

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.509.059>

Investigation of the Action of the Anolyte after Different Storage Times on the Gram-negative Bacteria

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ABSTRACT

Keywords

Electrochemically, sodium chloride, anolyte, *Pseudomonas aeruginosa*, *Salmonella enterica*, antibacterial activity.

Article Info

Accepted:

20 August 2016

Available Online:

10 September 2016

Studies were carried out to determine the sensitivity of pathogenic Gram-negative bacteria (*Salmonella enterica* ATCC and *Pseudomonas aeruginosa* strain №450) to electrochemically activated 3% aqueous sodium chloride solution (anolyte) in concentrations of 100%, 50%, 25% and 12,5% immediately after preparation (fresh) and after storage at room temperature for 7 days, 21 days and 28 days. As a control was used the disinfectant Virkon S in concentrations of 1%, 0,5%, 0,25% and 0,125%. It was found that the suspension of *P. aeruginosa* with density of 10^6 cells/ml was killed after 2 minutes under the influence of all tested concentrations of the fresh anolyte. The same strain in concentration of 10^8 cells/ml was killed within 2 minutes by 50% and 100% fresh anolyte, but the lower concentrations had such effect after 5 - 10 minutes. The anolyte stored 7 days had similar action. After 21 and 28 days the anolyte in all tested concentrations had fast bactericidal activity on *P. aeruginosa* in suspension of 10^6 cells/ml (in 2 minutes), but in such of 10^8 cells/ml single cells remained viable even after 10 minutes. *S. enterica* in both tested suspensions - of 10^6 and 10^8 cells/ml was inactivated within 2 minutes by all examined concentrations not only of the fresh anolyte, but of these stored for 7, 21 and 28 days. Virkon S in all tested concentrations killed within two minutes *P. aeruginosa* and *S. enterica* in both examined densities.

Introduction

The increasing resistance of pathogenic bacteria to antimicrobial agents is one of the biggest problems today. Bacteria develop resistance not only to antibiotics, but also to disinfectants and fighting them becomes more difficult. On the other hand, the protection of the environment from toxic effects of commonly used disinfectants in medicine, household, industry, agriculture

and other fields of human activity is an environmental issue with significant importance. Therefore the demand and application of efficient and at the same time safer from the environmental perspective antimicrobials is very topical. One promising modern approaches in this respect is the electrochemical treatment of water with electricity, which leads to the

preparation of electrochemically activated aqueous solutions (catholyte and anolyte) having specific physicochemical properties, achieved by changing the electrochemical properties of the water. These activated aqueous solutions - catholyte and anolyte can be used in medicine to treat various bacterial and viral diseases, as well as for disinfection of water (Karadzhev *et al.*, 2014; Ignatov *et al.*, 2015). There are reports for antibacterial and antiviral activity of anolyte. Studies of Atanasov *et al.*, (2014) show that the anolyte does not affect the growth of the cell culture PK-15, but shows a pronounced inhibitory effect on the development of the virus of classical swine fever in it. Gluhchev *et al.*, (2015) also found a significant inhibitory effect of the anolyte on the development of the of Classical swine fever (CSF) virus in cell culture, as well as of *Escherichia coli*. In the previous our studies (Popova *et al.*, 2016) have experimentally established a high antibacterial activity of the freshly prepared anolyte, which in a concentration of 100%, 50% and 25% kills for a short time (2 minutes) suspensions of Gram-negative bacteria: *Salmonella enterica*, *Escherichia coli* and *Pseudomonas aeruginosa* at a concentration of 10^6 cells/ml and suspensions of *S. enterica* with concentrations of 10^8 cells/ml. What is the period of retention of this antibacterial activity is not known, although it is important from a practical point of view.

Therefore in this work we aimed to investigate the effect of anolyte after different intervals of storage at room temperature on highly concentrated suspensions of pathogenic Gram-negative bacteria.

Materials and Methods

Anolyte (activated water). In the experiments was used anolyte containing Cl⁻, prepared by

electrochemical activation of distilled water with 3% NaCl, applied in various final concentrations from 12.5% to 100%. The antibacterial action of the anolyte was tested immediately after preparation (fresh anolyte) and after storage at room temperature for 7 days, 21 days and 28 days.

Control: Virkon S was used as a control in final concentrations from 1% to 0,125%.

Microorganisms: In the study were used suspensions with concentrations of 10^6 and 10^8 cells/ml of *Pseudomonas aeruginosa* № 450, isolated from an animal with chronic infection and showing multi resistance to antibiotics *in vitro*. Suspensions with concentrations 10^6 and 10^8 cells/ml of *Salmonella enterica* ATCC were also used. The suspensions were prepared in sterile saline solution by the optical method.

Nutrient media: Culture media from Scharlau - Antisel, Bulgaria were used - agar of Mueller Hinton for the preparation of 24-hour cultures of the bacterial strains and Cetrimide agar and Eosin Methylene Blue agar to determine the antimicrobial activity of the tested solutions respectively on *P. aeruginosa* and *S. enterica*.

Scaffold

Investigation of the antimicrobial activity of anolyte. Twice increasing dilutions of the anolyte were prepared in sterile distilled water and the concentrations obtained were respectively 100% anolyte, 50%, 25% and 12,5% in an amounts of 9 ml. To each of them was added a suspension of the tested microorganism at a concentration of 10^9 cells/ml in an amount of 1 ml at which was achieved a final concentration of 10^8 cells/ml. The same dilutions of the anolyte with concentrations of 100%, 50%, 25% and 12,5% in quantities of 9 ml were used for the examination of suspensions of the

microorganisms at a concentration of 10^6 cells/ml, and for this purpose to each dilution was added by 1 ml of the suspension at a concentration of 10^7 cells/ml. The following controls were put - sterile distilled water (without anolyte) with the same content of the tested bacterial strain, as well as 100% anolyte without microorganisms.

Study of the antimicrobial activity of Virkon S, used as a control for comparison of the effect of the anolyte. Twice increasing dilutions of Virkon S were prepared in sterile distilled water at concentrations of 1%, 0,50%, 0,25%, and 0,125% in amounts of 9 ml. To each of them was added a suspension of the tested microorganism at a concentrations of 10^9 cells/ml in amounts of 1 ml at which were achieved the final concentrations of 10^8 cells/ml. Tested were and controls - sterile distilled water (without Virkon S) with the same content of the studied bacterial strain and 1% Virkon S without microorganisms. After various time intervals for action of the anolyte and Virkon S (2 min, 5 min and 10 min) cultures of each sample were made on Cetrimide agar and Eosin Methylene Blue agar, which were incubated at 37 °C for 24-48 h under aerobic conditions. After the cultivation was reported the growth of the test bacteria treated with the test disinfectant, as well as the results of growth of the bacteria on the controls.

Results and Discussion

The results of the studies with *P. aeruginosa* are presented in Tables 1-4 and Figures 1, 2 and 3. From Table 1 it is seen that the suspension of *P. aeruginosa* 450 with density of 10^6 cells/ml was killed after 2 minutes under the influence of all tested concentrations of the fresh anolyte. The same strain in a concentration of 10^8

cells/ml was killed within 2 minutes under the action of 50% and 100% fresh anolyte, but with stand for 2 minutes in the solutions with a lower concentration. After 5 and 10 minutes of impact, however, the anolyte in all tested concentrations had a bactericidal effect on this strain in suspensions of density even 10^8 cells/ml.

From Table 2 and Figure 1 it is clear that the *P. aeruginosa* 450 at concentration of 10^6 cells/ml also was killed after 2 minutes under the effect of all tested concentrations of anolyte stored 7 days. In a hundred-fold higher concentration tested this strain was killed after 2 minutes under the effect of 100% anolyte stored 7 days but lasted for 2 minutes in a solution with its lower concentrations. After 5 and 10 minutes of impact the anolyte stored 7 days in all tested concentrations also had a bactericidal effect on this strain with density 10^8 cells/ml.

Interesting results showed the anolyte after 21 days of storage. As can be seen from Table 3 and Figure 2, in all tested concentrations it had a bactericidal activity on *P. aeruginosa* 450 with density 10^6 cells/ml. However, the same strain, tested in suspension with a hundredfold higher density, was affected to a lesser degree by the 21 days stored anolyte. Even after 10 min effect of undiluted anolyte and of that at concentration of 50% single cells of *P. aeruginosa* 450 remained viable. This strain when was tested in a high concentration (10^8 cells/ml) showed a higher stability and was not completely killed after 10 minutes exposure even to 100% anolyte stored 21 days. The effect of this elder anolyte was significant and greatly reduction of the amount of viable cells was achieved, but single cells remained viable.

The results presented in Table 4 and Figure

3 show that the freshly prepared solution of Virkon S, used as a control in all the tested concentrations exhibited bactericidal activity on *P. aeruginosa*, whose suspensions in both tested concentrations were killed even within two minutes exposure time.

As it is seen from Table 5, *S. enterica* ATCC was sensitive to all examined concentrations of the fresh anolyte even in suspension with the highest density of 10^8 cells/ml tested.

S. enterica ATCC, however, showed a high sensitivity to all tested concentrations (12,5% to 100%) of the anolyte stored for 7 days (Table 6) and even for 21 days (Table 7).

As can be seen from Table 7 and Figure 4, this long stored anolyte inactivated suspensions of this strain of *S. enterica* even at concentration of 10^8 cells/ml within 2 minutes.

The data in Table 8 show that the 28 days stored anolyte in all tested concentrations (from 12,5% to 100%) had a bactericidal

effect on the suspensions at a concentration of 10^6 cells/ml of *P. aeruginosa* 450 and of *S. enterica* even within 2 min exposure time.

The results presented in Table 9 show that the solutions of Virkon S, used as a control, in all the tested concentrations exhibited bactericidal activity on *S. enterica* ATCC, whose suspensions in both tested concentrations were killed even within two minutes exposure time.

From the presented results it is clear that not only the fresh anolyte, but also that after storage for 1 to 3 and even 4 weeks exhibit high bactericidal activity against pathogenic Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Salmonella enterica*, which are distinguished by particularly high resistance to disinfectants. Especially encouraging are the data obtained for *Salmonella enterica*. It appears to be very sensitive to all concentrations of the anolyte even after 4 weeks of its storage. In *Pseudomonas aeruginosa* the results depend on the density of the tested suspensions, as well as on the time of storage of the anolyte.

Table.1 Growth (quantity of colonies) of *P. aeruginosa* 450 at concentrations of 10^6 cells/ml and 10^8 cells/ml after various intervals of exposure of fresh anolyte, applied in different concentrations

Concentration of the anolyte in %	Exposure time – min					
	2		5		10	
	10^6	10^8	10^6	10^8	10^6	10^8
100	0	0	0	0	0	0
50	0	0	0	0	0	0
25	0	Single	0	0	0	0
12,5	0	Single	0	0	0	0
Control (bacteria without anolyte)	many	Many	many	Many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.2 Growth (quantity of colonies) of *P. aeruginosa* 450 at concentrations of 10⁶ cells/ml and 10⁸ cells/ml after various time intervals of exposure to the anolyte stored 7 days, applied in different concentrations

Concentration of the anolyte in %	Exposure time - min					
	2		5		10	
	10 ⁶	10 ⁸	10 ⁶	10 ⁸	10 ⁶	10 ⁸
100	0	0	0	0	0	0
50	0	single	0	0	0	0
25	0	single	0	0	0	0
12,5	0	single	0	0	0	0
Control (bacteria without anolyte)	many	many	many	Many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.3 Growth (number of colonies) of *P. aeruginosa* 450 at concentrations of 10⁶ cells/ml and 10⁸ cells/ml after various intervals of exposure to the anolyte, stored 21 days, applied at various concentrations.

Concentration of the anolyte in%	Exposure time - min					
	2		5		10	
	10 ⁶	10 ⁸	10 ⁶	10 ⁸	10 ⁶	10 ⁸
100	0	1	0	single	0	1
50	0	12	0	30	0	single.
25	0	30	0	36	0	many
12,5	0	Many	0	Many	0	many
Control (bacteria without anolyte)	many	Many	many	Many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.4 Growth (number of colonies) of *P. aeruginosa* 450 at concentrations of 10⁶ cells/ml and 10⁸ cells/ml after different intervals of impact of Virkon S at various concentrations

Concentration of Virkon S in %	Exposure time - min					
	2		5		10	
	10 ⁶	10 ⁸	10 ⁶	10 ⁸	10 ⁶	10 ⁸
1	0	0	0	0	0	0
0,5	0	0	0	0	0	0
0,25	0	0	0	0	0	0
0,125	0	0	0	0	0	0
Control (bacteria without Virkon S)	many	many	many	Many	many	many
Control (Virkon S without bacteria)	0	0	0	0	0	0

Table.5 Growth (number of colonies) of *S. enterica* ATCC at concentrations of 106 cells/ml and 108 cells/ml after various intervals of impact of fresh anolyte applied in different concentrations

Concentration of the anolyte in %	Exposure time - min					
	2		5		10	
	10 ⁶	10 ⁸	10 ⁶	10 ⁸	10 ⁶	10 ⁸
100	0	0	0	0	0	0
50	0	0	0	0	0	0
25	0	0	0	0	0	0
12,5	0	0	0	0	0	0
Control (bacteria without anolyte)	many	many	many	many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.6 Growth (number of colonies) of *S. enterica* ATCC at concentrations of 106 cells/ml and 108 cells/ml after various intervals of exposure to the anolyte stored 7 days and administered at various concentrations.

Concentration of the anolyte in %	Exposure time - min					
	2		5		10	
	10 ⁶	10 ⁸	10 ⁶	10 ⁸	10 ⁶	10 ⁸
100	0	0	0	0	0	0
50	0	0	0	0	0	0
25	0	0	0	0	0	0
12,5	0	0	0	0	0	0
Control (bacteria without anolyte)	Many	many	many	Many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.7 Growth (number of colonies) of *S. enterica* ATCC at concentrations of 106 cells/ml and 108 cells/ml after various intervals of exposure to the anolyte, stored 21 days, applied at various concentrations

Concentration of the anolyte in %	Exposure time - min					
	2		5		10	
	10 ⁶	10 ⁸	10 ⁶	10 ⁸	10 ⁶	10 ⁸
100	0	0	0	0	0	0
50	0	0	0	0	0	0
25	0	0	0	0	0	0
12,5	0	0	0	0	0	0
Control (bacteria without anolyte)	many	many	many	many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.8 Growth (number of colonies) of *P. aeruginosa* 450 and of *S. enterica* ATCC at concentrations of 10⁶ cells/ml after various intervals of exposure to the anolyte, stored 28 days and applied at different concentrations

Concentration of the anolyte in %	Exposure time - min					
	2		5		10	
	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>
100	0	0	0	0	0	0
50	0	0	0	0	0	0
25	0	0	0	0	0	0
12,5	0	0	0	0	0	0
Control (bacteria without anolyte)	many	Many	many	many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.9 Growth (number of colonies) of *S. enterica* ATCC at concentrations of 10⁶ cells/ml and 10⁸ cells/ml after different intervals of impact of Virkon S, applied at various concentrations

Concentration of the Virkon S in %	Exposure time - min					
	2		5		10	
	10 ⁶	10 ⁸	10 ⁶	10 ⁸	10 ⁶	10 ⁸
1	0	0	0	0	0	0
0,5	0	0	0	0	0	0
0,25	0	0	0	0	0	0
0,125	0	0	0	0	0	0
Control (bacteria without Virkon S)	many	many	many	Many	many	many
Control (Virkon S without bacteria)	0	0	0	0	0	0

Fig.1 Growth of *P. aeruginosa* 450 after impact of the anolyte stored 7 days, applied in various concentrations (100%, 50%, 25% and 12.5%) with a duration of 2 min (left), 5 min (in the middle) and 10 min (right).



Fig.2 Growth of *P. aeruginosa* 450 after impact of anolyte stored 21 days after its preparation, applied in different concentrations (100%, 50%, 25% and 12.5%) with a duration of 2 min (left), 5 min (in the middle) and 10 min (right) on suspensions of concentration of 10⁶ cells/ml (above) and 10⁸ cells/ml (below).



Fig.3 Growth of *P. aeruginosa* 450 after impact of Virkon S in various concentrations (1%, 0.50%, 0.25% and 0.125%) with duration of 2 min (left), 5 min (in the middle) and 10 min (right).



Fig.4 Growth of *S. enterica* at concentration of 10⁸ cells/ml after impact of anolyte stored 21 days after its preparation, applied in different concentrations (100%, 50%, 25% and 12,5%) with a duration of 2 min (left), 5 min (in the middle) and 10 min (right). Colonies are visible only in the field of control with bacteria, without anolyte (below sectors)



Comparatively less sensitivity to the long-term stored at room temperature anolyte, as well as when the tested strains of *Pseudomonas aeruginosa* are in suspensions with a high density (10⁸ cells/ml), probably is due to the presence of additional protective polysaccharide coat of the cells - glycocalyx.

After 7-day of storage the effect of anolyte is completely analogous to the freshly prepared one and it can be used with the same success for fast and safe disinfection of materials containing high concentrations (up to 10⁸ cells/ml) even of the most resistant to chemical effects Gram-negative bacteria in an aqueous medium such as *P. aeruginosa* and *S. enterica*. After this period of storage the effect of the anolyte with respect to these microorganisms is retained even up to 4 weeks after its preparation for the salmonellas, and for *P. aeruginosa* it is effective for materials, which contain not more than 10⁶ cells/ml in an aqueous medium. At higher concentrations of bacteria of this type the quantity of viable cells is significantly reduced within 10 minutes exposure time, but some of them remain viable.

These data categorically show that anolyte is sure disinfectant with rapid bactericidal

effect, which fully retains its high bactericidal activity within period of time for three weeks. After this period it can also be used successfully for the rapid (within 2-5 minutes) disinfection of materials containing Gram-negative bacteria in an aqueous medium at concentration up to 10⁶ cells/ml. For materials with a higher concentration of bacterial cells it is necessary a more prolonged exposure time of undiluted anolyte, and most sure is to be used a fresh anolyte or the one that has been stored no longer than seven days.

The active ingredient of anolyte is hypochlorous acid (HClO), which normally occurs in eukaryotic organisms and is involved in the reactions of the immune response (killing pathogens during phagocytosis). The surface activity of hypochlorous acid (in sufficient concentrations) is based on the destruction of the cell membrane of the microbial cells (Nixall, 2015). Obviously, this acid is kept in sufficient concentrations in the anolyte for the researched by us 4-week period.

The high efficiency of the anolyte with respect to some of the most resistant Gram-negative bacteria such as *P. aeruginosa* and *S. enterica* is an indicator of similar

performance with respect to the most of the others species of microorganisms of this group. However, further studies are needed to identify the spectrum of the effectiveness of the anolyte, as well as the timing of its bactericidal action to materials with different concentrations of microorganisms.

In conclusion, the anolyte is sure disinfectant with rapid bactericidal effect, that immediately after its preparation, as well as after storage for 1 week exhibits high bactericidal activity against pathogenic Gram-negative bacteria of the species which are distinguished by particularly high resistance to disinfectants such as *Pseudomonas aeruginosa* and *Salmonella enterica* at significant cell density (up to 10^8 cells/ml). The effect of the anolyte after 3 weeks of storage at room temperature is completely analogous to the freshly prepared one in respect of *S. enterica*, and for *P. aeruginosa* the effect is maintained in suspension with a cell density of up to 10^6 cells/ml. Anolyte after storage at room temperature for 4 weeks fully retains its high bactericidal activity against *S. enterica* with a high density of cells (up to 10^8 cells/ml). For *P. aeruginosa*, however, the results with the use of stored four weeks anolyte depend on the density of the tested suspensions.

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How to cite this article:

Teodora P. Popova, Toshka Petrova and Stoil Karadzhev. 2016. Investigation of the Action of the Anolyte after Different Storage Times on the Gram-negative Bacteria. *Int.J.Curr.Microbiol.App.Sci*. 5(9): 530-539. doi: <http://dx.doi.org/10.20546/ijcm.2016.509.059>