

In Vitro and *In Situ* Evaluation of the Efficacy and Efficiency of Products to Clean, Disinfection and Sterilization Process in Veterinary Environmental

Keywords: Antiviral; Cytotoxicity; Glutaraldehyde; Microbicide; Protease; Quaternary ammonium

Abstract

Pathogen microorganisms resistance presence in surgical instrument and places where process are performed are the most common right now, this kind of topics are very important and robust on humans, for this reason, the study of bacterial resistance is popular, when is compared with livestock areas, the issue is the same, but less study the trouble, this represent a big annual economic loss, as has been reported different livestock environments that the contamination levels can achieve 82% to periods of until 5 years, this indicates a need for research, for that reason the objective of this study was to evaluate the effectiveness and efficiency of products for the cleaning, disinfection and sterilization process in veterinary environments, the target in this research was to develop different methodologies, in order to carry out previous microbiological identifications, bioassays with four products: 1) monoenzymatic detergent (protease) and quaternary ammonium, 2) high-level disinfectant detergent based on potentiated glutaraldehyde (0,17%) and quaternary ammonium, 3) broad-area disinfectant detergent with quaternary ammonium grapefruit fragrance and 4) disinfectant detergent of furnishings based on quaternary ammonium. In addition, cytotoxic, extracellular and *in situ* activities were determined, from which it was possible to conclude that all evaluated products have bactericidal, fungicidal and virucidal activity, without affecting the cells or tissues where the microorganisms were found.

Introduction

The World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE) and international experts in public health, animal health and the environment bacterial resistance to antibiotics, along with rabies virus and animal influenza, as the three-major emerging global threats [1]. Since the 1970s, bacterial resistance has been followed by the appearance of new or occult diseases characterized by their zoonotic and pandemic character.

This phenomenon takes on greater dimensions in the hospital environment, due to the pressure exerted using antimicrobials, promoting the selection and accumulation of resistance genes among resident bacterial populations [2]. This situation has caused antibiotic resistance to be considered by some countries and international organisms mentioned above, a global challenge for two reasons: **a)** the ability of bacteria to transmit genetic information and **b)** the globalization that facilitates the possibility of spreading such resistance in short periods [3].

In hospitals, this resistance has a higher impact on morbidity and mortality rates, because the therapeutic options are limited and

increases costs for alternative therapy and hospital stay. Intra hospital infections are estimated to cost US \$ 28.4 to 33.8 billion in the United States and 70% of them are caused by resistant microorganisms at a time when infectious diseases are the leading cause of mortality in low-income countries, both in humans and animals [4].

At the end of the 20th century and until the present, the concern for microbial resistance at the veterinary level and the relationship between antibiotics for animal use and the impact on public health began in the developed countries. As a result, the OIE published in 2003 international standards on antimicrobial resistance, where it provides guidance for resistance monitoring and control program setting [5]. In 2008 a study of 31 accredited veterinary teaching hospitals in the United States by the American Veterinary Medical Association (AVMA), reports that 82% of these hospitals had out breaks of nosocomial infection during the previous 5 years [6].

In Germany, an increase in the prevalence of multidrug-resistant *Acinetobacter baumannii* has been identified in hospitalized animals in the veterinary clinic of the Justus-Liebig University of Giessen and other clinics in the city for a period of 9 years, where records from 2000 to 2008 of the Department of Microbiology of the Faculty of Veterinary Medicine of this University shows that of 137 hospitalized animals of different species, 56 acquired *Acinetobacter* spp. Infections, identified and differentiated by a combination of genotypic methods and susceptibility to antimicrobials [7].

Sources of infection associated with hospitals include medical staff, the patient's own flora and inanimate objects. Research conducted by the Veterinary College of Ontario, Canada (CVO) determine the prevalence of contamination of cell phones carried by CVO health science staff with methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), where 1.6% corresponded to MRSP and 0.8% to MRSA [7].

Agents that cause infectious diseases, or pathogens, are very varied. The most common are viruses, bacteria and protozoa, in addition to parasites that are not microorganisms, such as mites, helminths or worms. In veterinary area, the diagnosis of many of these infectious diseases is of great importance, since they allow the causal



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pathogen to be identified in time and ensure a correct treatment to guarantee the good health of the animal, which is the reason for the consultation [8,9].

Among the diseases most frequently diagnosed are: Feline Viral Leukemia, Canine Distemper, Canine Parvovirus, Ehrlichia, Anaplasma, Feline Immunodeficiency (FIV or VIF), Brucellosis, Equine Encephalitis, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Aphthovirus* and Aujeszky's Disease, intestinal parasites, ear infections, skin and urine infection. In the present, there is a variety of quick tests to identify the causative agent of the infection, which has made it possible to know the prevalence of many of these diseases, mainly due to the sterilization practices of the veterinary environment [10,11].

Infections from hospital origin are a major problem, which has been documented in the case of human medicine, however, has not been well documented in the case of veterinary medicine. Microorganisms associated with the hospital environment are generally resistant to a significant number of antibiotics and products used for cleaning and disinfection, causing significant costs associated with their treatment, as well as increasing hospitalization periods [12].

Therefore, it is essential to ensure a clean environment, providing the best attention possible to all patients, as well as protection to the staff that performs their tasks. The professionalization of production sectors and the scientific studies that prove their usefulness have led these sectors and administrations themselves to include programs of national scope, often mandatory, for the control and eradication of many infectious diseases. Biosecurity includes a set of measures focused at preventing the entry of pathogens into a farm and to curb or prevent their spread therein and to other neighbors, as well as to minimize the risk (if occur) for employees. Cleaning and disinfection practices are a major part of the programs and are becoming more important every day [12].

According to the foregoing, the present investigation aimed at evaluating the efficacy and efficiency of products for the cleaning, disinfection and high-level disinfection process in veterinary environments, whose main composition is quaternary ammoniums of the last generation, enzymes (protease) and Glutaraldehyde.

Materials and Methods

Study area

This study was carried out in 6 clinics and veterinary reference centers in Manizales city, which for confidentiality reasons, the information corresponding to the stuff or social name of the institutions that supported the research will not be divulged. In each place, the samples (swabs) were taken, for triplicate from areas such as external consultation, hospitalization, surgery, laboratory, nursery, hairdressing clinic and veterinary centers, following the notation and methodology of the Standard Methods for the Examination of Water and Wastewater [13].

Partial microorganism's identification

The prevalence of microorganisms was determined in the study places, which were responsible for the main clinical manifestations, specifically, in dogs, cats, among others. The identifications were made according to the notation and method of Madigan et al. [14].

In vitro bioassays

Cytotoxic activity: *In vitro* cytotoxic activity was evaluated with quaternary ammonium-based products, enzymes (Protease) and glutaraldehyde by *in vitro* bioassays at a concentration of 100,000 cells/ml in RPMI-1640 culture medium, each product **P1** (Bactericidal monoenzyme detergent, water soluble dose of 20 g), **P2** (High Level Disinfectant, Potentialized Glutaraldehyde at 0.17%, pH=6), **P3** (Disinfectant detergent, fifth generation quaternary ammonium 30,50 g/Kg, pH=5,0 in 750 ml) and **P4** (Surface disinfectant detergent, fifth generation quaternary ammonium, water soluble dose of 5 ml with grapefruit scent), which were prepared according to the manufacturer's data sheet. The effect was determined by spectrophotometry at 570 nm emission. As viability control, cultured cells were used in the absence of the products [12,15].

Antiviral activity: Evaluation of the antiviral activity was performed in chicken embryo culture tissue supplemented with SFB (10%), by determination of cellular viability by the Neutral Red method using plates of 96 wells [16,17]. In this assay 100 ml of culture medium containing formulations of the products to be evaluated were added. After 90 min of incubation at 37 °C, 10 ml/well of the viral suspensions (ATCC CCL-34 and ATCC VR-2209) were added. The cultures were incubated at 37 °C in 5% CO₂ atmosphere for 5 days [12,18].

Extracellular antiviral activity: To determine the direct inactivation of the ATCC CCL-34 and ATCC VR-2209 particles, equal volumes of each of the (independent) strains and double concentrations of the products to be evaluated were mixed and incubated for 30 min at 37 °C. At the end of this time, each mixture was added to chicken embryo cell cultures (50 ml / well) in 96-well plates, using 4 replicates per concentration. For 5 days of incubation at 37 °C in 5% CO₂ atmosphere, the antiviral activity was determined by the Neutral Red method (EC₅₀). The experiment was performed in triplicate [12,18].

In situ bioassay

Products were applied in each of the areas of veterinary centers and institutions environments. The formulations were made according to the technical data of the products; additionally the protocol of use was made according to the specifications and recommendations of the manufacturer. To evaluate the prevalence (presence/absence) of microorganisms, samples were collected (swab) before and after the application of the products, evaluating cleaning and disinfection by luminometry (System Sure Plus) [19,20].

The swabs were stored according to the notation and methodology of the Standard Methods for the Examination of Water and Wastewater, which were transported to the Applied Microbiology Laboratory of the Caldas University [13]. The samples were incubated on agar and nutrient broth, both formulated with the treatments of interest, for 24 hours at 37 °C. As a control of effectiveness, both agar and nutrient broth were formulated with Ampicillin-Sulbactam and Amphotericin B' and as viability control agar was formulated in the absence of the evaluated products [14,15]. After the incubation period, in the solid medium, the growth of microorganisms was evaluated by means of colonies [14]. In the liquid medium, microbial growth was assessed by turbidity measured by spectrophotometry according to the 0.5 scale of Mc Farland [21].

Results

Preliminary bacterial identification

Samples identification was obtained from critical areas (e.g. hospitalization, surgery, and outpatient clinic). Incubation was carried out at 35 °C for 72 hours on nutritive agar. At the end of this time, qualitative identification of these microorganisms was achieved by gram staining. Brown tones, characteristic of microorganisms of the genus *Bacillus* and *Staphylococcus*, were observed. Likewise, specific red/violet staining of gram positive bacteria was observed. Additionally, the position of the spore was recognized to identify the probable *Bacillus* species present in the analyzed samples (Figure 1). All the bacteria presented the spore in central position, of oval or cylindrical form and non-distended sporangium (Table 1).

The evaluated products presented antimicrobial potential, as well as the effectiveness controls (Ampicillin/Sulbactam and Amphotericin B). Higher inhibition was reported in contact with **P1**(Bactericidal monoenzyme detergent, water soluble dose of 20 g), **P2**(High-Level Disinfectant, Potentiated Glutaraldehyde at 0.17%, pH=6), **P3**(Disinfectant detergent, fifth generation quaternary ammonium 30,50 g/Kg, pH=5,0 in 750 ml) and **P4**(Surface disinfectant detergent, fifth generation quaternary ammonium, water soluble dose of 5 ml with grape fruit scent). The greater activity of the products contrasted with the controls is highlighted (Figure 2). Regarding the target, microbial growth is evidenced in the solution lacking the evaluated products and the effectiveness controls.

Bioassays

In vitro and in situ biological activity: The cultures of the product were performed in non-selective culture media (RPMI 1640, agar and nutrient broth) for *in vivo* and *in situ* analyzes. After the incubation time (24 hours and 5 days at 37 °C), bactericidal, fungicidal and antiviral activity of all the evaluated products was evidenced, being the mono-enzymatic product (Bactericidal monoenzyme detergent, water soluble dose of 20 g), the formulation that presented greater

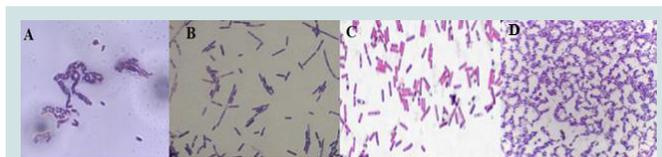


Figure 1: Gram staining identification of *Bacillus*.
A. Hospitalization, B. Surgery, C. External consult and D. Sacrificing room.

Table 1: Characteristics of representative species of the genus *Bacillus* taken from Madigan [14].

Oval or cylindrical spore, facultative aerobes; Hydrolyze casein and starch; Non-distended sporangium, thin spore wall		
Characteristic	Specie	Spore position
Mesophile	<i>B. licheniformis</i>	Central
	<i>B. cereus</i>	Central
	<i>B. anthracis</i>	Central
	<i>B. megaterium</i>	Central
	<i>B. subtilis</i>	Central
	<i>B. thuringensis</i>	Central

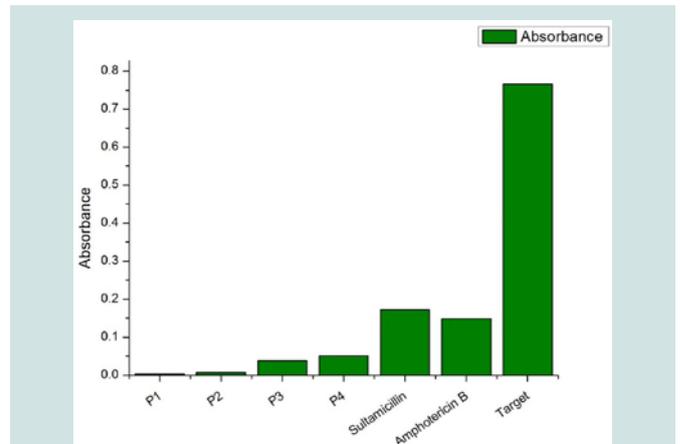


Figure 2: Antimicrobial activity of the treatments evaluated.
P1(Bactericidal monoenzyme detergent, water soluble dose of 20 g), **P2**(High-Level Disinfectant, Potentiated Glutaraldehyde at 0.17%, pH=6), **P3**(Disinfectant detergent, fifth generation quaternary ammonium 30,50 g/ Kg, pH=5,0 in 750 ml) and **P4**(Surface disinfectant detergent, fifth generation quaternary ammonium, water soluble dose of 5 ml with grape fruit scent).

activity, in relation to the time and the evaluated activity, followed by the potentiated glutaraldehyde based product (0.17%), furniture quaternary ammonium and surface disinfectant detergent.

The effectiveness controls used for antibacterial activity (Ampicillin/Sulbactam) and for the antifungal activity (Amphotericin B), formulated according to the notation and method proposed by the European Committee on Antimicrobial Susceptibility Testing, evidenced the absence of growth of the microorganisms collected by means of the swab, after 10 minutes of the bioassay [22].

In vitro antiviral activity: This experiment showed no growth of the evaluated strains (ATCC CCL-34 and ATCC VR-2209), reporting EC₅₀ (Mean Effective Concentration) from 0.0186 µg/ml to 0.0901 µg/ml of the evaluated products. According to the data of the LC₅₀ (Mean Lethal Concentration), the low toxicity generated on the chicken embryo cells, used for the establishment of the test organisms, was evidenced with respect to the untreated cultures (*p*<0.05) (Table 2).

The variability in virucidal activity may be due to the concentration variant of the components present in the products. Virulent extracellular action, given by the fact that at low concentrations of the product (0.0186 ± 0.1 mg/ml), indicates inhibition in viral replication. Due to the non-toxicity on the culture cells for the case of the mono-enzymatic detergent (water soluble dose of 20 g), it was not possible to calculate the Selectivity Index.

Respect to the cleaning and disinfection process evaluated *in situ* by luminometry, the elimination of microorganisms in different veterinary environments is evidenced (external consultation, hospitalization, surgery, laboratory, nursery and hairdressing), where the collected swabs of mesons, feeding areas (drinkers and feeders) and surgical instruments, reporting initial values of 3855 RLU (Prior to the use of the products) and 28.0 RLU in the end (after the application of the products, without the minimum contact time) (Table 3).

Table 2: *In vitro* cytotoxic and antiviral activity of the evaluated products.

Treatment	LC ₅₀ (µg/mL) ± SD	EC ₅₀ (µg/mL) 0 ± SD		Selectivity Index (SI)	
		ATCC CCL-34	ATCC VR-2209	ATCC CCL-34	ATCC VR-2209
P1	> 200	0,0186 ± 0,1	0,0201 ± 0,1	DN	DN
P2	32,60 ± 0,1	0,0370 ± 0,1	0,0264 ± 0,1	881,08	1,2 x 10 ³
P3	177,66 ± 0,1	0,0572 ± 0,1	0,0400 ± 0,1	3,1 x 10 ³	4,44 x 10 ³
P4	81,47 ± 0,1	0,0901 ± 0,1	0,0734 ± 0,1	904,21	1,1 x 10 ³

a. P1: Bactericidal monoenzyme detergent (water soluble dose of 20 g), P2: High-Level Disinfectant (Potentialized Glutaraldehyde at 0.17%, pH=6), P3: Disinfectant detergent (Fifth generation quaternary ammonium 30.50 g/Kg, pH=5.0 in 750 ml) and P4: Surface disinfectant detergent (Fifth generation quaternary ammonium, water soluble dose of 5 ml with grape fruit scent). b. LC₅₀: Mean Lethal Concentration; c. EC₅₀: Average Effective Concentration; d. DN: Data Not Calculated.

Table 3: Evaluation of cleaning and disinfection processes of evaluated products in different areas of clinics and veterinary centers environments.

Area	Sample unit	Start RLU	Final RLU			
			P1	P2	P3	P4
External consultation	Table	1835	0,0	0,0	0,0	0,0
Hospitalization	Cannula	629	0,0	0,0	0,0	0,0
	Table	1047	0,0	0,0	0,0	0,0
Surgery	Scalpel	1633	0,0	0,0	0,0	0,0
	Tweezers	1062	0,0	0,0	0,0	0,0
Laboratory	Plate	486	0,0	0,0	0,0	0,0
	Autoclave	1773	0,0	0,0	0,0	0,0
Animal care	Table	830	0,0	0,0	0,0	0,0
	Cage	3409	35,0	28,0	42,0	44,0
	Drinkers	3010	40,0	30,0	53,0	52,0
Hairdresser's shop	Dining room	3855	40,0	33,0	47,0	48,0
	Comb	496	0,0	0,0	0,0	0,0
	Tub	2841	0,0	0,0	0,0	0,0
	Pail	296	0,0	0,0	0,0	0,0

a. RLU: Relative Light Units; b. Products evaluated outside the time indicated by the data sheet for the processes of cleaning and disinfection (<1 min of application of the products).

Discussion

The present research demonstrates the *in vitro* biological activity of products for cleaning, disinfection and sterilization against bacteria, fungi and viruses that cause pathologies in veterinary, where one of the criteria most used to consider the effectiveness of an antiviral product, is to obtain a selectivity index value above 10,18.

The fact that the Selectivity Index (SI) is higher in the virucidal experiment, when the cells are incubated for 1 hour prior to infection, demonstrates the strong virucidal action of each product. These results indicate the ability to reduce the infective title in 2 logarithms (Log₂). Other possible mechanisms of inhibition are not eliminated, along with the fact of the quantity of active compounds in the evaluated products in the tests.

According to the data reported by luminometry, the high efficiency and efficacy in the elimination of organic matter and

microorganisms in different locations in the study places is evidenced, emphasizing that the products were not evaluated in the required time and indicated by the technical data, however, determined the decrease in the activity evaluated, where, for veterinary environments, the maximum luminometric measurement (3855 RLUs) verified a decrease in organic matter and microorganisms up to 98,76% (48, 0 RLUs), denoting a reduction in organic matter and microorganisms present on the surfaces.

The fact that at 2 minutes at the beginning of the bioassay and 3 days of treatment, the test to which the mono-enzymatic detergent was supplied in 20 g (0.5%) water-soluble dosage (manufacturer's recommendations), showed both an inhibition of the viruses, as a significant reduction of the replication with respect to the control group, this suggests the presence of a protective effect, before the aroused infection, which supports the antiviral activity obtained.

In future studies, *in vivo* evaluation is recommended, verifying the route of administration.

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