



REVIEW

From: Professor Darinka Nedelcheva Ilieva, PhD, Scientific specialty "Epizootology, Infectious Diseases and Prevention of the Infectious Diseases in Animals", National Diagnostic Research Veterinary Medical Institute, Sofia, member of the Scientific Jury, according to Order № ZPS-370/07/05/2023 of the Rector University of Forestry, Sofia.

On a dissertation on the topic: "Antimicrobial Action and Biological Effects of Electrochemically Activated Aqueous Solutions", author Toshka Evgenieva Petrova, M.Sc., full-time doctoral student at the Department of "Infectious Pathology, Hygiene, Technology and Control of Foods of Animal Origin", enrolled by Order No. ZSD-21/24.01.2020 of the Rector of UF, for awarding the Educational and Scientific Degree "Doctor" (PhD), Field of higher education 6. Agricultural Sciences and Veterinary Medicine, Professional direction 6.4. Veterinary Medicine, Scientific specialty "Epizootology, Infectious Diseases and Prevention of the Infectious Diseases in Animals".

Scientific supervisor: Prof. Dr. Teodora Popova, Ph.D., D.Sc., FVM, UF-Sofia

1. Brief presentation of the doctoral student.

Toshka Evgenieva Petrova was born in 1980 in Yambol. She graduated from a mathematics high school in the city of Yambol with intensive training in English and passed the state exam (written and oral) in English. She graduated in 2007 with a "Master's" degree in veterinary medicine with excellent results in the state exams - FVM, UF-Sofia. In 2008, he joined the Department of "Infectious Pathology, Hygiene, Technology and Control of Foods of Animal Origin" as an Assistant professor in the disciplines "State Veterinary Medicine and Public Health", "Veterinary Medical Legislation and Forensic Veterinary Medicine". In 2009, due to structural changes in the Faculty of Veterinary Medicine, she was an Assistant professor in the Department of "Surgery, Anesthesiology, Radiology, State Veterinary Medicine and Public Health" in the same disciplines. Since 2015, again due to structural changes, she was an Assistant professor at the Department of Animal Husbandry Sciences. For the period 2008-2016, she held the academic position of Assistant professor on a basic employment contract. Since January 2017, she has been a part-time assistant on an employment contract at the Department of Animal Husbandry Sciences, as well as an organizer of educational activities in the department and a part-time Assistant professor until now. She has command of English /independent level-B1/B2/ and works with a computer. From the presented certificate ed. from FVM-177/21.04.2023 for passed exams from the doctoral minimum, it is clear that the doctoral student passed 3 exams in 2020: in the disciplines "General Epidemiology and Preventive Medicine", "Infectious Bacterial Diseases of Domestic Animals" and "Methodical Foundations of Scientific Research" she has performed with excellent and very good success. The doctoral student was deducted with the right of defense, according to Order No. ZSD-128/24.04.2023, effective from 14.03.2023. On 22.05.2023, Dr. Petrova submitted to the department a project of the dissertation in electronic and paper format and all materials required in connection with art. 32, paragraph 5 of the Regulations for the development of the academic staff in UF. A report from the scientific supervisor of the doctoral student, Prof. Dr. Teodora Popova, was also submitted to the department, with a positive assessment and opinion that the doctoral student is ready for the preliminary defense of the doctoral dissertation. By decision of the department - Protocol No. 191/08.06.2023 a procedure for the preliminary discussion of T. Petrova's dissertation and the presentation to the Extended Departmental Council (EDC) on 22.06.2023 was revealed, and Order ZPS No. 327/16.06.2023 of the Rector of UF was issued. At the EDC, a positive decision was taken to defend the dissertation of Dr. Petrova, the composition of the Scientific jury and the date /30.11.2023/ of its final meeting were proposed. With Protocol No. 192/22.06.2023 ed. from the Department of "Infectious Pathology, Hygiene, Technology and Control of Foods of Animal Origin", a procedure was opened and a move was made for the official defense of the dissertation for the acquisition of the SED "Doctor". The Scientific jury was appointed by Order No. ZPS-370/07.05.2023 of the Rector of UF.

2. Actuality of the developed problem.

The dissertation work developed by Toshka Evgenieva Petrova presents data on the antimicrobial action and biological effects of electrochemically activated aqueous solutions (EHAAS). When water is treated with an electric current, electro- or electrochemically activated water solutions (catholytes and anolytes) with changed kinetic and electrochemical properties are obtained. In the available literature, there are scientific reports on experiments with electrolyzed water solutions (EWS) that began at the beginning of the 20th century, initially in Japan, Russia, China, and at a later stage in other countries, including Bulgaria. The development and research with EWS in the last century was related to the military-industrial manufacture, and for this reason some

data was classified and published decades later, as well as at the beginning of the new century. Electrolyzed aqueous solutions are better studied in medicine for the treatment of people with emerging diseases of a different nature, including in the treatment of infections caused by microbial agents (bacterial, viral and mycotic). It is characteristic of EWS that they exhibit a germicidal and disinfecting effect when treating resistant microorganisms. At the European Union level, they have been evaluated and approved for use as biocides, which are harmless and ecologically clean for humans, animals and the environment. EWSs are widely used in various spheres of the economy, and therefore at the current stage the devices for the production of activated water, the so-called electrolyzers continue to be improved by commercial manufacturers. Their principle of action is the same and is based on the physico-chemical laws of water electrolysis. Electrolyzers have electrodes (cathode and anode), membrane and chambers. The biological effects of activated water are due to their unique physico-chemical properties that cannot be obtained in any other way. Anolyte has reduced electronic activity with properties of an oxidizer (oxidant) with acidic pH values and biocidal properties, acts as an antimicrobial agent and is used to treat inflamed wound surfaces. Catholyte has increased electronic activity, alkaline pH indicators and according to the research of various authors, has a beneficial effect on living organisms and plants. Electrochemical activation can replace bulky chemical productions and the use of traditional chemical reagents through the metastability of activated water, where it passes into an intermediate energy state. That is why activated aqueous solutions of oxidizers or reducers after a certain period of time are naturally neutralized to neutral water, and it is extremely important to determine the duration of their activity. The measured pH values and oxidation-reduction potential (ORP) in the catholyte and anolyte from the moment of water activation change differently. The catholyte retains its high alkalinity $\text{pH} > 9$ for about 1 week, but its oxidation-reduction potential rapidly changes and after the second day is practically reduced to zero. In contrast, with the anolyte, pH values and ORP change insignificantly (about 10%) up to 1 year. From the data and facts presented above, it follows that the electrochemical activation of aqueous solutions is a highly effective technology for purifying and turning water into a suitable disinfectant for a variety of technological purposes.

Scientific research related to the study of EVR in veterinary medical practice and their disinfection effect on isolated in clinical conditions pathogenic bacterial strains, for the therapy of infectious disease processes in animals, as well as their biological effects after oral intake by mammals and birds are still not well studied. In this aspect, I believe that the dissertation work developed by full-time doctoral student Toshka Evgenieva Petrova is innovative and original.

3. Structure and content of the presented dissertation work.

The dissertation work submitted me for review is written on 210 pages, includes 38 tables and 58 figures. The literary index contains 348 author titles, of which 34 in Cyrillic and 314 in Latin, arranged in alphabetical order.

The dissertation includes the necessary sections, according to generally accepted requirements and is structured according to the scheme: Title page – 1 page; Table of Contents and Used Abbreviations – 4 pages; Introduction – 2 pages; Literature review – 45 pages; Conclusion – 2 pages; Purpose and tasks - 1 page; Materials and methods – 18 pages; Results – 65 pages; Discussion – 25 pages; Conclusions – 2 pages; Recommendations for practice - 1 page; Scientific contributions - 2 pages; Publications in connection with the dissertation work and Acknowledgments - 2 pages; Dissertation summaries in Bulgarian and English - 4 pages; Literature index - 36 pages.

The Literature review presents historical data and facts about electrolyzed aqueous solutions. It examines the types of devices for the production of EWS and EHAAS, their basic structure and the processes for obtaining anolytes and catholytes through electrolyzers. Data from the world literature are presented, that EWSs are harmless and are a good alternative to many chemical agents, they can be successfully used for disinfection, prevention and therapy of infectious processes and diseases in living organisms, since microbes do not develop resistance to them. The PhD student has thoroughly studied the available scientific literature. The Literature review is properly structured and closely related to the doctoral thesis being developed.

In the section "Aim and tasks" the aim is clearly formulated: study of the antimicrobial action and biological effects of EWS. In connection with the goal, the doctoral student has completed the planned 5 scientific tasks, namely: the antimicrobial effect of EWS (anolytes and catholytes) was investigated in laboratory conditions *in vitro* against pathogenic microorganisms of different groups, and their activity was monitored after different periods of storage at room temperature and in the dark; the antimicrobial effect of EHAAS was investigated in the treatment of materials naturally saturated with bacteria obtained from the sludge of an urban sewage treatment plant; the antimicrobial effect of anolyte as a disinfectant and antiseptic agent on various surfaces and materials was investigated; the biological effect of catholyte applied per os was followed in experiments with ducklings; studied the effect of anolytes and catholytes for the therapy of patients *in vivo* in bacterial, mycotic and associated with various microbial agents skin infections, some of which are persistent and difficult to treat with conventional antibiotic and chemotherapeutic agents due to manifested microbial multiresistance.

4. Evaluation of materials and methods of research.

The experiments were carried out methodically correctly in the presence of corresponding controls. The "Materials and Methods" section details the field and reference microbial strains used, apparatus (types of electrolyzers), equipment, diagnostic tests, experimental animals, disinfectants and electrolyzed aqueous solutions (EWSs). In laboratory conditions, the doctoral student has tested 17 types of EWSs; 4-electroactivated (anolytes and catholytes) and 13-electrochemically activated: 5 types of catholytes and 8 types of anolytes, applied in 4 different concentrations, 100%, 50%, 25 % and 12.5%, respectively. The action of the different types of anolytes and catholytes has been studied "*in vitro*" by classical microbiological methods, by isolating and cultivating the individual bacterial species under suitable conditions on solid and liquid selective nutrient media, suitable for the respective bacterial species. The antimicrobial effect of different EWSs has been traced against test microorganisms, namely, 4 types of Gram (-) and Gram (+) pathogenic bacteria, known for their greater resistance to inactivation with disinfectants and a total of 7 different reference and field strains, isolated from patients of the University Clinic for Small Animals, FVM, UF-Sofia. The period of preservation of the antimicrobial effect of the studied anolytes and catholytes (after their storage in the dark at room temperature for 7, 21, 28 and 29 days) has been monitored. In the "*in vitro*" studies, the following physical parameters of EWS have been measured; pH, ORP (mV), T°C; McFarland standard, an optical method for obtaining working suspensions of pure bacterial cultures with a certain concentration, has been used in the "*in vitro*" experiments. A suspension method has been applied, and the quantification of microorganisms has been carried out according to the classical method in serial tenfold increasing dilutions. For the identification of the isolated microorganisms, microscopic, cultural and biochemical studies have been carried out. Their action on organic and inorganic materials has been investigated in comparison with the bactericidal and antiseptic action of 3 types of broad-spectrum disinfectants.

In the "*in vivo*" study, a total of 66 animals have been used, of which 20 were birds of the Peking white duck species. In them, weight development and growth have been tracked by periodic measurement of live weight and secondary selection parameters of ducks from the experimental and control groups; nutrient uptake has been determined by calculating feed expenditure, crude protein and energy. When ducks received catholyte as the only drinking water, blood samples have been taken twice on the 12th and 30th day from the experimental and control groups, in order to evaluate the dynamics and changes in the values of the serum biochemical indicators of the blood, being selected the following parameters: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and alkaline phosphatase (ALP) have been selected for the study of liver activity, total protein (TP) and albumin (ALB) for the state of proteins in the blood, for kidney function - creatinine (CREAT) and urea (UREA) and for the mineral and electrolyte profile - calcium (Ca), phosphorus (P) and potassium (K) for the birds from the experimental and control groups.

In the “*in vivo*” studies, 21 dogs of different breeds and ages have been treated with anolyte for paw antiseptics, as well as 25 animals with clinical bacterial infections (conjunctivitis and otitis); infected skin wounds and abscess; dermatomycoses - ringworm; microsporia; microsporia complicated by secondary bacterial infection with *P. fluorescens* and mixed skin infection by *C. albicans* and *E. coli*. The identification of the isolated pathogenic bacterial strains has been made by MALDI Biotyper Identification – Bruker. Biochemical tests have been performed with Polymicrotest - Staphy Test 24 and using an automated system for the identification of microorganisms and pathogenic fungi MD Phoenix™ M50. The sensitivity of the isolated bacteria to antimicrobial agents have been performed according to the classic agar-gel diffusion method of Bauer et al. (1966) and by MD Phoenix™ M50. In dogs with dermatomycoses, the diagnosis has been made by clinical signs, after microscopy of hair samples taken from the affected areas and cultural examination on selective nutrient media (Endo agar, Sabouraud agar).

I find, that the research materials and experimental animals are diverse and sufficient to collect a large amount of data. The experiments have been carried out thanks to the new innovative technologies of the companies, which with innovative thought and skills continue to improve the devices for obtaining EWSs.

5. Illustration and presentation of the results obtained.

The data obtained are presented in text, in tabular form and demonstrated with coloured figures. From the “Results” section, it can be concluded that the doctoral student successfully completed the assigned tasks, achieving satisfactory results. The data are formed statistically and show that EWSs and EHAASs - anolytes and catholytes have an antimicrobial effect “*in vitro*” against pathogenic bacteria. Suspensions from pure cultures of field strain of *E. coli* O45, 3 field strains of *P. aeruginosa* (No 318 and No 450) and *P. aeruginosa* isolated from a horse, two reference strains of *S. aureus* (ATCC 3350 and Cowan) and ref. strain of *S. enterica* (ATCC 1304) were used in the study. The strains were prepared prepared in sterile physiological solution according to McFarland standart to obtain a starting suspension with a concentration of 10^7 cells/ml and 10^9 cells/ml, in which a final working concentrations of 10^6 cells/ml and 10^8 cells/ml have been achieved and plating respective controls. Experiments with anolytes and catholytes “*in vitro*” have been carried out and their antimicrobial action has been determined against test microorganisms in comparison with the disinfectant *Virkon S*, which is a biocide and meets the criteria for widespread use, is safe for animal and human health, and is harmless to the environment. *Virkon S* solutions inactivate microorganisms through their oxidizing action and low pH values. *Virkon S* is recommended for use in anti-epizootic disinfection practice. The PhD student has also investigated the antimicrobial effect of EHAAS after 4 weeks of storage at room temperature and in the dark. In the dissertation work, the disinfection action of an anolyte containing Cl⁻ obtained by electrochemical activation of sterile distilled water with 3% NaCl has been studied in sludge from a city wastewater treatment plant. In the dissertation, the antimicrobial effect of anolyte has been studied in the treatment of various surfaces and materials of inorganic and organic matter. When studying the disinfection action of anolyte on laboratory surfaces (sinks, worktops and tables) after 10 min of exposure, the anolyte has showed an antimicrobial effect similar to the control disinfectant (chlorhexidine).

The dissertation student has examined skin surfaces and the antiseptics of dogs' paws after a walk treated with an acidic anolyte, whose inactivating action has been found to be similar to an alcohol-based disinfectant proposed for use in veterinary practice in connection with the Covid-19 pandemic with a broad bactericidal spectrum of action up to 99.9%. Encouraging results have been obtained in connection with the studies to determine the therapeutic effect of catholyte and anolyte “*in vivo*”, applied alone or in combination in patients with inflammatory reactions in the organism - bacterial conjunctivitis; polybacterial otitis; infected skin wounds, dermatomycoses and associated infections, clinically manifested on the basis of bacterial and mycotic agents.

At the current stage, the development of a number of diseases is associated with the destructive action of oxidants - free radicals (ROS - reactive oxygen species), whose targets are cells, their structures, DNA, etc., as a result of which various pathological processes develop. Alkaline EWSs are recommended for controlling the negative effects of oxidative stress and inflammation. It is known, that catholyte is physiologically functional water, it has antioxidant and immunostimulating properties. The dissertation presents data from experiments on the biostimulating effect of electroactivated alkaline water (catholite), taken *per os "ad libitum"* as the only water, to improve the productivity and growth indicators of ducks of the White Peking duck breed from 8 weeks of age during 1 month, with values of serum biochemical indicators of blood preserved within the physiological range for both groups (experimental and control).

The results presented in the dissertation are obtained from the study of appropriate materials by performing a wide range of methods. I believe, that they are the personal work of the doctoral student.

6. "Discussion" section.

The discussion shows the literary knowledge of the PhD student, her ability to analyze and summarize the information when compared with the results achieved by other researchers, which is a prerequisite for forming correct conclusions, recommendations and contributions.

7. Conclusions, recommendations and contributions

In connection with the defense of the dissertation "Antimicrobial action and biological effects of electrochemically activated aqueous solutions", Toshka Petrova has signed a declaration of authorship for the acquisition of SED "Doctor".

In the dissertation formulated 9 conclusions, 5 recommendations and 12 original contributions with a purposefulness on veterinary practice, which reflect the results obtained and should be accepted.

Notes regarding the dissertation.

The PhD student has largely complied with some notes and guidelines, that were given to her during the approbation of the dissertation. In Toshka Petrova's draft dissertation there were spelling and technical errors, that she had to correct before the public defense of the dissertation. There are no corrections on page 126 and on page 129, where two different figures are marked with №38. On page 130 and page 132 also two different figures are marked with №44.

I recommend, that PhD student prepare a correction sheet before the dissertation is deposited in NACID. The remarks and recommendations made do not concern essential parts of the dissertation, they aim to improve its shaping and vision.

Publications in connection with the dissertation

The dissertation student has a total of 4 collective scientific articles published in English during the period 2016-2022; 3 publications in the Journal "Tradition and Modernity in Veterinary Medicine", FVM, UF, Sofia and 1 publication in "Bulgarian Journal of Soil Science, Agrochemistry and Ecology", with which she has fulfilled the Minimum national requirements for the acquisition of the SED "Doctor", Field of Higher education 6. Agricultural Sciences and Veterinary Medicine, professional direction 6.4. Veterinary Medicine.

The doctoral student has participated in scientific forums and has presented-3 certificates from online International Scientific Conference, "Tradition and Modernity in Veterinary Medicine" during 2021 and 2022; certificate of participation in collective development of the 5th Eastern European Regional Veterinary Conference, Slovenia, 2022; certificate of collective participation in two posters of the XV Congress of Microbiologists in Bulgaria with International Participation, 2022; certificate of participation in collective development of an International Congress, Istanbul, 2022. There are no presented numerical indicators-citations, IF, etc.

Abstract

The Abstract is an integral part of the dissertation and reflects the sequence of the research conducted, the results achieved, the conclusions drawn, recommendations for veterinary practice and the original contributions achieved.

Conclusion:

The dissertation is up-to-date, contains original contributions aimed at veterinary medical science and practice. The set tasks have been completed, as a result of which the goal has been achieved. For the first time in the field of veterinary medicine in the country, in experiments conducted “*in vitro*” and “*in vivo*”, electro- or electrochemically activated aqueous solutions (anolytes and catholytes), obtained by a modern approach and innovative technology using electrolyzers, were studied. The author of this dissertation, M. Sc. Toshka Petrova has obtained encouraging results, that EWSs have a germicidal and disinfecting effect in the treatment of resistant test microorganisms Gram (+) and Gram (-) pathogenic bacteria, as well as on field and reference strains. For the first time, the doctoral student has investigated the therapeutic effect of catholyte and anolyte “*in vivo*”, administered alone or in combination in patients with inflammatory reactions in the body - bacterial conjunctivitis; polybacterial otitis; infected skin wounds, dermatomycoses and associated infections clinically manifested on the basis of bacterial and mycotic agents. The biostimulating effect on the organism of catholyte, taken *per os* “*ad libitum*”, given as the only drinking water for one month, has been proven, which improved productivity and growth indicators in the White Peking duck breed.

The doctoral student has fulfilled the Minimum national requirements, according to the Law on the Development of Academic Staff of the Republic of Bulgaria, Regulations for its implementation and Internal Rules for Development of the Academic Staff of the University of Forestry, Sofia. This gives me the reason to positively evaluate in my review the dissertation work on the topic: "**Antimicrobial Action and Biological Effects of Electrochemically Activated Aqueous Solutions**" and to recommend to the honorable Scientific Jury to award the M. Sc. Toshka Evgenieva Petrova, full-time doctoral student at the department "Infectious Pathology, Hygiene, Technology and Control of Food of Animal Origin", the Educational and Scientific Degree "Doctor" (PhD) in the Field of higher education 6. Agricultural Sciences and Veterinary Medicine, Professional direction 6.4. Veterinary Medicine, Scientific specialty "Epizootology, Infectious Diseases and Prevention of the Infectious Diseases in Animals".

21.09.2023
Sofia

REVIEWER:

(Prof. Dr. Darinka Ilieva, PhD)