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EFFECTS OF ANOLYTE WATER ON THE CLASSICAL SWINE FEVER VIRUS: EFFECTS OF OXIDANTS AND pH

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author SK designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EI and II managed the analyses of the study. Author AA managed the literature searches. All authors read and approved the final manuscript.

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Review Article

ABSTRACT

The aim of the analyses was to examine the interaction between anolyte and its compounds and classical swine fever virus. The results on the anti viral action of acidic electrochemically activated water solution anolyte are prersented. It was produced in the anode chamber of the electrolitic cell (electrolyzer). The main stage of the electrochemical treatment of water occurs in the electrolysis' cell, consisting of cathode and anode separated by a special semipermeable membrane (diaphragm). The diaphragm separates water to alkaline fraction (catholyte) and acidic one (anolyte). The classical swine fever (CSF) virus was treated with anolyte under laboratory conditions in the cell culture and suspensions. The assay of determination of Colony Forming Units (CFU) was used. The results were achieved with immunoperoxidase technique after inoculating them with cell cultures. There presence of viral antigens was measured. It was found that anolyte did not affect the growth of the cell culture PK-15. The viral growth during the infection of a cell monolayer with a cell culture virus was affected in the greatest degree by the anolyte in 1:1 dilution. The results were less in other dilutions.

Keywords: Acidic medium; electrolysis; biocidal.

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1. INTRODUCTION

The electrochemically activated water is produced in electrolyzer, consisting of a cathode and an anode chambers separated by a special semipermeable membrane. The electrolysis separates water of two factions. The alkaline fraction is catholyte and acidic fraction – anolyte. When the electric current is passing through the water, the flow of electrons from the cathode as well as the removal of electrons from the water at the anode is accompanied by series of oxidation reduction reactions on the surface of the electrodes. As a result, new chemical compounds are being formed such as H^*, OH^-, O_2, H_2, H_2O_2 and others, and different intermolecular interactions occurp which are presented below.

During the production of electrochemically activated water, gaseous hydrogen (H_2) is generated at the cathode. Gaseous oxigen (O_2) is produced at the anode. Water also contains a certain amount of hydronium ions (H_3O^+) depolarizing at the cathode with formation of the atomic hydrogen [1,2]:

$$H_3O^+ + e^- \rightarrow H^* + H_2O, \quad (1)$$

In an alkaline environment a disruption of H_2O molecules, accompanied by formation of atomic hydrogen and hydroxide ion (OH^-) occurs [3,4]:

$$H_2O + e^- \rightarrow H^* + OH^-, \quad (2)$$

The reactive hydrogen atoms were adsorbed on the surface of the cathode, and molecular hydrogen H_2, released in the gaseous form after recombination:

$$H^* + H \rightarrow H_2, \quad (3)$$

The proofs for atomic (nascent) hydrogen were given in 2019 in catholyte water by Mehandjiev, Vassileva et al. with reaction with potassium permanganate (KMnO4) [5].

In an alkaline environment, the OH- ions moving under the electrophoresis from the cathode to the anode. In anolyte water was formed oxygen (O_2):

$$4OH^- \rightarrow O_2 + 2H_2O + 4e^- \quad (4)$$

In an acidic environment, this process is accompanied by the destruction of H_2O molecules:

$$2H_2O - 4e^- \rightarrow O_2 + 4H^+ \quad (5)$$

The normal redox potentials of these reactions compiles +1,23 V and +0,403 V, respectively, but the process takes place in certain conditions of electric overload.

Similar reactions can take place at the anode. Their mechanism follows the next scheme [6].

$$OH^- \rightarrow O^* + 2e^- + H^+ \quad (6)$$

$$O^* + O^* \rightarrow O_2 \quad (7)$$

$$OH^- \rightarrow OH^* + e^- \quad (8)$$

$$OH^* \rightarrow O^* + e^- + H^+ \quad (9)$$

$$2OH^* \rightarrow H\text{-}O\text{-}O\text{-}H \rightarrow (H_2O_2) \quad (10)$$

In bulk oxidative processes products of electrolysis of water – oxygen (O_2), hydrogen peroxide (H_2O) and hydrochlorine acid (HClO) has a play a special role. During the electrolysis, an extremely reactive compound H_2O_2 is formed [7,8]. Its formation occurs due to the hydroxyl radicals (OH*) which are products of the discharge of hydroxyl ions (OH^-) at the anode:

$$2OH^- \rightarrow 2OH^* \rightarrow H_2O_2 + 2e^- \quad (11)$$

where OH^* is the hydroxyl radical.

The chlorine-anion is transformed to Cl_2:

$$2Cl^- \rightarrow Cl_2 + 2e^- \quad (12)$$

Gaseous Cl_2 forms highly active oxidants: Cl_2O; ClO_2; ClO^-; HClO; Cl^*; HO_2^*. The parameters of pH, ORP and the electrical conductivity of the anolyte/catholyte depend on different factors including the ratio of water volumes in the two electric chambers, the material of electrodes, NaCl concentration, the temperature, electric voltage and processing time.

As a result of the anode (anolyte) treatment the water obtains acidic reaction. The results with electrical conductivity are:

The anolyte has antibacterial, antiviral, antifungal, anti-allergic, anti-inflammatory, antiedematous and antipruritic effect. For antibacterial effects there are proofs for Gram-neagtive bacteria [9], *Staphylococus aereus* [10], *Pseudomonas aeruginosa* [11,12], *E. coli* [12,13]. For the fungi there are evidences for effects on *Candida albicans* [14,15]. Other research show effects on different pathogens [16,17].

The biocide elements in the anolyte are not toxic to somatic cells, as represented by oxidants, such as those ones produced by the cells of higher organisms.

Classical swine fever (CSF) is caused by a virus of the genus *Pestivirus* of the family *Flaviviridae*. CSF virus

retains its virulence for a long period of time in frozen meat and organs – from a few months up to one year; in salted meat – up to three years; in dried body fluids and excreta – from 7 to 20 days. In rotting organs it dies for a few days and in urine and faeces – for approx. 1–2 days. In liquid fertilizer it can withstand 2 weeks at 20° C, and over 6 weeks at 4° C. Its thermal resistance may vary depending on the strain type, but the inactivation is dependent mostly on the medium containing the virus [18,19].

Although the CSF virus loses its infectivity in cell cultures at 60 ^{0}C for 10 min, it is able to withstand at least 30 min at t = 68 ^{0}C in defibrinated blood. It is relatively stable at pH = 5–10, and the dynamic of the inactivating process below pH = 5 depends on the temperature.

It is accepted that 2 % solution of sodium hydroxide (NaOH) is more suitable for the disinfection of spaces contaminated with CSF virus [20].

In 2014 Bulgarian team (Karadzhov, Ivanova et al.) showed high effect of anolyte against CSF virus, similar to those of a chemical antiviral compound, shown in 2016 by Korean team (Cha, Chun-Nam et al.) [21,22].

The aim of the present study was to show the mechanism for antiviral treatment on CSF virus with water anolyte.

2. MATERIALS AND METHODS

2.1 Electrolyzer

To obtain catholyte and anolyte, water electrolysis was performed. The functional scheme of the electrolyzer is presented in Fig. 1.

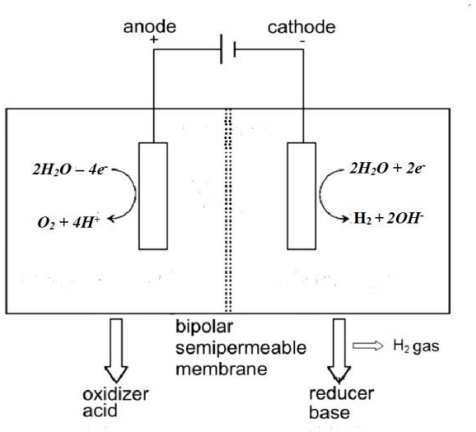

Fig. 1. Scheme of the water electrolyzer

The main stage of the electrochemical treatment of water occurs in the electrolysis' cell, consisting of cathode and anode separated by a special semipermeable membrane (diaphragm). The diaphragm separates water to alkaline fraction (catholyte) and acidic one (anolyte). When the electric current passes through the water, the flow of electrons from cathode as well as the removal of electrons from water at the anode is accompanied by series of redox reactions on the surface of the cathode and anode.

The experiments were conducted with the anolyte obtained by the electrolysis apparatus "Wasserionisierer Hybrid PWI 2100". The device was equipped with four titanium electrodes coated with platinum. Chemically pure sodium chloride (NaCl) with 0.3 % solution of in distilled water was used. The anolyte had pH = 3.2±0.03 ORP = +1070±10.7 mV and electrical conductivity 1471±14.7 µS/cm. The parameters of control sample water were pH = 7.4±0.07 and ORP = +255±2.6 mV and electrical conductivity 114.2±1.1 µS/cm.

2.2 Oxidation Reduction Potential (ORP)

The device – HANNA Instruments HI221 meter equipped with Sensorex sensors – was used to measure Oxidation Reduction Potential (ORP) in mV, and pH.

Range of HANNA Instruments HI221 meter is as follows:

pH - (2.00-16.00 ±0.01)
ORP (±699.9±0.01 – ±2000±0.1) mV.

2.3 Electric Conductivity

ADWA AD330 device
EC probe AD76309

Range
0.00±19.99 µS/cm; 0.00 to 9.99 ppm
20.0 to 199.9 µS/cm; 10.0 to 99.9 ppm
200 to 1999 µS/cm; 100 to 999 ppm
2.00 to 19.99 mS/cm; 1.00 to 9.99 ppt
20.0 to 199.9 mS/cm; 10.0 to 99.9 ppt
-9.9 t0 120.0 °C

Sensitivity
0.01; 0.1; 1 µS/cm; ppm
0.01; 0.1; 1 mS/cm; ppt
0.1 °C

Accuracy
EC±1%; TDS±1%; temperature ±0.5 °C

2.4 Prepartion of Classical Swine Fever (CSF) Virus

The studies of antiviral activity of the anolyte were performed at the National Reference Laboratory of Classical and African Swine Fever, section "Exotic and Especially Dangerous Infections" of the National Diagnostic and Research Veterinary Medical Institute (Sofia, Bulgaria). The interaction of the anolyte with the virus suspension was carried out at a t= 22^0C.

A cell culture of porcine origin sensitive to the CSF virus was used, that was the PK-15. Inoculation of the cell cultures was carried out with the standard cell culture test virus 2.3 (Bulgaria) with a cell titre $10^{7.25}$ $TCID_{50}$/ml and organ suspension. The suspension was from internal organs (spleen, kidney, lymph node) of wild boar originating from the last outbreak of CSF in Bulgaria in 2009. The titer of the established virus in the suspension was $10^{4,75}$ $TCID_{50}$ ml.

2.5 Cell Culrure for Research of CSF Virus

To estimate the virucidal activity, the cell culture was prepared for inoculation. It was treated with the following dilutions of the anolyte and sterile distilled water: 1:4 (20 %), 1:3 (25 %), 1:2 (33.33 %), 1:1 (50 %). The proportion of inocula with dilutions was 1:1. The virus suspension was 100 µl (containing $10^{6.25}$ $TCID_{50}$ of cell culture test virus and $10^{3,75}$ $TCID_{50}$ of organ suspensions). The appropriate anolyte concentration also was 100 µl. The time of action was conformed to the period necessary to detect any virus present on the cell culture. The exposure period for the infection of the cell monolayer was 1 hour.

Different dilutions were used to establish the virucidal activity of the anolyte on the CSF virus in the suspension. The inoculum was mixed with the concentrated anolyte. The anolyte-inoculum ratios were 15:1; 7:1; 3:1 and 1:1. The growth of the virus does not dependces on a cytopathic effect. Immunoperoxidase plates dyeing for research were used. The viral antigen was detected after binding to a specific antibody labeled with peroxidase. The organs exude 1 cm^3 of tissue, which was homogenized in a mortar with 9 ml of the cell culture medium containing antibiotics. The aim was to obtain 10 % organ suspension. For improvement of the homogenization sterile sand was added. The study was made at room temperature. The centrifugation process was for 15 min at 2500 g. The supernatant was used for the infection of the cells. In case of cytotoxic effect, the parallel dilutions of the homogenates were prepared in proportions 1:10 and 1:100. From the suspensions into multi well (24-well) plates were added 200 µl of the inoculum to cells with coverage of 50–80 %. Cell cultures were incubated at t = 37° C for 1 h in order to "capture" an eventual virus if presented, then they were rinsed once with PBS and fresh media were added. Alternatively, the plate was filled directly (cell suspension), since the preliminary studies had found that the anolyte did not induce a cytotoxic effect.

Cell cultures were incubated for 72–96 h at t = 37° C in a CO_2 incubator. The procedure with the positive and negative control samples was similar. The positive control sample was a reference strain of the virus of CSF. The immunoperoxidase technique was used. The fixation of the plates was carried out thermally for 3 h at t = 80 ^{0}C in a desiccator. In the processing was used a primary monoclonal antibody C 16 diluted in proportion 1:50, and secondary antibody RAMPO diluted in proportion 1:50. For the immunoperoxidase staining was used 3 % solution hydrogen peroxide (H_2O_2). AEC (dimethylformamide and 3-amino-9-ethylcarbazole) in acetate buffer was used also. The antibody-antigen complex was visualized by the peroxidase reaction with the substrate.

A polymerase chain reaction (PCR) was performed in real time scale. The cell culture and organ suspensions were examined for the presence of CSF viral genome by the polymerase chain reaction in real time. The research (real-time RT-PCR, one step, Taqman), one-step according to Protocol of the Reference Laboratory for CSF of EU. For RNA extraction was used the test QIAamp Vital RNA Mini Kit, Qiagen Hilden (Germany). The initial volume of elution – 60 µl was used and for the biological material it was 140 µl.

For amplification of PCR the test Qiagen OneStep RT-PCR Kit in a total volume of 25 µl, and template volume of 5 µl was used. Primers A 11 and A14, and probe TaqMan Probe–FAM–Tamra were included in the reaction.

PCR experiments were carried out with a thermo cycler machine "Applied Biosystems 7300 Real Time PCR System" with the temperature control for reverse transcription at t = 50 ^{0}C – 30:00 min, inactivation of reverse transcriptase and activation of Taq at t = 95° C – 15:00 min, denaturation at t = 95° C – 00:10 min, extension at t = 60° C – 00:30 min for 40 cycles.

The second study on the antimicrobial activity of anolyte/catholyte was performed at the Institute of Molecular Biology of the Bulgarian Academy of Sciences (BAS). The two solutions were prepared using the Activator-I, developed at the Institute of Information and Communication Technologies at BAS. For this, drinking water without additional

quantity of NaCl was used. This led to pH = 3,0 and ORP = +480 mV for the anolyte, and pH = 9,8 and ORP = -180 mV for the catholyte.

Bacterial strain used in these experiments was *E. coli* DH5α with genotype: *fhuA2 lac(del)U169 phoA glnV44 Φ80′ lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.*

The assay of determination of Colony Forming Units (CFU) was used to assess cellular viability. The conditions for the bacterial cultures growth were as follow. The bacterial cells were cultivated on the LB-medium (pH = 7,5) with 1 % bactotryptone; 0,5 % yeast extract; 1,0 % NaCl at t = 37° C. After overnight cultivation of bacteria 100 µl samples of culture liquids were taken, centrifuged for 1 min at 10000 g and the pellet of bacterial cells was resuspended in 100 µl of the anolyte or the catholyte. Bacterial samples re-suspended in non-activated water were used as control samples. Different dilutions of cells were spread on LB-agar Petri plates. After the overnight incubation at t = 37° C the appeared bacterial colonies were counted. The viable cells were calculated as a percentage from the CFU. The CFU obtained from culture liguids treated with non-electrochemically activated water were accepted as 100 %.

The experiments were performed three times.

3. RESULTS AND DISCUSSION

3.1 Research into the Effects of Electro-activated Aqueous Sodium Chloride (Anolyte) on the Classical Swine Fever Virus

As shown in Fig. 2, the cytoplasm of cells infected by the virus was stained in the dark reddish brown color (positive reaction), whereas in the uninfected cells it was colorless. That indicates on the presence of viral antigen in the samples.

Table 1 summarizes the results of different experiments of the virucidal action of the anolyte on the cell culture suspension of the CSF virus upon infecting cell monolayer PK-15. As is shown in Table 1, upon treatment of the viral inoculum with the anolyte in a 1:1 dilution, there was no viral growth in the four infected wells of the plate, upon 1:2 dilution there was no growth in two of the wells, the other two were reported as positive. Upon treatment with the anolyte at dilutions 1:3 and 1:4, the result was identical: no growth in one of the inoculated wells of the plate, and poor growth – in the other three. The results obtained by infection of a cell monolayer and cell suspension were identical.

Table 2 summarizes the results of studies aimed at the evaluation of the virucidal effect of the anolyte on organ suspension containing CSF virus upon infecting a cell monolayer PK-15 with the virus. According to the data received, upon treatment of the viral inoculum (organ suspension) with the anolyte in all dilutions, there was no viral growth in the four infected wells of the plate.

3.2 Mechanism of Antiviral Interaction of Anolyte on CSF Virus

Evidently, the anolyte has a destructive influence on the envelope of the CSF virus, where the main antigens (proteins) are localized. Studies of the viral inocula used in the tests by means of polymerase chain reaction (PCR) in real time demonstrate the presence of a genome (RNA) in them, also after the treatment with the anolyte. Some shortening of the time is proved (the decreased number of amplification cycles), required for the formation of a fluorescent signal, respectively, a positive reaction for genome, closely correlated with the exposure under the treatment of the viral inocula. The longer the exposure of processing with the anolyte, the sooner the presence of the RNA virus in the PCR was detected. This may serve as an indirect indication that anolyte destroys the virus envelope, which, in its turn, facilitates the extraction of RNA and it is more rapid reading by the fluorescent signal [21]. We attributed essential significance to the fact that we determined the concentration limit (25 %) of the well demonstrating virucidal activity. In this aspect the further studies on reducing the time of action, and the conducting of experiments in the presence of biofilms which protect viruses would be promising.

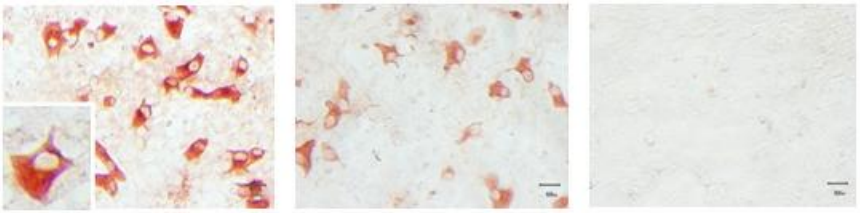

Fig. 2. The established presence of viral antigen in cell cultures (left) and a negative control sample (right)

Table 1. Virucidal action of the anolyte on cell culture suspensions of the CSF virus upon infecting cell monolayer PK-15

Contamination (µl) of CC with:	Dilutions of anolyte (100 µl)	Total volume of the inoculum (µl)	Concentration of anolyte in %	Number of wells:	Result: positive/ negative:
Virus 200	–	200	–	4	4/0
Virus 100	1:1	200	25	4	0/4
Virus 100	1:2	200	16,51	4	2/2
Virus 100	1:3	200	12.5	4	3/1
Virus 100	1:4	200	10	4	3/1

Table 2. The virucidal action of the anolyte on organ suspensions containing CSF virus upon infecting cell monolayer PK-15

Contamination (µl) of CC with:	Dilutions of anolyte (100 µl)	Total volume of the inoculum (µl)	Concentration of anolyte in %	Number of wells:	Result: positive/ negative:
Virus 200 µl	–	200	–	4	4/0
Virus 100 µl	1:1	200	50	4	0/4
Virus 50 µl	3:1	200	75	4	0/4
Virus 25 µl	7:1	200	87	4	0/4
Virus 12,5 µl	15:1	200	94	4	0/4

The chemical reactions in anolyte are:

$$2H_2O - 4e^- \rightarrow O_2 + 4H^+$$
$$2OH^* \rightarrow H\text{-}O\text{-}O\text{-}H \rightarrow (H_2O_2)$$

There is presence of hydrogen peroxide (H_2O_2) in anode chamber [23]. The oxygen atom in the compound is in a negative first oxidation state. It is a very strong oxidizer.

The valid formula for the concentration of hydrogen ions in the anolyte with pH=3.2 is

$C_{H+} = 10^{-3.2}$ mol/l

There are analysis performed for dependence of pH and concentration of H_2O_2. For pH 3.2 it was 5.2 mg/l [24].

In anode chamber are formed reactive oxygen spieces as Cl_2O; ClO_2; ClO^-; HClO; Cl^*; HO_2^*. They have oxdant effects with reactions [25].

$$Cl_2 + {}^- OH \rightarrow Cl^- + HOCl$$
$$HOCl \rightarrow H^+ + OCl^-$$

The research shows the reactions in anodic chamber with anti-bacterial and anti-viral effects for CSF.

4. CONCLUSION

The anolyte in a 1:1 dilution inactivates the CSF virus and prevents the viral growth in the cell cultures. The effect of higher dilutions of anolyte is lower. The virucidal effect of non diluted anolyte on organ suspension containing CSF virus also is proved. This effect is probably due to destructive influence of anolyte on the envelope protein of the CSF virus because of the content of O_2 and reactive oxygen spieces (HClO, HO_2' OH^*), as well as Cl_2 and HCl in it.

The team Ignatov, Gluhchev, Neshev, Mehandjiev created cluster model of waters Anolyte and Catholyte [26, 27].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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