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ORIGINAL ARTICLE

Study of disinfectants bactericidal properties against *Campylobacter* spp. at sub-zero temperatures

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To provide an effective complex of veterinary and sanitary measures in farms, regardless of the livestock management and weather conditions, it is necessary to expand the range of disinfectants with expressed bactericidal properties. The research aimed to improve the disinfectant formulation and develop a method for determining its bactericidal properties at sub-zero temperatures. Based on the results, a method for determining the disinfectant activity at sub-zero temperatures was developed, which includes determining the bactericidal properties of the disinfectant against bacterial cells by suspension method, that includes several stages: preparing of bacterial suspension, preparing of test objects, contaminating of test objects and disinfecting at the temperature range from -20 to -1 °C. The developed disinfectant showed effectiveness at low temperatures and consists of active substances (glutaraldehyde, formaldehyde) at a concentration of 0.5% and antifreeze at a concentration of 20.0%. Bactericidal properties of new disinfectant have shown activity against *Campylobacter fetus* subsp. *venerealis* (*Cfv*) and *Campylobacter fetus* subsp. *fetus* (*Cff*) cultures at a concentration of 0.5% at an exposure of 30 minutes at temperatures -20°C. The obtained data supplemented the methodological approaches regarding testing new antimicrobial drugs and expanding the arsenal of effective disinfectants for veterinary medicine.
Keywords: disinfectant, antifreeze, low temperatures, concentration, exposure, campylobacteria.

Introduction

The effective operation of any livestock complex is impossible without strict compliance with the proper veterinary management level and regulated zoo-sanitary standards for keeping farm animals (Hassink et al., 2017; Shkromada et al., 2019; Palii et al., 2019). Animal productivity and profitability of agricultural production directly depend on the farm's epizootic status (Buller et al., 2018). Several specific prophylaxis tools allow controlling the epizootic situation of many animal infectious diseases of bacterial and viral etiology (McVey & Shi, 2010; Lund et al., 2014; Chambers et al., 2016). Along with this, non-vaccine preventable diseases are becoming widespread today, preventing and controlling, which is possible only through the use of non-specific agents (Paliy et al., 2020a). The central role belongs to the complex of veterinary-sanitary measures, particularly to disinfection of livestock facilities (Bruins & Dyer, 1995; Zavgorodniy et al., 2013). Disinfection is a crucial process during obtaining high-quality livestock products (Paliy et al., 2018b). Disinfection's role increases significantly with the ubiquitous circulation of antibiotic-resistant pathogens (Randall et al., 2004; Wellington et al., 2013; Hadzevych et al., 2019). Environmental factors play a leading role in spreading pathogens (Paliy et al., 2019b; Palii et al., 2018c; 2020). For disinfection, a large number of disinfectants of both local and foreign manufacturing from different groups of chemical compounds (Hao et al., 2013; Paliy et al., 2016; 2019a), as well as physical means (Yoon et al., 2004; Zavgorodniy et al., 2019; Rodionova et al., 2020b). However, not all of them are effective in industry conditions due to the high biological load, temperature difference (Paliy, 2018; Iñiguez-

Moreno et al., 2018). The long-term disinfectant use for disinfection at bacteriostatic concentrations leads to increasing microbial resistance to their bactericidal activity (Hoff & Akin, 1986; Bridier et al., 2011).

A significant number of pathogenic microorganisms can withstand the effects of low temperatures for a long time (Ukhovskyi et al., 2019), and therefore disinfection and quarantine measures in the winter have their characteristics. The temperature of the environment and the treated surfaces is one of the essential factors that affect disinfection effectiveness. The disinfecting effect of some biocidal products is reduced at sub-zero temperatures. This is because, at low temperatures, the diffusion rate of chemical molecules between the working solution of the disinfectant and the decontaminated substrate decreases, and the interaction conditions between the microbial cell and the disinfectant deteriorate (Ivanov et al., 2018). The main etiological agents of gastroenteritis in humans and animals in the world are *Campylobacter* spp. There are many various sources of Campylobacter infection (Whiley et al., 2013). These bacteria colonize the intestine, and thus, infected animals and humans without clinical signs remain asymptomatic carriers (Silva et al., 2011). Consumption of contaminated meat, vegetables, and fruits and contact with infected animals are major risk factors for campylobacteriosis (Suzuki & Yamamoto, 2009; Szosland-Fałtyn et al., 2018; Ibrahim et al., 2019). An additional problem is an increase in the proportion of *Campylobacter* spp. isolates resistant to antibiotics, which raises serious concerns about antimicrobials' efficacy in treating this disease (Szczepanska et al., 2017; Osimani et al., 2017). Thus, high resistance (from 88.6% to 100%) of campylobacteria to macrolides, tetracycline, quinolones, and chloramphenicol was described. A lower prevalence of resistance to β-lactams (47%) and gentamicin (12.9%) was observed as well (Gharbi et al., 2018). It is proved that the *Campylobacter* spp. can spread rapidly and characterize by a significant survival time in the facilities (Cools et al., 2005). Biofilm formation, non-culture, and close interaction with other microorganisms may contribute to their survival outside of the host (Bronowski et al., 2014). Proper cleaning and disinfection of the environment is an essential measure in campylobacteriosis eradication (de Castro Burbarelli et al., 2017; Sibanda et al., 2018).

For more rational and reasonable use of disinfectants, as well as to prevent the formation of increased resistance of microorganisms to disinfectants, it is necessary to expand the range of drugs with necessary bactericidal properties and their practical application schemes (Boyce, 2016; Montagna et al., 2019; Paliy et al., 2020e).

Materials and methods

The study aimed to improve domestic disinfectants' formulation and develop a method for determining their bactericidal properties at sub-zero temperatures. Studies were conducted in the specialized laboratories of the National Research Center "Institute of Experimental and Clinical Veterinary Medicine" (Kharkiv).

Improvement of the disinfectant formulation and study of its bactericidal properties was carried out according to the guidelines "Methods for determining and evaluating the safety and quality of disinfectants, detergents, and disinfectants used in the production, storage, transportation and sale of animal products" (2010). The bactericidal activity of the disinfectant was determined by the suspension method using test strains *Campylobacter fetus* subsp. *venerealis* (*Cfv*) SV and *Campylobacter fetus* subsp. *fetus* (*Cff*) 3-1943 by growing from 3 to 12 days at temperature of 37.0±1.0 °C under microaerophilic conditions (in the presence of 15% CO_2) on tryptic soy agar (TSA), meat-liver peptone semi-liquid agar (0.15-0.2%) (MLPSLA) containing 0.02% cystine and meat-liver peptone agar (MLPA) containing 0.02% cystine and 0.5% yeast extract. The culture of test strains in concentration 2×10^9 CFU/ml was transferred into tubes with disinfectant or sterile isotonic solution (control). The contents of the vials were mixed thoroughly. After disinfectant application during 10, 30, 60, and 120 minutes, 10 cm^3 of suspension samples were centrifuged at 1500 rpm for 20-30 minutes. To stop the disinfectant's action, the pellet was washed twice with a sterile isotonic solution by centrifugation at 1500 rpm for 20-30 minutes. Control samples were also washed twice with a sterile isotonic solution in the same mode. Washed bacteria were plated on TSA, MLPSLA, and MLPA and incubated at a temperature of 37.0±0.5°C with the colony growth control every 24 hours. The absence or presence of colony growth in the experimental plates and colony growth in the control plates confirmed the presence or absence of bactericidal activity of the disinfectant, respectively. This was followed by studying the disinfectant properties using different surfaces: 1×2 cm batiste strips, 12×12×2 cm wooden bars, and cement tiles used repeatedly in the experiments.

A bacterial suspension and sterile sludge were applied to the experimental and control test objects separately at the rate of 0.5 ml of sludge per 1 ml of two billion suspensions. After that, the test objects were placed in the freezer and kept at different temperatures: -20, -15, -10, -5, and -1°C. The test objects were then evenly treated with a disinfectant solution. A sterile isotonic solution was applied to the control test objects in the same volume.

At the end of the disinfectant solution application (after 30 or 60 minutes), the test object was scraped and washed with the sterile isotonic solution into sterile Petri dishes. The Petri dishes contents were transferred to centrifuge tubes and centrifuged at 1500 rpm for 20-30 minutes. The sediment in the test tube was washed twice with a sterile isotonic solution to stop the disinfectant's action. After centrifugation, the pellet with an isotonic solution was plated on MLPA. After the same time, scraping and rinsing were performed on each control test object, washed twice by isotonic solution, centrifuged at 1500 rpm during 20-30 minutes, and tubes with media were inoculated by pellet followed by incubation at 37.0±0.5 °C. All experiments were performed in triplicate. The bactericidal action (mode of application) of the disinfectant was considered established if during the study the absence of culture growth proved on the experimental plates and the presence of bacteria growth on the control plates.

Results

Based on the scientific data analysis, today, the range of disinfectants from different chemical groups at low temperatures is quite limited and does not meet modern agricultural production requirements. The most common antifreeze added to disinfectants is propylene glycol and ethylene glycol (Arakelova & Troickaia, 2009; Tatarchuk, 2014), but their use is limited high toxicity (LaKind et al., 1999; Brooks & Wallace, 2002; Starek-Świechowicz & Starek, 2015).

Considering the results of previous research and the literature analysis, we have identified the most promising chemical components for domestic frost-resistant disinfectant design. For this purpose, a composition containing a mixture of aldehydes, antifreeze, and water was created (Table 1).

Table 1. The composition of the disinfectant

Application	Chemical component	Wt %
1	Glutaraldehyde	0.1-1.0
2	Formaldehyde	0.1-1.0
3	Sodium formate	10.0-30.0
4	H_2O	up to 100

Glutaraldehyde is a reliable disinfectant in public and veterinary medicine for infectious diseases of various etiologies. Aldehyde-containing compounds, along with chlorine-containing oxidants, are the most common disinfectants in Ukraine. The bactericidal activity of aldehydes is characterized by their ability to react with many substances of bacterial cells, especially proteins, reducing the synthesis of DNA and RNA. Simultaneously, there is a denaturation of proteins with the formation of new compounds with their characteristic properties; there is a decrease in α-aminobutyric acid transport (Sehmi et al., 2016; Migneault et al., 2018). Formaldehyde (formic acid aldehyde) is characterized by high antimicrobial activity. This agent inactivates microorganisms due to its high reactivity to amino acids and proteins, nucleoids, and nucleic acids (Szende & Tyihak, 2010; Chen et al., 2016). Sodium formate is used as an antifreeze and functional additive to solutions of chemical compounds that are intended for use at sub-zero temperatures (Achkeeva et al., 2014). According to the results, the tested composition does not change its physicochemical properties at temperatures from minus 20 to 0°C.

The next stage of our research was to determine the bactericidal activity of the disinfectant against Campylobacter. The local strains *Cff* and *Cfv* are certified and stored in the national collection of microorganisms at the NSC "IECVM". Test strains were incubated during 5-6 hours at a temperature of 37.0±0.5°C. Control of purity of cultures' growth was carried out by microscopy of slide smears after Gram and Stamp's staining. Pure 3-day cultures were washed from the agar surface with sterile saline. 5.0 ml of the investigated disinfectant solutions were inserted into sterile bacteriological tubes. Test cultures were added to the test tubes in the required amount so that the final concentration of microorganisms was 2×10^9 CFU/ml following the McFarland Standard. As a control, similar dilutions of the test culture in sterile saline were used. Commercial disinfectant "Virocid" was used for comparing bactericidal activity. Its working solution was prepared using propylene glycol. This preparation was used against campylobacter-contaminated wooden test objects at different temperatures (Table 2).

Table 2. Bactericidal properties of the disinfectant "Virocid" against campylobacteria at different temperatures

Disinfectant	Test culture	Application mode	Temperature, °C					Control
			-20	-15	-10	-5	-1	
Virocid (in distilled water)	*Cfv*	0.5% / 1 hour	+	+	+	+	+	+
	Cff		+	+	+	+	+	+
Virocid (10.0% solution of propylene glycol)	*Cfv*	0.5% / 1 hour	+	+	+	+	–	+
	Cff		+	+	+	+	–	+
Virocid (20.0% solution of propylene glycol)	*Cfv*	0.5% / 1 hour	+	+	–	–	–	+
	Cff		+	+	–	–	–	+
Virocid (40.0% solution of propylene glycol)	*Cfv*	0.5% / 1 hour	–	–	–	–	–	+
	Cff		–	–	–	–	–	+

Note: "-" no bacteria growth; "+" bacteria growth.

Table 3. Experimental compositions of the disinfectant

Application	Chemical components	wt%
1	Glutaraldehyde	0.1
	Formaldehyde	0.1
	Sodium formate	10.0
	Water	89.8
2	Glutaraldehyde	0.25
	Formaldehyde	0.25
	Sodium formate	20.0
	Water	79.5
3	Glutaraldehyde	0.5
	Formaldehyde	0.5
	Sodium formate	20.0
	Water	79.0
4	Glutaraldehyde	1.0
	Formaldehyde	1.0
	Sodium formate	30.0
	Water	68.0

Data from Table 2 showed that the disinfectant "Virocid" at a concentration of 0.5% and exposure of 1 hour does not become effective at sub-zero temperatures against test strains. With the addition of 10% or 20% propylene glycol, the bactericidal activity of disinfectant was registered only at minus 1 °C and minus 10 °C, respectively. Nevertheless, the bactericidal properties of "Virocid" at minus 15-20 °C exhibits by adding 40% propylene glycol at a concentration of 0.5% during 1-hour exposure.
Our studies' next step was to determine the developed frost-resistant compositions' bactericidal properties (Table 3). Previously, the experimental composition's bactericidal properties against campylobacteria *in vitro* were determined (Table 4 & 5).

Table 4. Bactericidal action of disinfectant on *Campylobacter fetus spp. venerealis LBV*

Medium	Application				Control
	№ 1	№ 2	№ 3	№ 4	
		10 minutes of exposure			
TSA	Tg	Tg	Tg	–	Tg
MLPA	Tg	Tg	Tg	–	Tg
MLPSLA	Tg	Tg	Tg	–	Tg
		30 minutes of exposure			
TSA	Tg	–	–	–	Tg
MLPA	Tg	–	–	–	Tg
MLPSLA	Tg	–	–	–	Tg
		60 minutes of exposure			
TSA	Tg	–	–	–	Tg
MLPA	Tg	–	–	–	Tg
MLPSLA	Tg	–	–	–	Tg
		120 minutes of exposure			
TSA	Tg	–	–	–	Tg
MLPA	Tg	–	–	–	Tg
MLPSLA	Tg	–	–	–	Tg

Note: "-" no bacteria growth; "Tg" typical growth.

Table 5. Bactericidal action of disinfectant on *Campylobacter fetus spp. fetus LBF*

Medium	Application				Control
	№ 1	№ 2	№ 3	№ 4	
		10 minutes of exposure			
TSA	Tg	Tg	Tg	–	Tg
MLPA	Tg	Tg	Tg	–	Tg
MLPSLA	Tg	Tg	Tg	–	Tg
		30 minutes of exposure			
TSA	Tg	–	–	–	Tg
MLPA	Tg	–	–	–	Tg
MLPSLA	Tg	–	–	–	Tg
		60 minutes of exposure			
TSA	Tg	–	–	–	Tg
MLPA	Tg	–	–	–	Tg
MLPSLA	Tg	–	–	–	Tg
		120 minutes of exposure			
TSA	Tg	–	–	–	Tg
MLPA	Tg	–	–	–	Tg
MLPSLA	Tg	–	–	–	Tg

Note: "-" no bacteria growth; "Tg" typical growth.

The first composition at exposures from 10 minutes to 120 minutes and compositions 2 and 3 at exposures of 10 minutes do not have bactericidal activity against test strains (Tables 4, 5). Composition 4 at exposure to 10-120 minutes and compositions 2 and 3 at the exposure of 30-120 minutes neutralized test strains. The next step in our research was to determine the developed tool's disinfectant properties for disinfection of test objects at sub-zero temperatures (Table 6).
We found that the disinfectant compositions 2, 3, 4 disinfect entirely test objects contaminated with test cultures during 30 minutes of incubation. We determined that the developed agent is effective at low temperatures with active substances and antifreeze at a rate of 0.5% and 20.0%, respectively. Previous studies have shown that this chemical composition demonstrates tuberculocidal activity against atypical mycobacteria of different groups (Paliy et al., 2020d). Based on the obtained results, a new frost-resistant disinfectant (Paliy et al., 2017a) was proposed, as well as a method for determining the activity of disinfectants at sub-zero temperatures (Paliy et al., 2017b).

Table 6. Disinfecting effect of the preparation against campylobacteria at sub-zero temperatures

Application	Time of exposure, min	Temperature, °C	Experiment	Control
№ 2	30	0	–	+
		-5	–	+
		-10	–	+
		-20	–	+
	60	0	–	+
		-5	–	+
		-10	–	+
		-20	–	+
№ 3	30	0	–	+
		-5	–	+
		-10	–	+
		-20	–	+
	60	0	–	+
		-5	–	+
		-10	–	+
		-20	–	+
№ 4	30	0	–	+
		-5	–	+
		-10	–	+
		-20	–	+
	60	0	–	+
		-5	–	+
		-10	–	+
		-20	–	+

Note: "-" no bacteria growth; "+" bacteria growth.

Discussion

Livestock production involves using some chemical compounds for both prevention and control of animal diseases and obtaining high-quality products (Stegniy et al., 2019; Paliy et al., 2020b). Recently, organic production has become popular, which has its characteristics and strict restrictions on the use of chemical derivatives (Rodionova et al., 2020a). As an alternative to chemical disinfection, the effectiveness of sunlight has been proven as a possible option for inactivation of resistant pathogenic microorganisms in water for irrigation of crops, even in the presence of organic matter. (Aguas et al., 2017). Physical means of decontamination have become widely used (Rodionova & Paliy, 2016; Zavgorodniy et al., 2019). Proper application of disinfectants in agricultural environments minimizes antibiotic resistance and does not reduce the susceptibility of disinfectants to the pathogens of infectious diseases (Maertens et al., 2019). Also, there are reports of the emergence of resistance to disinfectant microorganisms (Randall et al., 2007). The problem of biofilm formation in livestock facilities and their role in spreading disease among animals (Bronowski et al., 2014; Kukhtyn et al., 2019; Kolchyk et al., 2020) and even humans (Jamal et al., 2018) deserve special attention. Their increased resistance to the various detergents and disinfectants' action has been proven (Vickery et al., 2012). Disinfection plays an essential role in parasitic infestation control in animals (Paliy et al., 2020c).

Extensive introduction into disinfectant manufacturing requires implementing successive stages of their testing in both laboratories (Bondarchuk et al., 2019; Kovalenko et al., 2020) and production conditions (Paliy et al., 2015). The quality and effectiveness of disinfection are influenced by a number of factors, including the properties of the environment and the biological properties of the pathogen (Gerba, 2015; Romanko et al., 2016; Paliy et al., 2018a). Several disinfectant compositions have been proposed for the disinfection of livestock facilities in the winter. It was found that the addition to polyhexamethylene guanidine chloride antifreeze urea, the onset temperature of crystallization of 1.0% aqueous solution of the drug is minus 16 °C (Lysytsya et al., 2012). For disinfection of permafrost soil contaminated with the pathogen of tuberculosis, the action of several aldehyde compounds (Parasod, phosphorus, metaphor) and bleach was studied (Prokopeva, 2004). Reliable disinfection of glacier surfaces from feed storage in cage farming in Yakutia contaminated with *Sal. abortion equi* БН-12, *Str. aqui* Н-34 and *Bac. subtilis* ТНП-3 is achieved using peracetic acid, electrochemically activated "Anolyte" with a content of 0.1 mg/mL of active chlorine (Tarabukina et al., 2007). It was found that the preparation of 1.0% working solutions of the disinfectant "Ecocide C" on 40% propylene glycol prevents their freezing and changes in appearance, providing effective disinfection at sub-zero temperatures down to minus 18 °C for four days (Tatarchuk, 2014). For the use of the drug "Virocid" at sub-zero temperatures (minus 20 °C), the disinfectant solution is prepared on a 40% aqueous solution of propylene glycol or ethylene glycol (Arakelova & Troickaia, 2009). In winter, "Ecobiocide M" is used to disinfect vehicles (Butko et al., 2009).

Nowadays, the production of an effective disinfectant, "Cryodez," for disinfection of technological equipment at the food and processing industry enterprises and agro-industrial complex in cold conditions (Glazova, 2007). The studies of the dependence of the biocidal characteristics of "Dezefect", "Dezefect-Sanita," and "Dezefect-Forward" on the degree of freezing of samples of these preparations during their transportation and storage. It was found that cold and prolonged freezing and periodic sampling did not affect the biocidal activity of disinfectants (Chencov, 2002). The high disinfecting activity of DZPT-2 at low

temperatures during the disinfection of test objects contaminated with the African swine fever virus (Paliy et al., 2020a) has been proven. A promising area is the combination of physical and chemical disinfectants to inactivate objects with different physical properties (Rutala & Weber, 2013).

Conclusions

A method for determining the activity of disinfectants at sub-zero temperatures has been developed, which includes determining the bactericidal properties of the preparation against test bacteria by the suspension method, preparing bacterial suspension and test objects, test objects contaminating and disinfecting them at different sub-zero temperature conditions: -20, -15, -10, -5, and -1°C. A new disinfectant has been proposed that is effective at low temperatures as a composition of active substances (glutaraldehyde, formaldehyde) and antifreeze at a concentration of 0.5% and 20.0%, respectively.

Our studies of the bactericidal properties of the new disinfectant have shown its effectiveness against *Campylobacter fetus* subsp. *venerealis* and *Campylobacter fetus* subsp. *fetus* strains at a concentration of 0.5% and exposure time of 30 minutes at low temperatures down to minus 20 °C.

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