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Comparative Study of the Effect of Electrochemically Activated Water Solutions on *Pseudomonas aeruginosa*

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Abstract

Studies have been carried out to determine the sensitivity of *Pseudomonas aeruginosa* to activate aqueous solutions (anolytes and catholytes) *in vitro*. The solutions were obtained by electrochemical activation of water with 0.8% NaCl, as well as with combination of 0.4% NaCl and 0.4% Na_2CO_3 and were tested at final concentrations of 50% and 100%. The disinfectant Virkon S was used as a control. After different intervals of exposure of a *P. aeruginosa* suspension with a final concentration of 10^6 cells/ml to the action of the solutions, samples were seeded on Cetrimide agar and cultured for 24 hours at 37°C. An inhibitory effect on *P. aeruginosa* was established depending on the type and final concentration of the activated solutions. Electrochemically activated aqueous solutions with 0.8% NaCl, as well as 0.4% NaCl and 0.4% Na_2CO_3 exert inhibitory action on a *P. aeruginosa* suspension with a concentration of 10^6 cells/ml
Keywords: anolyte, catholyte, *P. aeruginosa*, antibacterial activity

Резюме

Проведени са изследвания за определяне на чувствителността на *Pseudomonas aeruginosa* към активирани водни разтвори (анолити и католити) *in vitro*. Разтворите са получени чрез електрохимично активиране на вода с 0.8% NaCl, както и с комбинация от 0.4% NaCl и 0.4% Na_2C0_3, изпитани в крайни концентрации от 50% и 100%. За контрола е използван дезинфектантът Virkon S. След различни интервали на въздействие на разтворите върху суспензия на *P. aeruginosa* с крайна концентрация 10^6 клетки/ml са правени посявки на Цетримид агар и култивирани 24 часа при 37°C. Установено е инхибиторно действие върху *P. aeruginosa* в зависимост от вида и крайната концентрация на активираните разтвори. Електрохимично активираните водни разтвори, получени с 0.8% NaCl, както и с комбинация от 0.4% NaCl и 0.4% Na_2CO_3, оказват инхибиторно действие върху суспензия от *P. aeruginosa* с концентрация 10^6 клетки/ml.

Introduction

The anolytes and catholytes obtained by electrochemical activation are safe for humans, animals and the environment. This is so because the active substances contained in them are chloro-oxygenic and peroxide compounds, which relatively quickly change their concentration. Those substances are in a metastable state for a time interval of different duration for the anolyte and the catholite afterwards reverting to a state of inactive water electrolyte (Bakhir, 2009a, b; Gluhchev *et al.*, 2018). No resistance is built up by the microorganisms against the electrochemically activated water solutions (EAASs) because these solutions have a constantly changing dynamic composition depending on the salts used. An active ingredient in these solutions is chlorine, but these waters appear to be stronger disinfectants compared to conventional chlorine preparations. When applied, they destroy bacteria, viruses and spores without any by-product formation unlike conventional chlorine preparations. Their properties depend on the redox - potential and their content of chlorine or iodine (Ignatov *et al*., 2015; Gluhchev *et al*., 2018).

The growing prevalence of strains of pathogenic bacteria, resistant to antibiotics, and the rapidly developing resistance to commonly used dis-

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infectants is a serious problem today. Gram-negative bacteria display higher resistance to chemical effects, which significantly impedes the measures aimed at effectively controlling their presence. Some of them, especially *Pseudomonas aeruginosa,* adapt particularly quickly to chemical and disinfection solutions (Alibert-Franco *et al*., 2009; Popova, 2016). Due to the increasingly frequent isolation of multi-resistant to antimicrobial means strains of this microorganism, the aim of the present study was to investigate the action of electrochemically activated aqueous solutions received with NaCl and Na_2CO_3 against *P. aeruginosa in vitro*.

Materials and Methods

Activated aqueous solutions: Activation time - 12 min

Anolytes: Anolyte prepared with 0.8% NaCl; Anolyte prepared with a combination of 0.4% NaCl and 0.4% Na_2CO_3; Anolyte prepared without salt addition.

Catholytes: Catholyte prepared with 0.8% NaCl; Catholyte prepared with a combination of 0.4% NaCl and 0.4% Na_2CO_3; Catholyte prepared without salts.

Control. The broad-spectrum disinfectant Virkon S, applied at a final concentration of 0.5%, was used as a positive control.

Microorganisms. A virulent strain of *P. aeruginosa*, isolated from a horse with sinusitis, multi-resistant to antibiotics of most groups and sensitive only to certain aminoglycosides (gentamicin and amikacin) and to quinolones was used.

Nutritient media. Media from BUL BIO NCIPD - Sofia, Bulgaria were used: Mueller Hinton agar to receive 24-hour cultures of bacterial strains, Mueller Hinton broth for liquid cultures, as well as *P. aeruginosa* selective Cetrimide agar for determination of the effect of the solutions tested for antimicrobial activity against this bacterial species.

Experimental designs

Experiment 1. Three milliliters of the EAAS tested (anolyte or catholyte) and 1 ml of *P. aeruginosa* broth culture were added to 2 ml of 3-fold concentrated nutrient broth. The final concentration of the EAAS was 50%. The next steps were homogenization and culturing for 24-48 hours at 37°C.

Experiment 2. A suspension of *P. aeruginosa* at a concentration of 10^7 cells/ml in an amount of 1 ml was added to 9 ml of each EAAS (undiluted) tested, resulting in a final concentration of 10^6 cells/ ml. After various time intervals of action of the EAAS (2 min, 5 min, 10 min and 15 min), cultures of each of the samples were made on Cetrimide agar and incubated at 37°C for 24-48 h under aerobic conditions.

The following *controls* were set: sterile distilled water (without anolyte or catholyte) with the same concentration of the tested bacterial strain, 100% anolyte and 100% catholyte without microorganisms, distilled water with 0.5% Virkon S; Mueller Hinton broth in an amount of 5 ml with 1 ml broth culture of the examined microorganism.

Each experiment was performed in triplicate.

Results

The physical indicators pH, oxidation - reduction potential (ORP) and temperature of the EAASs tested are presented in Table 1.

The data in the table show that the anolyte

Table 1. Physical indicators of the anolytes and catholytes used

Starting composition		pH	ORP, mV	t° C
Aqueous sodium chloride solution 0.8%	before electrolysis	8,92	218 mV	22,1 °C
	anolyte	2,47	1000 mV	20, 7 °C
	catholyte	11,91	-368 mV	20,7 °C
Aqueous solution of sodium chloride (0.4%) and sodium carbonate (0.4%)	before electrolysis	11, 33	109 mV	18,5 °C
	anolyte	10,74	439 mV	20,6 °C
	catholyte	11,95	-323 mV	19,7 °C
Water without salts	before electrolysis	9,57	302 mV	18,7°C
	anolyte	3,55	743 mV	22,5°C
	catholyte	10,03	-214 mV	22,5°C

ORP - oxidation-reduction potential

Table 2. Growth of *P. aeruginosa* in broth after exposure to EAASs administered at 50% concentration and growth of *P. aeruginosa* on Cetrimide agar (amount of colonies) after various intervals of exposure to EAASs applied at a concentration of 100%

Sample №	Type of activated solution	Growth in broth	Growth on Cetrimide agar			
			Time of impact - min			
			2	5	10	15
1	anolyte of NaCl	-	-	-	-	-
2	anolyte of Na_2CO_3	-	-	-	-	-
3	0,5% Virkon S in distilled water	+	-	-	-	-
4	catholyte of NaCl	+	+	+	-	-
5	anolyte without salts	+	+	+	+	+
6	catholyte without salts	+	+	+	+	+
7	catholyte of Na_2CO_3	-	-	-	-	-
K	untreated control	+	+	+	+	+

EAASs - electrochemically activated aqueous solutions

obtained with 0.8% NaCl was characterized by the highest ORP (1000 mV), followed by the salt-free anolyte (743 mV) and that with a combination of NaCl and Na_2CO_3 (439 mV). Among the catholytes, the lowest ORP was registered in that obtained with 0.8% NaCl (-368 mV), followed by catholyte with combination of NaCl and Na_2CO_3 (-323 mV) and the salt-free one (-214 mV). The pH value was the lowest in the anolyte with NaCl (2.47), and the highest - in the catholyte obtained with a combination of NaCl and Na_2CO_3 (11.95), very similar to this of catholyte of 0.8% NaCl (11.91).

The results of the microbiological investigations for anti-bacterial activity of the EAASs against *P. aeruginosa* in broth at a final concentration of 50% of experiment 1 and on Cetrimide agar of experiment 2 are shown in Table 2 and some of them - in Fig. 1 and Fig. 2.

From the results presented, it can be seen that the anolyte prepared with 0.8% NaCl, as well as that with a combination of 0.4% NaCl and 0.4% Na_2CO_3, applied at a final concentration of 50% and 100%, have a bactericidal effect on the multi-resistant strain of *P. aeruginosa*. Similar results were also found in samples with catholyte of Na_2CO_3. *P. aeruginosa* was killed within 2 minutes under the influence of these EAASs. When using 0.5 % Virkon S dissolved in distilled water, however, growth was observed in some of the broth cultures of *P. aeruginosa*, although colonies were not detected on the selective medium even after 48 hours of cultivation. Catholyte with NaCl at a final concentration of 100% had a bactericidal action on *P. aeruginosa* within 10 minutes, but at a twice lower concentration (in broth) the effect was not bacteri-

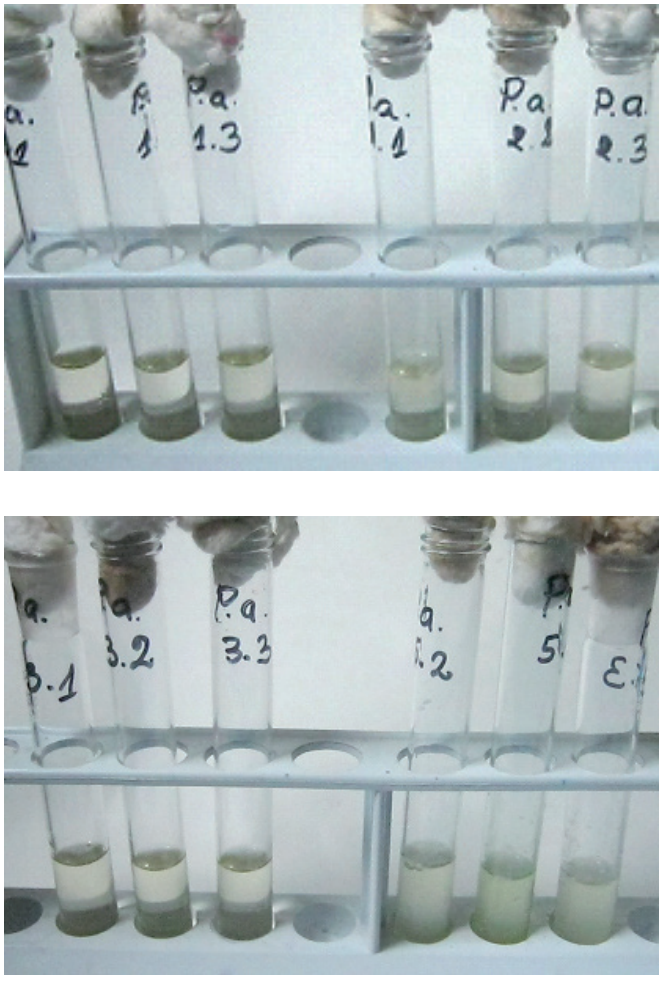

Fig. 1. Growth of *P. aeruginosa* in broth after exposure to EAASs: 50% anolyte of NaCl (1-1, 2 and 3), anolyte of Na_2CO_3 in a concentration of 50% (2-1, 2 and 3), 0,5% Virkon S in distilled water (3-1, 2 and 3) and anolyte without salts (5 - 1, 2 and 3).

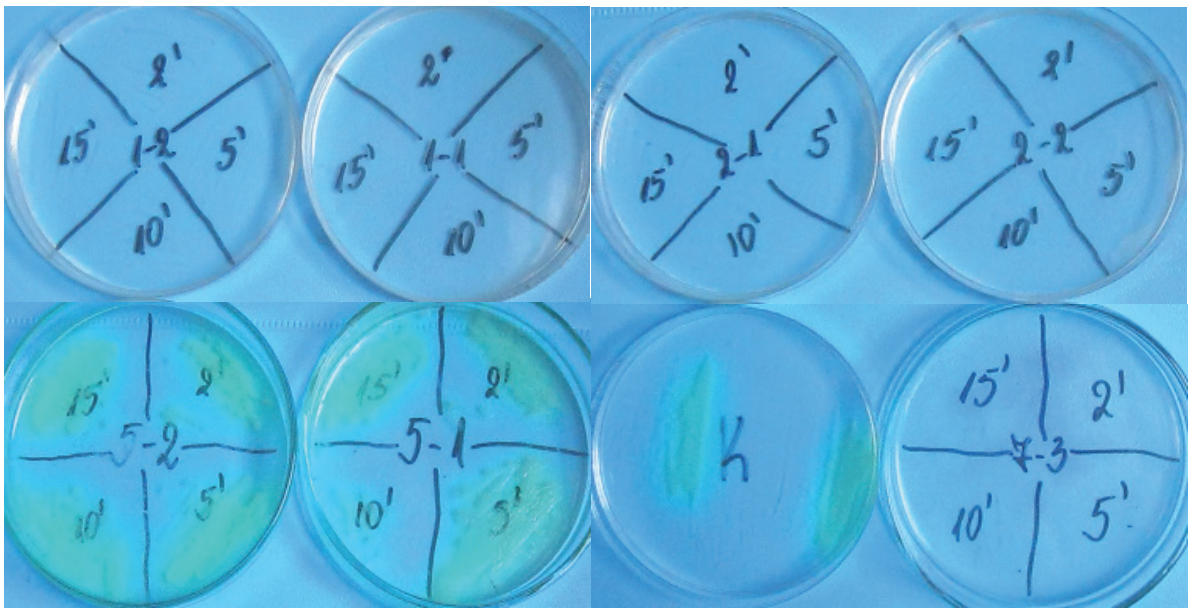

Fig. 2. Growth of *P. aeruginosa* on Cetrimide agar after treatment with EAASs. From left to right above: anolyte of NaCl (1 - 1, 2); anolyte of Na_2CO_3 (2-1, 2); below: anolyte without salts (5 – 1, 2); catholyte of Na_2CO_3 (7 - 3); untreated control (K).

cidal. In the samples with anolyte prepared without salts, as well as in the salt-free catholyte, colonies and growth in the broth were also detected.

Discussion

The results of the present study, demonstrating high antimicrobial activity of EAASs, are consistent with the investigations of other authors. Gurgulova *et al*. (2010, 2011), Popova *et al*. (2016a, c) and others reported significant bactericidal activity of such solutions against Gram-negative and Gram-positive bacteria, including spore-forming bacteria. Tasheva *et al.* (2010) found antimycotic effect of EAASs, and Atanasov *et al.* (2014), Karadzhov *et al*. (2014) and others - antiviral activity. These and other literature data have reported on the antimicrobial activity of anolytes but there is no conclusive evidence about such activity of catholytes. Our studies indicate for the first time a bactericidal action of catholytes, including catholyte prepared with a combination of 0.4% NaCl and 0.4% Na_2CO_3 without dilution and 50% diluted, as well as of that with 0.8% NaCl without dilution but not of the catholyte prepared without salts.

Our previous studies (Popova *et al*., 2016 a, b) provided evidence of high activity of anolyte obtained with 3% NaCl against *P. aeruginosa*. The results of the current research show that when using a significantly lower NaCl concentration (0.8%), as well as 0.4% NaCl and 0.4% Na_2CO_3, a significant bactericidal effect was also achieved against a virulent multi-resistant strain of *P. aeruginosa*, but EAASs without salts did not have such activity against a strain with such properties.

The antimicrobial activity of EAASs directly depends on their oxidation-reduction potential as it is manifested only in its certain range. Their properties change depending on their ORP. According to the studies of Gluhchev *et al*. (2018), characteristic of the anolyte and the catholyte is that the former possesses reduced electron activity and has pronounced oxidizing properties, while the latter has increased electron activity and exhibits properties of a regenerator (reducer). In this respect, the results obtained by us are consistent with those of Dimitrova *et al*. (2013), according to which solutions with the highest ORP inhibit bacterial cells to the highest extent, which can be explained by the high ORP and oxidative stress on the cellular membrane structures, as a result of which they are damaged.

The results from our present studies show that even at a concentration of 50%, the anolytes tested and the catholyte of Na_2CO_3 are a reliable means of safe decontamination of materials containing a virulent multi-resistant to antimicrobials strain of *P. aeruginosa*. Our research also categorically shows that EAASs activated with the addition of a relatively low amount of NaCl as well as with Na_2CO_3 can be used as disinfectants and antiseptics with great success. This disinfection is efficient and safe.

Conclusions

Anolytes obtained by activation with 0.8% NaCl, as well as with a combination of 0.4% NaCl and 0.4% Na_2CO_3, exhibit high antimicrobial activity on the tested multi-resistant virulent strain of *P. aeruginosa*. Applied without dilution, they exert a bactericidal effect within 2 minutes, and they also show a bactericidal effect at a concentration of 50%. The anolyte without salts does not have such action.

Of the investigated catholytes, the highest bactericidal activity was exhibited by the solution activated with a combination of 0.4% NaCl and 0.4% Na_2CO_3. At 100% concentration, its bactericidal action was detected within 2 minutes. The twice lower concentration also manifested bactericidal effect.

The catholyte produced by activation with 0.8% NaCl exhibited high antimicrobial activity when administered without dilution, as the multi-resistant virulent strain of *P. aeruginosa* perished for 10-15 minutes under its action. However, no such effect was observed with the 50% concentration. The salts-free catholyte also does not have such effect.

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