Assessment of the Antimicrobial Effect of Non-Thermal Plasma Activated Water Against Coagulase-Positive Staphylococci

Irina LIPOVAN[1], Andra Cristina BOSTĂNARU[2], Valentin NĂSTASĂ[2], Eugen HNATIUC[3], Vasile VULPE[1], Mihai MAREŞ[2]*

[1, 2] Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad", Iasi - Romania

[3] Technical University Gh. Asachi, Iasi-Romania

**Corresponding author: mmares@uaiasi.ro*

Bulletin UASVM Veterinary Medicine 72(2) / 2015,
Print ISSN 1843-5270; Electronic ISSN 1843-5378
DOI:10.15835/buasvmcn-vm: 11528

Abstract

Coagulase-positive staphylococci, the main etiologic agents of pyodermatitis have a well-known ability to develop multi antibiotic resistance mechanisms. Non-thermal plasma activated water (PAW) is a new generation of disinfectants/decontaminants so-called "non-conventional" highly efficient against the microorganisms of medical interest.

Since the plasma discharge in water gives it specific physical and chemical properties, including bactericidal activity, the purpose of our paper was to evaluate the degree of reduction of the microbial burden after the exposure to PAW of coagulase-positive staphylococci suspensions.

Evaluation of the antimicrobial effect was performed on 38 strains of coagulase-positive staphylococci isolated from skin lesions in humans and pets. In order to obtain PAW, a GlidArc reactor was used. A defined volume of staphylococcal suspension (10 ml) with 3 McFarland density (approx. 10^9 CFU/ml) was mixed with 90 ml PAW and after various contact times (3, 5, 7 and 10 minutes) known volumes of the mixture (0.1 and 1.0 ml) were transferred to Baird-Parker Agar plates in order to determine the number of CFU/ml. In parallel, the initial burden was determined by serial dilution method. The reduction of the bacterial burden was calculated as the log [(CFU/ml before treatment)/(CFU/ ml after treatment)].

After 3 minutes contact with PAW, the average burden reduction was 5.00 $\log_{10.}$ After 10 minutes of treatment, the initial burden has been reduced with an average of 7.61 logarithmic units.

PAW proved to be a powerful anti-staphylococcal agent and further *in vivo* studies are warranted to demonstrate its therapeutic benefits in different clinical dermatological conditions.

Keywords: *dermatology, disinfection, non-thermal plasma activated water, Staphylococcus*

INTRODUCTION

Plasma, the fourth form of the matter, can exist in a variety of states: low-pressure plasmas, atmospheric pressure plasmas and high-pressure plasmas. On the other hand, studies are currently conducted on the thermal and non-thermal plasmas. The difference between the two varieties resides in the relative temperature of electrons, ions and neutrals that is at a very high level in the variant thermal plasmas. It is the reason why its application is limited (Bensignor, 2000).

Non-thermal plasma activated water (PAW) is currently studied as a method of decontamination and disinfection. Various studies worldwide have as the subject the use of plasma in the food industry (Brisset *et al.*, 2012); the antimicrobial treatment

(Carp, 2014); military equipment (Deilmann *et al., 2008*); heat-sensitive materials (Dexi *et al., 2011*); periodontal diseases and the surface of teeth (Grundmann *et al., 2006*; Guguianu, 2002).

The use of PAW in dermatology represents a viable solution relating to the mechanisms of resistance to antiseptics and disinfectants. Adaptive mechanisms and those of bacteria resistance to antiseptics and disinfectants are known, resistance being a natural (intrinsic) property or a property acquired due to mutation or the acquisition of plasmids (self-replicating and extrachromosomal DNA) or transposons (chromosomal transposons or integration plasmids). (Heinlin *et al.,* 2010; Kamgang-Youbi *et al.,* 2008)

In the global "fight" with staphylococcal skin infections, a special case is represented by the methicillin-resistant *Staphylococcus aureus* (MRSA), considered as a global threat. (Kamgang-Youbi *et al.,* 2008); the development of some alternative topical treatments being salutary.

This study analyzes the effectiveness of the antimicrobial action of PAW on the coagulaso-positive *Staphylococcus spp.*, the main etiological agent of pyoderma in dogs, having a high frequency in cats. (Kamgang-Youbi *et al.,* 2009; Laroussi *et al.,* 2003).

MATERIALS AND METHODS

In this study, a new algorithm was used, algorithm proposed by the team of Hatniuc and of his collaborators, based on a multielectrod system, using a type of GlidArc electrochemical reactor with auxiliary electrodes.

The antibacterial effect of PAW was tested on a number of 38 strains of staphylococci from people and pets. The strains were isolated from purulent collections from subjects diagnosed with folliculitis, furunculosis, juvenile acne, abscesses, otitis externa.

The phenotypic identification based on the cultural and biochemical characteristics (pigmentation, catalase, mannitol fermentation, hemolysis) of the staphylococci strains pointed out that, out of the 38 strains, 27 were *Staphylococcus aureus*, and 11 were non-*S.aureus* strains. Pathogenicity was tested by conducting the testing of the coagulation of citratated rabbit plasma (BioRad). As a positive witness(better control), in this study, the strain of *Staphylococcus aureus* ATCC 25923 was used. The 38 strains were identified as being coagulase-positive, 23 of them as coagulating in 30 minutes, and 15 in 24 hours.

An inoculum equivalent to the 3 McFarland standard (aprox.10^9 cells/ ml) was obtained from the bacterial culture, incubated at 35ºC ± 1ºC for 24 hours, under conditions of aerobiose. The initial bacterial concentration was assessed accurately by using the method of serial dilutions, up to 10^{-7}, in duplicates. For the last two dilutions, 10^{-6} and 10^{-7} respectively, an quantity equivalent to 100µl was discharged in Petri plates with Plate Count Agar and distributed evenly using sterile, glass beads. The plates were incubated at 35ºC ± 1ºC, under conditions of aerobiose for 24 hours, after which the CFUs/ ml were calculated using the following formula:

$$CFU/ml = \Sigma(n \cdot d)/N$$

Where "n" is the number of colonies counted on the Petri plate; "d" is the dilution factor, "N" is the number of Petri plates taken into account for each dilution. The initial CFU determined in this way was used as control for the calculation of reductions.

For each strain, 90 ml of PAW were put in contact with 10 ml of initial inoculum. At different times of contact, 3, 5, 7 and 10 minutes, respectively, the known volumes of mixture (0.1 and 1.0 ml) were distributed on Petri plates with the agar Baird-Parker medium. 0.1ml was discharged on the surface of the medium and evenly distributed by using sterile glass beads. 1.0 ml was introduced on the Petri plate, over which the molten medium was cooled to to 45° C and poured; it was incorporated and carefully homogenized. The plates that were obtained in this way, were incubated at 35ºC ± 1ºC for 24 hours, under conditions of aerobiose.

After the expiry of the incubation period, for each plate were counted the remaining colonies and the CFU/ ml was calculated again. The reduction of the bacterial burden was calculated as the log [(CFU / ml before treatment) / (CFU / ml after treatment)].

RESULTS AND DISCUSSION

Water that is activated with plasma acquires an acid Ph and contains among other groups: hydrogen peroxide (H_2O_2), nitrate (NO_3) and nitrite (NO_2) anions, responsible for the degradation of the bacterial wall, by means of an oxidative effect

on proteins, lipids, DNA (McDonnel and Russell, 1999; Naitali *et al.,* 2010).

The efficacy of inactivation by using this method depends on abiotic (temperature, the hardness of water, the amount of organic substances and nutrients for the bacterium) and biotic (the structure of the cell wall, the presence or absence of capsules, the ability to sporulate) factors (Oehmigen *et al.,* 2010, Paterson, 2008).

Previous studies point out that the antimicrobial action of PAW increases in the following order: yeast, gram-positive and gram-negative bacteria (Roth, 2005; Sladek *et al.,* 2007). The factors that may affect the action on bacteria depend on:

- the thickness of the bacterial cell wall (in Gram + = 20-80nm, in Gram- = 10-15nm) (15);
- its rigidity (in Gram +, the wall is rigid, in comparison with Gram- in the case of which the wall is elastic) and/ or
- the chemical structure of the wall (GLP – the glucidic and lipidic polypeptidic structure in Gram- and the glucidic and polypeptidic structure in Gram+); the existence of some strains of the capsule. (Ursache *et al.,* 2014; Vleugels *et al.,* 2005).

In this study, the antibacterial activity of PAW was analyzed on a total number of 38 strains of staphylococci, isolated from clinical cases of pyoderma and otitis externa in humans and animals and 1 ATCC 25923 *Staphylococcus aureus* strain. Reduction was calculated at 3 and 10 minutes respectively, the results being graphically illustrated in Fig. 1.

After 5 and 7 minutes treatment, the staphylococcal burden reduction didn't differ significantly from that occurred after 3 minutes. An obvious reduction - that means more than 5log, has been observed only after 10 minutes contact with PAW. We consider that PAW acts against staphylococci following two steps: first one being the penetration through the cell wall and second one involving the impairment of cell content (i.e. DNA, proteins) which is practically responsible for bacterial death.

At the 3-minute treatment of the strains with PAW, the values of reduction ranged from 0 to 7.38 with an average of 5 log; and every 10 minutes, the minimum and maximum reduction was 4.99; 10.11 respectively; with an average of 7,61 log. The PAW action on the strains of coagulase-positive staphylococci proved to have a heterogeneous

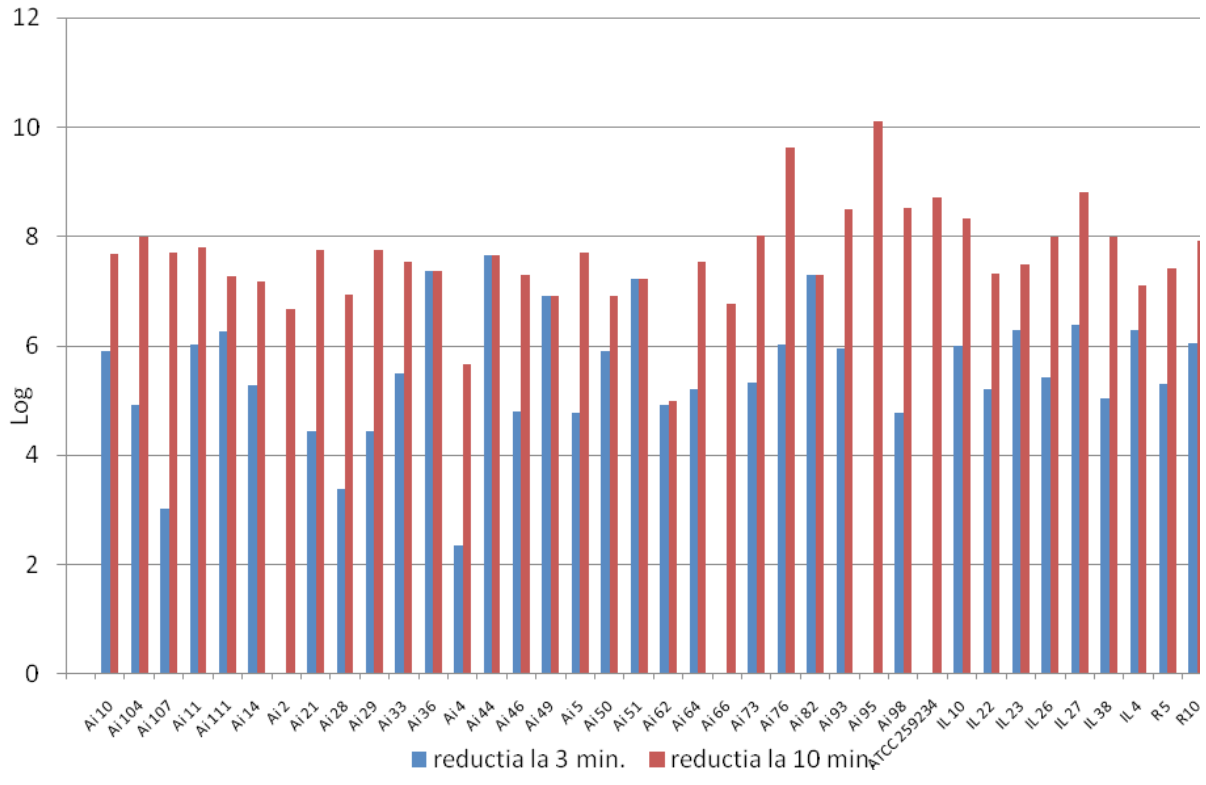

Fig. 1. The reduction of *Staphylococcus spp*. After the PAW treatment at 3 and 10 minutes

character. The heterogeneous behavior is more pronounced within 3 minutes from treatment compared to within 10 minutes from treatment. For the treatment time of 3 minutes, 26 strains of Staphylococci were placed in the average range of reduction of 5 ± 1 log, reduction compared to the 34 strains, for the treatment time of 10 minutes, in the average range of reduction 7.61 ± 1 log. This demonstrates that the bactericidal action is proportional to the treatment time. In four (Ai 2, 66, 95 and ATCC 259234) out of the 39 strains, although within 10 minutes the reduction was complete, there was a reduction 0 noticed within 3 minutes from the treatment with POW. In 23 out of the total number of strains tested, the „cid" effect was complete within 10 minutes from the treatment with PAW. In the case of these strains, it was noticed that the total inactivation was independent of the initial inoculum CFUs, before the treatment with POW, these ones ranging between $4,7^{8}x10^{8}$ and $8,3x10^{10}$.

The (eventual) correlation of the degree of the tested reduction of *Staphylococcus* species, will be achieved after carrying out the molecular biology tests and identifying the genotype.

CONCLUSION

In this study, PAW obtained by using the GlidArc reactor, the bacterial load dropped by an average of 5 log at a duration of 3 minutes of contact and of 7.61 log of 10 minutes of contact.

The behavior of those 39 strains (38 from clinical isolates and 1 ATCC) was found to be heterogeneous. At 3 minutes of contact of the Staphylococcus culture with PAW, 26 strains had a reduction in the average range of 5 ± 1 log., while after 10 minutes of contact, 34 strains had a reduction in the average range of of 7.61 log.

After 10 minutes of contact, 23 strains were totally inactivated, irrespective of the initial load of the inoculum used.

The results of the "in vitro" study on the antibacterial action of PAW on the strains of *Staphyloccoccus spp*. recommend this method as a disinfection variant due to the economic advantages. As regards the use of PAW in dermatology, *in-vivo* future clinical trials in histochemistry, histopathology, toxicity, etc. will prove the impact on the body to which this treatment applies.

REFERENCES

1. Bensignor E (2000). Atlas des piodermites canines. Ed MED'COM, 19-21.
2. Brisset JL, Naitali M, Herry JM, Hnatiuc E, Kamgang G. (2012). Kinetics and Bacterial Inactivation Induced by Peroxinitrite in Electric Discharges in Air. Plasma Chem Plasma Process 32(4):675-692.
3. Carp Carare C (2014).Microbiologie generala. Ed "Ion Ionescu de la Brad",Iasi,97-99.
4. Deilmann M, Halfmann H, Bibinov N, Wunderlich J, Awakowicz P(2008). Low-pressure microwave plasma sterilization of polyethylene terephalate bottles. J Food Prot 71:2119-23.
5. Dexi L, Zilan X, Tianfeng D, Xincai Z, Yingguang C, Xinpei L(2011). Bacterial-killing effect of atmosphericpressure non-equilibrium plasma jetand oral mucosa response.J Huazhong Univ Sci Technol Med Sci 31(6):852-856.
6. Grundmann H, Aires de Sousa M, Boyce J, Tiemersma E (2006). Emergence and resurgence of meticilin-resistant Staphylococcus aureus as a publichealth,threat. Lancet 368: 874–885.
7. Guguianu E (2002). Bacteriologie Generala. Ed Venus, Iasi, 49-53.
8. Heinlin J, Isbary G, Stolz W, Morfill G, Landthaler M, Shimizu T, Steffes B, Nosenko T, Zimmermann L, Karrer S (2010). Plasma applications in medicine with a special focus on dermatology.
9. Kamgang-Youbi G, Herry JM, Bellon Fontaine MN, Doubla A, Naitali M (2008). Impact on disinfection efficiency of cell load and of planktonic/adherent/detached state;case of Hafnia alvei inactivation by POW. Appl Microbiol Biotechnol 81:449-457.
10. Kamgang-Youbi G, Herry JM, Meylheuc T, Brisset JL, Fontaine MN, Doubla A, Naitali M (2009). Microbial inactivation using plasma-activated water obtained by electric discharges. Applied Microbiology 48:13-18.
11. Laroussi M, Mendis D, Rosenberg M (2003). Plasma interactions with microbes. New Journal of Physics 5(41):1–10.
12. McDonnel G, Denver Russell A (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. Clinical Microbiology Reviews, 158-166.
13. Naitali M, Kamgang-Youbi G, Herry JM, Bellon Fontaine MN, Brisset JL (2010). Combined Effects of Long-Living Chemical Species during Microbial Inactivation Using Atmospheric Plasma-Treated water. Applied and Environmental Microbiology, 7662-7664.
14. Oehmigen K, Hahnel M, Branderburg R, Wilke C, Weltmann K and Woedtke T(2010). Plasma Process Polym, 7-250.
15. Paterson S (2008). Skin diseases of the Dog and Cat, second edition. Blackwell Publisching, 27-28.
16. Roth JR (2005). Potential industrial applications of the one atmosphere uniform glow discharge plasma operating in ambient air. Phys Plasma doi:10.1063/1.1882293.
17. Sladek REJ, Filoche SK, Sissons CH, Stoffels E (2007). Treatment of Streptococcus mutans biofilms with a nonthermal atmospheric plasma. Lett Appl Microbiol 45:318-323.

18. Ursache M, Moraru R, Hnatiuc E, Nastase V, Mares M (2014). Comparative assessement of the relation between energy consumption and bacterial burden reduction using plasma activated water. Optimization of Electrical and Electronic Equipment (OPTIM), 2014 International Conference, 1035-1041.

19. Vleugels M, Shama G, Deng X, Greenacre E, Brocklehurst T, Kong M (2005). Atmospheric plasma inactivation of biofilm-forming bacteria for food safety control. IEEE Trans Plasma Sci 33:824-8.