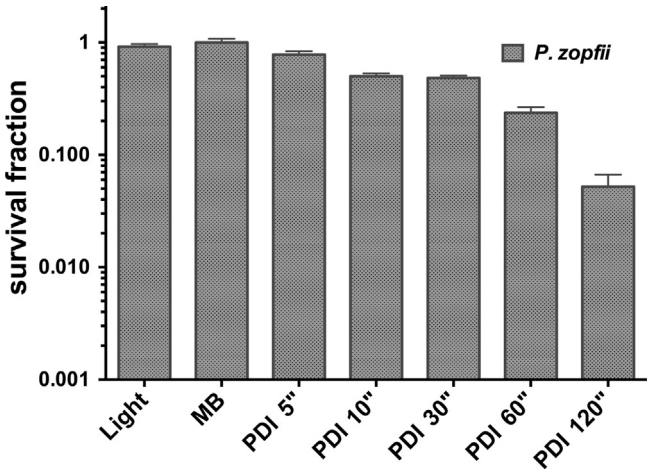

Fig. 2. Photodynamic inactivation of *Prototheca zopfii*. Algae were incubated with 50 μM methylene blue (MB) for 5 min and irradiated with a red LED (100 mW/cm^2). Data are the means of nine independent experiments and the bars are the SD.

After treatments, samples were serially diluted in sterile PBS and seeded in their respective proper agar plates (as mentioned before) according to the technique described by Jett et al. [33] for cfu/mL quantification. Plate dishes were incubated at 37 °C for 24 h (*S. aureus*, *S. dysgalactiae*, *S. agalactiae* and *P. zofpii*) and 48 h (*C. bovis*) to allow colony visualization.

All microbiological experiments were conducted in triplicates, and repeated among three different experimental days, which totalized $n = 9$ for all groups.

2.4. Statistical analysis

Mean values for microbial counts with the corresponding standard deviations were calculated. The survival fraction from each group was tested for significant differences by using ANOVA followed by Tukey test. $P < 0.05$ was considered statistically significant. Statistical comparisons between means were performed by the software Origin 8.5 (Origin-Lab, Northampton, MA USA).

3. Results

The *in vitro* susceptibility screening for the tested antimicrobial drugs is presented at (Table 1). Figs. 1 and 2, displays the susceptibility of tested microorganisms. We can observe that light and MB groups did not show any significant microbial inactivation. By the other hand, among the bacteria exposed to PDI, a significant inactivation was already observed after 5 s of irradiation (energy density of 0.5 J/cm^2) when compared to the C, L, and MB groups. However, *P. zopfii* only began to show slight inactivation after 10s of irradiation (energy density of 1 J/cm^2), thus indicating that the alga is less sensitive to PDI when compared to the evaluated bacteria (Figs. 1 and 2).

It can also be perceived from Fig. 1 that microbial sensitivity to PDI is dependent on irradiation parameters and microbial species. Following 5 s or 10 s of irradiation (0.5 J/cm^2 and 1 J/cm^2) bacterial load was reduced about 1–3.5 logs. *C. bovis* showed the highest PDI susceptibility when compared to the other analyzed species. After 30 s of irradiation (3 J/cm^2), bacterial viability was totally reduced for *S. aureus*, *S. dysgalactiae* and *C. bovis*. No significant differences were observed for *S. agalactiae* and *P. zopfii* microbial reduction between 10 and 30 s of irradiation. Following 60 s of irradiation, *S. agalacitae* displayed a burden reduction of 3 logs compared to control while *P. zopffi* reduced about 0.5 log. Complete *S. agalactiae* inactivation was achieved at 120 s (12 J/cm^2) of irradiation and *P. zopfii* at 180 s (18 J/cm^2) (Fig. 2).

4. Discussion

Regardless of the abundant literature about the use of PDI against pathogenic microorganisms such as *S. aureus*, no studies have yet described the use of PDI against *S. dysgalactiae*, *S. agalactiae*, *C. bovis*, *P. zopfii* species, which are all involved in the etiology of bovine mastitis. Moreover, no studies have demonstrated the potential of using PDI against pathogenic algae, which emphasizes the novelty of this investigation.

Usually, in natural circumstances, concentrations of pathogens are lower than those used in this experiment. However, we used a concentration of 10^7 cfu/mL, since it is standard in other antimicrobial assays, such as antibiograms and minimal inhibitory concentration. As the antimicrobial activity is confirmed for this situation, lower microbial concentrations will represent a much easier challenge.

Although the aim of the study was not to determine the drug resistance of certain pathogens in susceptibility tests, the relatively high occurrence of different resistance patterns in these microorganisms indicates that alternative therapeutic approaches with broad action spectrum, such as PDI, may be considered as an important strategy to manage infectious bovine mastitis.

The pathogenic bacterium *S. agalactiae* presented higher resistance in the susceptibility tests in vitro when compared to the other bacteria, and also showed higher resistance to PDI. Several studies have shown that multiresistant microorganisms can be just as susceptible to PDI as similar non-multiresistant strains [34]. However, according to Tegos and Hambin [35] and George et al. [36], multiresistant strains can be more difficult to eradicate with PDI compared to antibiotic-susceptible strains. These authors attribute this difference to the fact that mechanisms of drug resistance in microorganisms, as efflux pumps could bind phenothiazinium PSs, resulting in a lower amount of PS accumulating within the bacterial cell. Therefore, the correlation between high resistance in antibiogram and photoinactivation assays deserves further investigation in view of our findings.

Among the microorganisms assessed in this study, two are worthy of deeper discussion. The first is the *Staphylococcus* genus, and in particular *S. aureus*, which is known for its high resistance to antibiotics, thus emphasizing the importance of further investigations to control the spread of these bacteria in the mammary gland [37,38].

Table 1
Antimicrobials tested on clinical isolate of *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Staphylococcus aureus* and *Corynebacterium bovis*.

Antimicrobial drug	*Strep. agalactiae*	*Strep. dysgalactiae*	*Staph. aureus*	*C. bovis*
Amikacin	R	R	S	S
Ampicillin	R	R	R	R
Cephalexins	S	S	S	S
Cephalothin	S	S	S	S
Ceftiofur	S	S	S	S
Clindamycin	S	S	S	S
Chloramphenicol	S	S	S	S
Enrofloxacin	S	S	S	S
Erythromycin	S	S	S	S
Gentamicin	R	R	S	S
Oxacillin	R	R	S	R
Penicillin	R	R	R	R
Sulfamethoxazole + trimethoprim	R	S	S	S
Tetracycline	S	S	S	S
Vancomycin	S	S	S	S

R = resistant; S = susceptible.

The second is the *Prototheca* genus, which merits special attention mainly due to the lack of efficient treatments for infections of the mammary gland, from which these pathogens were originally isolated [25]. Our findings show that *P. zopfii* is less susceptible to PDI when compared to the assessed bacteria. Such results are also observed when comparing PDI sensitivity of fungi in relation to bacteria [39]. This fact is commonly associated to the greater complexity of eukaryotic cells, explained by the hazard of damages in eukaryotic fungi is furtherly reduced by the presence of a membrane that envelopes the nucleus and may act as a barrier to the penetration of dyes or their photoproducts. In addition, it is known that *Prototheca* genus is capable of producing endospores [40]. So, the difficulty of certain exogenous molecules face in diffusing into spores can be related to the impermeable nature of the spore coat and it could be responsible for much of the resistance of spores to reactive oxygen species [41,42].

The results of the present study show that, under the conditions employed, both pathogenic agents were susceptible to the action of PDI, thus demonstrating their potential for clinical application.

Despite the successful results and the promising application of the technique, some studies have shown that PDI does not always lead to a complete microbial reduction. Nevertheless, its ability to reduce the microbial burden is undeniable, thus allowing patient recovery when combined with existing therapies. Chibebe et al. [43,44] observed that a strain of *Candida albicans* and *Enterococcus faecium* respectively resistant to fluconazol and vancomycin showed increased susceptibility to antimicrobial compounds after small doses of PDI, suggesting that the possible combination of the two treatments could increase therapeutic efficiency, treatment success, and clinical recovery.

Regarding photodynamic-induced inactivation, there is still controversy related to its potentially deleterious effects on healthy cells of an exposed host organism. Although few studies have focused on the potential effects of PDI with phenothiazinium dyes on healthy cells or the immune system, some studies have investigated the action of PDI on fibroblasts, keratinocytes, or neutrophils [45–47]. Tanaka et al. [47] evaluated the action of PDI with different PSs on methicillin-resistant *Staphylococcus aureus* (MRSA) and neutrophils. The authors concluded that the dose necessary to inactivate MRSA was much lower than that causing neutrophil damage. Moreover, the neutrophils did not show noticeable morphological changes during irradiation with MB, and neutrophil viability was continuously maintained at a high level (>80%) with an energy flow of 5 J/cm^2 or 20 J/cm^2. This led the authors to propose that MB-mediated PDI did not cause cytotoxic effects in neutrophils under the optimal concentrations used to inactivate MRSA. Similarly, Noodt et al. [48] assessed the action of MB derivatives-induced PDI in fibroblasts *in vitro*. The results obtained led the authors to conclude that the enzyme activity of the Golgi apparatus, lysosomes, and endoplasmic reticulum was not affected. Moreover, according to Zeina et al. [49], the action of MB-mediated PDI on human keratinocytes caused low toxicity in these cells.

It is known that antineoplasic photodynamic therapy (PDI) is capable of activating and recruiting several types of immune cells in mammals, including neutrophils, dendritic cells, macrophages, T-lymphocytes, and mast cells. However, these studies focused on the application of PDI, with different PS and light sources [50]. Thus, elucidating the effects of PDI on isolated leukocytes of bovine milk and mammary gland tissue under specific experimental conditions sufficient to inactivate pathogens is of major importance. This assay would determine the survival threshold of these cells as well as the possible changes in their viability induced by PDI. With this information, it would be possible to estimate an optimized and safe PDI protocol for future clinical trials, inactivating pathogens while sparing immune cells.

In conventional treatment of bovine mastitis, topical antibiotic is applied through the teat canal of the mammary gland after milking. For this reason, we believe that for PDI clinical trials, PS should be administered after milking, as well as a conventional treatment. New light sources are constantly being developed and in of them can be coupled to an optical fiber to deliver light in a specific target, as for example in root canal decontamination [51]. Other devices with diffusing fibers have been developed to irradiate cavities such as bladder, esophagus, lung and stomach [52–55]. Unlike the efficient tools already developed and tested in the fields of medicine, physiotherapy, dentistry, and even veterinary medicine for the treatment of different diseases, a proper tool for the treatment of bovine mastitis should be developed to irradiate all the structures involved, since the microorganisms might be distributed throughout the entire mammary gland.

This is an initial study that aimed to verify the potential of PDI to inactivate relevant microorganisms associated to bovine mastitis. Future studies should consider an important factor with regard to the use of PDI as a clinical treatment for bovine mastitis is that the milk solids creates a physical barrier to light, which may block its transmission by scattering and absorption phenomena [56], resulting in a problematic dosimetry calculation. Clinical trials have demonstrated that light itself might also influence glandular tissues, resulting in an increase of glandular secretions [57,58]. It makes interesting to carry out further investigations to assess the effects of this technique on mammary gland tissue in respect to possible metabolic changes or damage in the tissue itself and, consequently, on the production of milk.

The use of PDI in veterinary medicine is still being investigated, studies have shown promising results using the technique in the treatment of different diseases [59–62]. We believe that further investigations need to be carried out and that they must extrapolate the *in vitro* assays.

5. Conclusion

Neither light nor MB inactivates the assessed pathogens tested in the parameters of this study. PDI was found to effectively inactivate all microorganisms in a dose-dependent way, regardless of their resistance phenotype and appears to be a promising option for the development of new therapeutic approaches to treat bovine mastitis. However, more studies are necessary to investigate the therapeutic protocols postulated under the present laboratory conditions and their application to clinical trials, in which the natural occurrence of the disease may be explored.

References

[1] A. Bradley, Bovine mastitis: an evolving disease, Vet. J. 164 (2002) 116–128.
[2] R.R. Grummer, Impact of changes in organic nutrient metabolism on feeding the transition dairy cow, J. Anim. Sci. 73 (1995) 2820–2833.
[3] F.J. Mulligan, M.L. Doherty, Production diseases of the transition cow, Vet. J. 176 (2008) 3–9.
[4] A.M. Della Libera, F.N. de Souza, C.F. Batista, B.P. Santos, L.F. de Azevedo, E.M. Sanchez, S.A. Diniz, M.X. Silva, J.P. Haddad, M.G. Blagitz, Effects of bovine leukemia virus infection on milk neutrophil function and the milk lymphocyte profile, Vet. Res. 46 (2015) 2–8.
[5] K. Kimura, J.P. Goff, M.E. Kehrli, J.A. Harp, B.J. Nonnecke, Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows, J. Dairy Sci. 85 (2002) 1437–1444.
[6] S.J. LeBlanc, K.D. Lissemore, D.F. Kelton, T.F. Duffield, K.E. Leslie, Major advances in disease prevention in dairy cattle, J. Dairy. Sci. 89 (2006) 1267–1279.
[7] J.R. Roberson, Treatment of clinical mastitis, Vet. Clin. North Am. Food Anim. Pract. 28 (2012) 271–288.
[8] R. Deb, A. Kumar, S. Chakraborty, A.K. Verma, R. Tiwari, K. Dhama, U. Singh, S. Kumar, Trends in diagnosis and control of bovine mastitis: a review, Pak. J. Biol. Sci. 16 (2013) 1653–1661.
[9] D.E. Bauman, W.B. Currie, Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis, J. Dairy Sci. 63 (1980) 1514–1529.
[10] J. Kneebone, P.C. Tsang, D.H. Townson, Short communication: rapid antibiotic screening tests detect antibiotic residues in powdered milk products, J. Dairy Sci. 93 (2010) 3961–3964.
[11] T. Whittem, J.H. Whittem, P.D. Constable, Modelling the concentration-time relationship in milk from cattle administered an intramammary drug, J. Vet. Pharmacol. Ther. 35 (2012) 460–471.
[12] F. Conzuelo, S. Campuzano, M. Gamella, D.G. Pinacho, A.J. Reviejo, M.P. Marco, J.M. Pingarrón, Integrated disposable electrochemical immunosensors for the simultaneous determination of sulfonamide and tetracycline antibiotics residues in milk, Biosens. Bioelectron. 15 (2013) 100–105.
[13] J.S. Moon, A.R. Lee, H.M. Kang, E.S. Lee, M.N. Kim, Y.H. Paik, Y.H. Park, Y.S. Joo, H.C. Koo, Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea, J. Dairy Sci. 90 (2007) 1176–1185.
[14] V. Saini, J.T. McClure, D. Léger, G.P. Keefe, D.T. Scholl, D.W. Morck, H.W. Barkema, Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms, J. Dairy Sci. 5 (2012) 4319–4332.
[15] H. Jamali, B. Radmehr, S. Ismail, Short communication: prevalence and antibiotic resistance of *Staphylococcus aureus* isolated from bovine clinical mastitis, J. Dairy Sci. 97 (2014) 2226–2230.
[16] M.R. Hamblin, T. Hasan, Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem. Photobiol. Sci. 3 (2004) 436–450.
[17] G. Jori, M. Magaraggia, C. Fabris, M. Soncin, M. Camerin, L. Tallandini, O. Coppellotti, L. Guidolin, Photodynamic inactivation of microbial pathogens: disinfection of water and prevention of water-borne diseases, J. Environ. Pathol. Toxicol. Oncol. 30 (2011) 261–271.
[18] J.L. Wardlaw, T.J. Sullivan, C.N. Lux, F.W. Austin, Photodynamic therapy against common bacteria causing wound and skin infections, Vet. J. 192 (2012) 374–377.
[19] A.S. Garcez, S.C. Núñez, N.J. Azambuja Jr., E.R. regnani, H.M. Rodriguez, M.R. Hamblin, H. Suzuki, M.S. Ribeiro, Effects of photodynamic therapy on Gram-positive and Gram-negative bacterial biofilms by bioluminescence imaging and scanning electron microscopic analysis, Photomed. Laser Surg. 31 (2013) 519–525.
[20] M. Wainwright, L. Amaral, The phenothiazinium chromophore and the evolution of antimalarial drugs, Trop. Med. Int. Health 10 (2005) 501–511.
[21] T. Dai, Y.Y. Huang, M.R. Hamblin, Photodynamic therapy for localized infections-state of the art, Photodiagn. Photodyn. Ther. 6 (2009) 170–188.
[22] L.I. Souto, C.Y. Minagawa, E.O. Telles, M.A. Garbuglio, M. Amaku, R.A. Dias, S.T. Sakata, N.R. Benites, Relationship between occurrence of mastitis pathogens in dairy cattle herds and raw-milk indicators of hygienic-sanitary quality, J. Dairy Res. 75 (2008) 121–127.
[23] R.P. Martins, J.A.G. da Silva, L. Nakazato, V. Dutra, E.S. Almeida Filho, Prevalence and infectious etiology of bovine mastitis in the microregion of Cuiabá, MT, Brazil, Ciênc. Anim. Bras. 11 (2010) 181–187.
[24] G.M. Costa, L.V. Paiva, H.C. Figueiredo, A.R. Figueira, U.P. Pereira, N. Silva, Population diversity of Staphylococcus aureus isolated from bovine mastitis in Brazilian dairy herds, Res. Vet. Sci. 93 (2012) 733–735.
[25] H. Sobukawa, M. Watanabe, R. Kano, T. Ito, M. Onozaki, A. Hasegawa, H. Kamata, In vitro algaecide effect of disinfectants on *Prototheca zopfii* genotypes 1 and 2, J. Vet. Med. Sci. 73 (2011) 1527–1529.
[26] J.L. Gonçalves, S.H. Lee, E.P. Arruda, D.P. Galles, V.C. Caetano, C.A. Fernandes de Oliveira, A.M. Fernandes, M. Veiga Dos Santos, Biofilm-producing ability and efficiency of sanitizing agents against *Prototheca zopfii* isolates from bovine subclinical mastitis, J. Dairy Sci. (2015), in press.
[27] P.R. Murray, E.J. Baron, J.H. Jorgensen, M.L. Landry, M.A. Pfaller, Manual of Clinical Microbiology, 9th ed., ASM Press., Washington, DC, U.S.A, 2007.
[28] R.S. Pore, Prototheca Krüger, in: C.P. Kurtzman, J.W. Fell (Eds.), The Yeasts: A Taxonomic Study, Elsevier, 1998, pp. 883–887.
[29] A.W. Bauer, W.M. Kirby, J.C. Sherris, M. Turck, Antibiotic susceptibility testing by a standardized single disk method, Am. J. Clin. Pathol. 45 (1966) 493.
[30] CLSI (Clinical, Laboratory Standards Institute), Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animal. Approved Standard, third ed., Clinical and Laboratory Standards Institute, Wayne, PA, U.S.A, 2008, ISBN: 1- 56,238-660-3CLSI document M31-A3.
[31] R.A. Prates, E.G. Silva, A.E. Yamada, L.C. Suzuki, C.R. Paula, M.S. Ribeiro, Light parameters influence cell viability in antifungal photodynamic therapy in a fluence and rate fluence-dependent manner, Laser Phys. 19 (2009) 1038–1044.
[32] M.A. Pfaller, L. Burmeister, M.S. Bartlett, M.G. Rinaldi, Multicenter evaluation of four methods of yeast inoculum preparation, J. Clin. Microbiol. 26 (1988) 1437–1441.
[33] B.D. Jett, K.L. Hatter, M.M. Huycke, M.S. Gilmore, Simplified agar plate method for quantifying viable bacteria, Biotechniques 23 (1997) 648–650.
[34] S.P. Tseng, L.J. Teng, C.T. Chen, T.H. Lo, W.C. Hung, H.J. Chen, P.R. Hsueh, J.C. Tsai, Toluidine blue O photodynamic inactivation on multidrug-resistant *Pseudomonas aeruginosa*, Lasers Surg. Med. 41 (2009) 391–397.
[35] G.P. Tegos, M. Hamblin, Phenotiazinium antimicrobial photosensitizers are substrates of bacterial multidrug resistance pumps, Antimicrob. Agents Chemother. 50 (2006) 196–203.
[36] S. George, M.R. Hamblin, A. Kishen, Uptake pathways of anionic and cationic photosensitizers into bacteria, Photochem. Photobiol. Sci. 8 (2009) 788–795.
[37] J. Sommerhäuser, B. Kloppert, W. Wolter, M. Zschöck, A. Sobiraj, K. Failing, The epidemiology of *Staphylococcus aureus* infections from subclinical mastitis in dairy cows during a control programme, Vet. Microbiol. 96 (2003) 91–102.
[38] M. Pol, P.L. Ruegg, Relationship between antimicrobial drug usage and antimicrobial susceptibility of Gram-positive mastitis pathogens, J. Dairy Sci. 90 (2007) 262–273.
[39] T. Dai, B.B. Fuchs, J.J. Coleman, R.A. Prates, C. Astrakas, T.G. St Denis, M.S. Ribeiro, E. Mylonakis, M.R. Hamblin, G.P. Tegos, Concepts and principles of photodynamic therapy as an alternative antifungal discovery platform, Front. Microbiol. 3 (2012) 120.
[40] J. Leonel Gonçalves, S. Hwa In Lee, E. de Paula Arruda, D. Pedroso Galles, V. Camargo Caetano, C.A. Fernandes de Oliveira, A.M. Fernandes, M. Veiga Dos Santos, Biofilm-producing ability and efficiency of sanitizing agents against *Prototheca zopfii* isolates from bovine subclinical mastitis, J. Dairy Sci. 98 (2015) 3613–3621.
[41] P.G. Calzavara-Pinton, M. Venturini, R. Sala, A comprehensive overview of photodynamic therapy in the treatment of superficial fungal infections of the skin, J. Photochem. Photobiol. B. Biol. 78 (2005) 1–6.
[42] T.N. Demidova, M.R. Hamblin, Photodynamic inactivation of *Bacillus spores*, mediated by phenothiazinium dyes, Appl. Environ. Microbiol. 71 (2005) 6918–6925.
[43] J. Chibebe Junior, B.B. Fuchs, C.P. Sabino, J.C. Junqueira, A.O. Jorge, M.S. Ribeiro, M.S. Gilmore, L.B. Rice, G.P. Tegos, M.R. Hamblin, E. Mylonakis, Photodynamic and antibiotic therapy impair the pathogenesis of *Enterococcus faecium* in a whole animal insect model, PLoS One 8 (2013) e55926.
[44] J. Chibebe Junior, C.P. Sabino, X. Tan, J.C. Junqueira, Y. Wang, B.B. Fuchs, A.O. Jorge, G.P. Tegos, M.R. Hamblin, E. Mylonakis, Selective photoinactivation of *Candida albicans* in the non-vertebrate host infection model *Galleria mellonella*, BMC Microbiol. 13 (2013) 217.
[45] N. Soukos, M. Wilson, T. Burns, P. Speight, Photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts and *Streptococcus sanguis* evaluated *in vitro*, Lasers Surg. Med. 18 (1996) 253–259.
[46] R. Haddad, O. Kaplan, E. Brazovski, M. Rabau, S. Schneebaum, A. Shnaper, Y. Skornick, H. Kashtan, Effect of photodynamic therapy on normal fibroblasts and colon anastomotic healing in mice, J. Gastrointest. Surg. 3 (1999) 602–606.
[47] M. Tanaka, M. Kinoshita, Y. Yoshihara, N. Shinomiya, S. Seki, K. Nemoto, T. Hirayama, T. Dai, L. Huang, M.R. Hamblin, Y. Morimoto, Optimal

photosensitizers for photodynamic therapy of infections should kill bacteria but spare neutrophils, Photochem. Photobiol. 88 (2011) 227–232.
[48] B.B. Noodt, G.H. Rodal, M. Wainwright, Q. Peng, R. Horobin, J.M. Nesland, K. Berg, Apoptosis induction by different pathways with methylene blue derivative and light from mitochondrial sites in V79 cells, Int. J. Cancer 75 (1998) 941–948.
[49] B. Zeina, J. Greenman, D. Corry, W.M. Purcell, Cytotoxic effects of antimicrobial photodynamic therapy on keratinocytes *in vitro*, Br. J. Dermatol. 146 (2002) 568–573.
[50] A.P. Castano, P. Mroz, M.R. Hamblin, Photodynamic therapy and anti-tumour immunity, Nat. Rev. Cancer 6 (2006) 535–545.
[51] C.P. Sabino, A.S. Garcez, S.C. Núñez, M.S. Ribeiro, M.R. Hamblin, Real-time evaluation of two light delivery systems for photodynamic disinfection of *Candida albicans* biofilm in curved root canals, Lasers Med. Sci (2014), in press.
[52] R.A. Ganz, J. Viveiros, A. Ahmad, A. Ahmadi, A. Khalil, M.J. Tolkoff, N.S. Nishioka, M.R. Hamblin, *Helicobacter pylori* in patients can be killed by visible light, Lasers Surg. Med. 36 (2005) 260–265.
[53] H. Kato, Our experience with photodynamic diagnosis and photodynamic therapy for lung cancer, J. Natl. Compr. Cancer Netw. 1 (2012) 3–8.
[54] M.J. Bader, H. Stepp, W. Beyer, T. Pongratz, R. Sroka, M. Kriegmair, D. Zaak, M. Welschof, D. Tilki, C.G. Stief, R. Waidelich, Photodynamic therapy of bladder cancer—a phase I study using hexaminolevulinate (HAL), Urol. Oncol. 31 (2013) 1178–1183.
[55] T. Yano, K. Hatogai, H. Morimoto, Y. Yoda, K. Kaneko, Photodynamic therapy for esophageal cancer, Ann. Transl. Med. 2 (2014) 29.
[56] B.M. Miller, A. Sauer, C.I. Moraru, Inactivation of *Escherichia coli* in milk and concentrated milk using pulsed-light treatment, J. Dairy Sci. 95 (2012) 5597–5603.
[57] A. Simões, M.D. Platero, L. Campos, A.C. Aranha, C.D.E.P. Eduardo, J. Nicolau, Laser as a therapy for dry mouth symptoms in a patient with Sjögren's syndrome: a case report, Spec. Care Dentist. 9 (2009) 134–137.
[58] B. Lončar, M.M. Stipetić, M. Baričević, D. Risović, The effect of low-level laser therapy on salivary glands in patients with xerostomia, Photomed. Laser Surg. 29 (2011) 171–175.
[59] C. Fabris, M. Soncin, F. Corsi, I. Cattin, G. Jori, Treatment of spontaneous piodermitis in dogs by PDT using porphyrin-type photosensitisers, Photodiagnosis Photodyn. Ther. 11 (2011) 138.
[60] J. Buchholz, H. Walt, Veterinary photodynamic therapy: a review, Photodiagn. Photodyn. Ther. 10 (2013) 342–347.
[61] F.P. Sellera, C.P. Sabino, M.S. Ribeiro, L.T. Fernandes, F.C. Pogliani, C.R. Teixeira, G.H.P. Dutra, C.L. Nascimento, Photodynamic therapy for pododermatitis in penguins, Zoo Biol. 33 (2014) 353–356.
[62] C.L. Nascimento, M.S. Ribeiro, F.P. Sellera, G.H. Dutra, A. Simões, C.R. Teixeira, Comparative study between photodynamic and antibiotic therapies for treatment of footpad dermatitis (bumblefoot) in Magellanic penguins (*Spheniscus magellanicus*), Photodiagn. Photodyn. Ther. 12 (2015) 36–44.