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Investigation of the Biocidal Effect of Electrochemically Activated Aqueous Sodium Chloride Solution on Gram-negative Pathogenic Bacteria

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A B S T R A C T

Studies were carried out to determine the sensitivity of pathogenic Gram-negative bacteria to electrochemically activated 3% aqueous sodium chloride solution (anolyte). From the resulting 100% anolyte in the experiments were used different final concentrations – 100%, 50%, 25% and 12,5%. As a control was used the disinfectant Virkon S, applied at final concentrations of 1%, 0,5%, 0,25% and 0,125%. Tested were *Escherichia coli* O45, *Salmonella enterica* ATCC and two strains of *Pseudomonas aeruginosa* (№318 and №450). It has been found that anolyte at a concentrations 25-100% fully inactivates suspensions of *E. coli* with a density of 10^6 cells/ml for 2 min. At decreasing the concentration below 25% the inactivation time was increased. Anolyte in all tested concentrations (12,5 to 100%) inactivated the suspensions of 10^6 cells/ml of both examined strains of *P. aeruginosa* (318 and 450) within 2 min. Also anolyte in concentrations from 12,5 to 100% completely inactivated suspensions of *S. enterica* with a densities of 10^6 cells/ml and 10^8 cells/ml for 2 min. The same effect had and Virkon S in all tested concentrations (from 1% to 0,125%). The results of studies show that anolyte exhibits very high and rapid bactericidal activity against Gram-negative bacteria. In suspensions with a concentration 10^6 cells/ml they die within 2 minutes in the presence of anolyte. The antibacterial activity of anolyte is completely analogous with that of the control preparation Virkon S. These results convincingly demonstrate that anolyte is promising antibacterial agent with very high activity.

Introduction

The increasing prevalence of strains of pathogenic bacteria, multi resistant to antibiotics, and fast developing resistance to commonly used disinfectants is a serious problem worldwide. Gram-negative bacteria are characterized by a higher resistance to chemical influences due to the protective properties of their outer lipoprotein envelope. Such bacteria with great ecological importance are *Escherichia coli*, *Salmonella enterica* and *Pseudomonas aeruginosa*. The latter is distinguished by a

particularly quick adaptation to chemical impacts and disinfectant solutions, and not only does not dies in them, but in many cases even develops therein (Popova, 2009; Alibert-Franco et al., 2009; Nallathamby et al., 2010). On the other hand the environmental pollution by chemicals used to combat microorganisms in all spheres of human activity, acquires an increasingly large scale. This leads to disruption of the ecological balance and biodiversity in the nature.

In recent years, there are reports that the electrochemically activated aqueous solutions of sodium chloride are promising as a wide spectrum and environmentally safe biocide. The scope of their action includes not only bacteria, but also spores, viruses (Atanasov et al., 2014; Ignatov et al., 2015) and fungi. Bacteria are affected successfully even when they are in the form of biofilm. There are evidences that the antimicrobial effect of these solutions is higher than that of the alcohols and it is commensurate with the effect of the sodium hydroxide. These can be used for disinfection of surfaces, floors, workspaces, tools, packaging, hands, etc., as their action is effective and without side effects (RADICAL WATERS, 2015).

According to Bahir (2009 a, b) anolyte is harmless to humans and the environment. Contained therein ozone, atomic oxygen, hydrogen peroxide, chlorine dioxide and others define it as extremely reliable biocide that excels the action of many of the previously known disinfectants. It destroys bacteria, viruses and spores. After using it quickly decays to environmentally friendly ingredients, unlike the widely used chlorine products.

As the scientific reports of testing the effect of anolyte on pathogenic microorganisms are scarce, in this work we set the goal to perform tests to determine its antimicrobial effect on some Gram-negative bacteria which are particularly important for infectious pathology and the environment.

Materials and Methods

Anolyte. Tested was the effect of anolyte obtained by electrochemical activation of 3% NaCl, dissolved in sterile distilled water and administered in four different final concentrations: 100%, 50%, 25% and 12,5%.

Control. The commercial disinfectant Virkon S was used as a control, administered in four final concentrations: 1%, 0,5%, 0,25% and 0,125%.

Microorganisms. In the studies were used suspensions with concentrations of 10^6 cells/ml of *Escherichia coli* O45 and of two strains of *Pseudomonas aeruginosa* (№318 and №450), all isolated from animals with chronic infections and showing multi resistance to antibiotics *in vitro.* Suspensions with concentrations of 10^6 and 10^8 cells/ml of *Salmonella enterica* ATCC also were 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 preparation of 24-hour cultures of the test strains, Eosin Methylene Blue agar (selective for gram-negative bacteria) for determining the effect of the tested solutions of anolyte and Virkon S for antimicrobial activity against *E. coli* and *S. enterica*, as well as Cetrimide agar to determine the sensitivity of *P. aeruginosa* to the tested solutions.

Experimental Staging

Investigation of the antimicrobial activity of the anolyte. Prepared were two fold dilutions of the anolyte in sterile distilled

water, as respectively were prepared concentrations of the anolyte 100%, 50%, 25% and 12.5% in an amounts of 9 ml. To each of them was added a suspension of *S. enterica* ATCC with a concentration 10^9 cells/ml in an amount of 1 ml, at which was achieved a final concentration 10^8 cells/ml. The same dilutions of the anolyte in concentrations of 100%, 50%, 25% and 12.5% in quantities of 9 ml were used and for the study the suspension of *S. enterica* ATCC with concentration 10^6 cells/ml, and for this purpose to each thereof was added by 1 ml of a suspension at a concentration of 10^7 cells/ml. To investigate the sensitivity of *E. coli* O45 and *P. aeruginosa* (№318 and №450), in separate rows of these dilutions of the anolyte (by 9 ml each) were added suspensions of the respective strains at concentrations of 10^7 cells/ml in an amount of 1 ml to each of them, whereby was achieved a final concentration of 10^6 cells/ml. The following controls were put - sterile distilled water (without anolyte) with the same content of each of the tested bacterial strains and 100% anolyte without microorganisms.

Investigation of antimicrobial activity of Virkon S, used as a control for comparison of the effect of the anolyte. Prepared were twofold dilutions in sterile distilled water at concentrations of 1%, 0,50%, 0,25% and 0,125% in an amounts of 9 ml. To each of them was added a suspension of *S. enterica* ATCC with 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 Virkon S with concentrations of 1%, 0,50%, 0,25% and 0,125% in quantities of 9 ml were used for the study and of suspensions of *S. enterica*, *E. coli* and *P. aeruginosa* (№318 and №450) with a density of 10^6 cells/ml, as for that purpose to each of them was added by 1 ml of suspension with concentrations of 10^7 cells/ml. Were placed and controls - sterile distilled water (without Virkon S) with the same content of each tested bacterial strain, as well as 1% Virkon S without microorganisms.

After various time intervals for impact of the anolyte and Virkon S (2 min, 5 min and 10 min) from each of the samples were made cultures on selective for gram-negative bacteria nutrient medium Eosin Methylene Blue agar, and for *P. aeruginosa* on Cetrimide agar, which were cultured at 37°C for 24 - 48 h under aerobic conditions. After the cultivation was read the growth of the used bacteria treated with the tested disinfectants, and of the set controls.

Results and Discussion

The results of the tests carried out to determine the sensitivity of *E. coli* to the anolyte, are presented in Table 1 and some of them – in Figure 1. Data show that the anolyte at a concentrations 25 - 100% inactivates *E. coli* for 2 min. At decreasing the concentration below 25% is achieved a strong reduction of the microorganisms, but with increased time of exposure - 5 to 10 min.

Analogous results were observed in tests for sensitivity to Virkon S at various concentrations, shown in Table 2.

The data in Table 3 show that the anolyte in all tested concentration (12,5 to 100%) inactivates the suspensions with a concentrations of 10^6 cells/ml of both tested strains of *P. aeruginosa* (№318 and №450) within 2 min. This can be seen and from Figure 2.

The antibacterial activity of the anolyte is completely analogous with that of the control preparation Virkon S, tested at

concentrations from 0,125 to 1%. The data can be seen from Table 4.

The results of research carried out to determine the sensitivity of *S. enterica* to the investigational chemical means are presented in Table 5 and some of them – in Figures 3 and 4. The data show that the anolyte at concentrations of 12,5 to 100% inactivates suspensions of *S. enterica* with densities 10^6 cells/ml and 10^8 cells/ml for 2 min. The same effect has and Virkon S in all tested concentrations (from 1% to 0,125%), as the results obtained in its application can be seen from Table 6.

From the figures it can be seen that there is growth only in the sectors of the cultures of the controls without anolyte and Virkon S.

From the presented results of the current research is seen that the anolyte in concentrations of 25 to 100% inactivates all tested Gram-negative bacteria after only two minutes of impact. *S. enterica* and both tested strains of *P. aeruginosa* (№318 and №450) die within two minutes even after administration of 12,5% anolyte but at this concentration a single *E. coli* cells remain viable even after 5 and 10 minutes.

The results of the studies show that the anolyte is a reliable agent for the destruction of Gram-negative bacteria, including highly resistant species such as *E. coli*, *S. enterica* and *P. aeruginosa.* Obviously the sanitary indicative species *E. coli* shows a little bit less sensitivity to this disinfectant and at contamination with *E. coli* it should be used at a concentration of at least 25%. Our results are to some extent in line with those of Gluhchev et al. (2015). However, they have established higher sensitivity of *E. coli* to the catholyte, rather than to the anolyte. Surprisingly, a higher sensitivity than *E. coli* to the anolyte in our studies showed both tested strains of *P. aeruginosa*, although this kind is distinguished with the highest resistance to chemical influences among the Gram-negative bacteria. The high efficiency of the anolyte to it in all concentrations tested is particularly favorable and promising from a practical viewpoint. *S. enterica*, which is also characterized by substantial resistance to chemical agents, exhibits a high sensitivity to the anolyte in all tested concentrations. Even the smallest tested concentration of 12,5% kills *S. enterica* not only when it is in a suspension with a density of 10^6 cells/ml, but in the hundred-fold more concentrated suspension with a cell density of 10^8 cells/ml.

Our results obtained when testing the antimicrobial activity of anolyte fully correspond to those in the study of the control preparation Virkon S, which was administered in conventional concentrations used in the practice. Its effect against the three examined bacterial species *E. coli*, *S. enterica* and *P. aeruginosa* is completely sure in all tested concentrations from 0,125 to 1%. Even in the suspension of *S. enterica* with a density of 10^8 cells/ml, all cells were killed within 2 minutes after administration of Virkon S with final concentrations of 0,125 to 1%. Virkon S is a versatile broad spectrum disinfectant. It is balanced, stabilized composition comprising peroxygen compounds, surfactant, organic acids and an inorganic buffer. According to the manufacturer, Virkon S kills a large number of pathogens, including many bacterial species, viruses and fungi in the absence of data to build resistance (DuPont, 2005, 2009). Therefore we chose this preparation as a control in our investigations of the antibacterial activity of the anolyte.

The results obtained in these studies demonstrate the high efficiency of the anolyte as a strong disinfectant with very fast and sure effect with respect to some of the most important pathogenic Gram-negative bacteria.

Table.1 Study of the Action of the Anolyte on *E. coli* in a Suspension with a Concentration 10^6 Cells/ml

Concentration of the anolyte in %	Growth of *E. coli* (number of colonies) after various times of exposure to the anolyte		
	2 min	5 min	10 min
100	0	0	0
50	0	0	0
25	0	0	0
12,5	40	5	6
Control without anolyte	many	many	many
Control (anolyte without *E. coli)*	0	0	0

Table.2 Growth (Number of Colonies) after Various Intervals of Action of Virkon S with Different Concentrations on a Suspension of *E. coli* with Density 10^6 Cells/ml

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

Table.3 Growth (Number of Colonies) after Various Intervals of Action of Anolyte, Applied in Different Final Concentrations on Suspensions of *P. aeruginosa* №318 and *P. aeruginosa* №450 with Density 10^6 Cells/ml

Concentration of the anolyte in %	**Exposure time – min**					
	2		**5**		**10**	
	№318	№450	№318	№450	№318	№450
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 without anolyte	many	many	many	many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.4 Growth (Number of Colonies) after Various Intervals of Action of Virkon S in different Final Concentrations on Suspensions of *P. aeruginosa* №318 and *P. aeruginosa* №450 with Density 106 Cells/ml

Concentration of Virkon S in %	Exposure time - min					
	2		5		10	
	№318	№450	№318	№450	№318	№450
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 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* with Densities 10^6 Cells/ml and 10^8 Cells/ml after Different Intervals of Action of Anolyte 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 without anolyte	many	many	many	many	many	many
Control (anolyte without bacteria)	0	0	0	0	0	0

Table.6 Growth (Number of Colonies) after Various Intervals of Action of Virkon S in different Concentrations on Suspensions of *S. enterica* with Densities 10^6 cells/ml and 10^8 Cells/ml

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 without Virkon S	many	many	many	many	many	many
Control (Virkon S without bacteria)	0	0	0	0	0	0

Figure.1. Growth of *E. coli* (a Suspension with a Concentration of 10^6 Cells/ml) on Eosin Methylene Blue Agar after Administration of Anolyte at Various Concentrations: 1 – 100%; 2 – 50%; 3 – 25%; 4 –12,5; 5 - 6,25%; K – Control (Anolyte without *E. coli*)

Figure.2 Cultures of *P. aeruginosa* №318 (above) and №450 (below) on Cetrimide Agar after the Action of Anolyte with Different Concentrations (100%, 50%, 25% and 12,5) with Duration 2 min, 5 min and 10 min. Growth is Seen only in the Sectors of the Controls without Anolyte

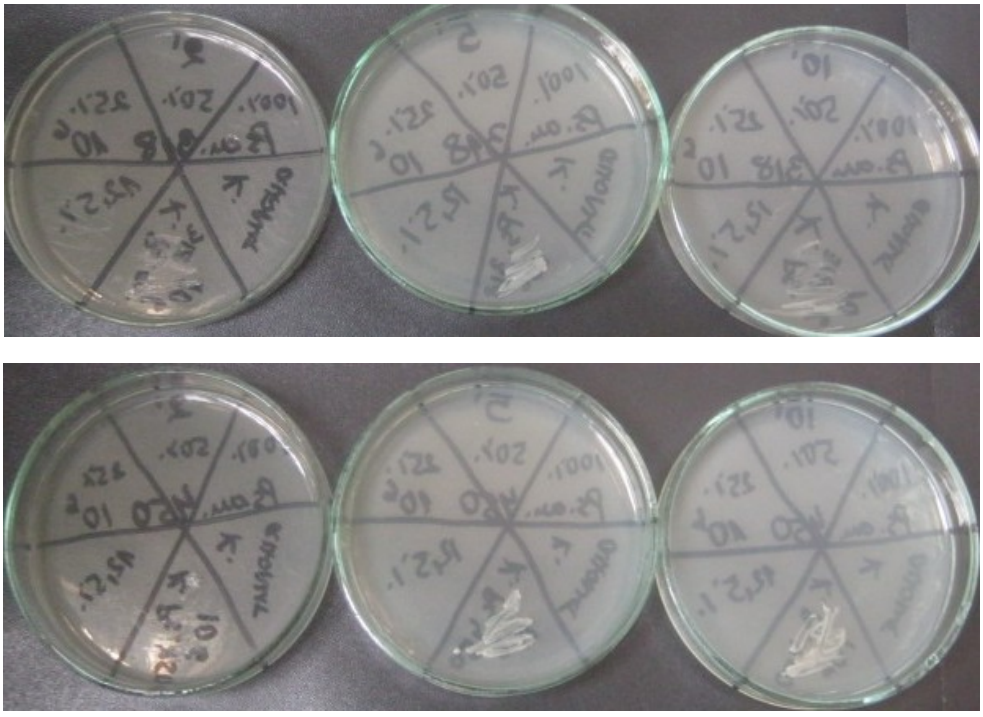
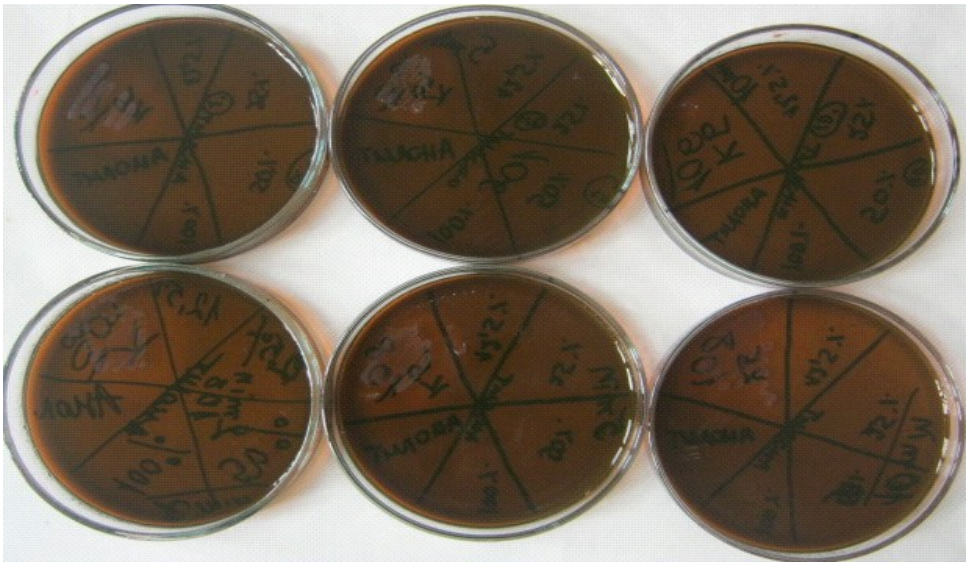


Figure.3 Cultures of *S. enterica* (above – a Suspension with Density 106 cells/ml, below – a Suspension with Density 108 Cells/ml) on Eosin Methylene Blue Agar after Administration of the Anolyte at Various Concentrations (100%, 50%, 25% and 12,5) with Duration 2 min, 5 min and 10 min. Growth is Seen only in the Sectors of the Controls without Anolyte



It can be successfully applied at concentrations of 25-100%, destroying Gram-negative bacteria in suspensions with a density of 10^6 cells/ml very quickly for no more than two minutes. When using a lower concentration, 10 minutes are insufficient for the complete destruction of all cells of *E. coli* when the treated suspension has a concentration of 10^6 cells/ml. Its effect against the three used bacterial species *E. coli*, *S. enterica and P. aeruginosa* is completely sure in all tested concentrations of 0,125 to 1%. Also upon administration of Virkon S with final concentrations of 0,125 to 1% all bacterial cells are killed within 2 minutes even in the suspension of *S. enterica* with a density of 10^8 cells/ml.

In conclusion, anolyte, administered at a concentrations of 100%, 50% 25% and 12,5%, kills in a short time (2 min) suspensions of *S. enterica* and *P. aeruginosa* with a density of 10^6 cells/ml and suspensions of *S. enterica* with concentrations of 10^8 cells/ml. *E. coli* in a suspension with a density of 10^6 cells/ml dies in 2 minutes under the effect of the anolyte at a concentrations of 25%, 50% and 100%, but after administration of a 12,5% anolyte single cells remain viable even after 5 and 10 minutes.

The disinfectant Virkon S at concentrations from 0,125 to 1% kills suspensions of *S. enterica* and *P. aeruginosa* with a density of 10^6 cells/ml, as well as such of *S. enterica* with concentrations of 10^8 cells/ml after 2 minutes, and suspension of *E. coli* with a density of 10^6 cells/ml - for 5 minutes.

Anolyte exhibits sure and quick effect against different Gram-negative microorganisms, which are particularly important for the infectious pathology and the environment. Its effect is similar to that of one of the most reliable disinfectants Virkon S, but administered at higher concentrations.

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