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## Microflora of boxes for holding veterinary patients in clinics

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A significant element of the prophylaxis of nosocomial infection in veterinary clinics is monitoring ambient objects, air, equipment, and instruments. In order to determine the role of boxes for keeping ill animals as a source of transmission of pathogens of nosocomial infections in veterinary clinics, we studied the microflora of surfaces of boxes and bioaerosol prior and after sanitation. For this purpose, we collected rinses from the surfaces of plastic and steel boxes, air samples prior to morning sanitation, after cleaning and wiping the surfaces with water and detergents and after disinfection. From the surfaces of the boxes for holding animals, we mostly isolated bacteria of *Staphylococcus* spp., *Streptococcus* spp., *Micrococcus* spp., *Corynebacterium* spp., *Enterococcus* spp. and *Bacillus* spp. Gram-negative species we found were bacteria of *Escherichia* spp., *Acinetobacter* spp. and *Enterobacter* spp. After wet cleaning and disinfection of plastic boxes, we detected species of *Staphylococcus* spp. and *Enterococcus* spp. in 5.4% of the samples, *Micrococcus* spp. in 8.1% and *Bacillus* spp. in 2.7%. Gram-negative bacteria of *Enterobacter* spp. were found in 2.7% of the samples. At the same time, the number of microorganisms in samples in which the bacteria were found after disinfection on the surfaces of stainless-steel boxes was 2.0 times lower than in such from the surfaces of plastic boxes. We determined that after wet disinfection of boxes' surfaces, there occurred decrease in the microbial number in the air, equaling 3.7 times on average, compared with prior to disinfection. The basis of the air microflora after disinfection comprised species of *Micrococcus* spp., *Corynebacterium* spp. and *Staphylococcus* spp., which can be airborne-transmitted. Bacteria that were isolated from the boxes after disinfection (*Micrococcus* spp., *Staphylococcus* spp.) formed highly dense biofilms, which probably ensure the survival of the microbial cells, thus making the boxes a probable source of nosocomial infection.

**Keywords:** nosocomial infection; veterinary clinics; microflora composition; biofilm; companion animals.

### Introduction

Recently, more and more urban dwellers get animal companions for positive emotions and communication (Stull & Weese, 2015; Lee et al., 2022). Other than bringing positive emotions to their owners, pets suffer from diseases, get traumatized in various ways, and need prophylaxis procedures, and therefore are patients of veterinary clinics (Nath et al., 2022). Sometimes, during complex prophylaxis, animals are hospitalized in clinics so they stay under professional veterinary monitoring (Sellera et al., 2021). Therefore, it is important to control the microflora circulating in the rooms and boxes for animals by their quantitative and qualitative compositions so as it takes no negative effect on patients' health, especially those that underwent surgery and are weakened (Loncaric et al., 2019).

The literature sources report (De Kraker et al., 2011; Smith et al., 2019; Kiser et al., 2021) that in rooms of veterinary clinics, particularly procedure and surgical rooms, there circulates nosocomial infection that infects "new" patients. This prolongs the period for which the animals stay in hospitals and leads to significant increase in the use of antimicrobial agents for the treatment of such infections (Stull & Weese, 2015; Morrissey et al., 2016). Pathogens of nosocomial infections that most often infect dogs and cats in veterinary clinics include the following microorganisms. *Staphylococcus aureus* is one of the main pathogens infecting hospital patients (Hamilton et al., 2013; Hritcu et al., 2020), and especially dangerous are *S. aureus* that have become resistant to methicillin (MRSA) (Feßler et al., 2018; Krapf et al., 2019; Elnageh et al., 2020). Despite the fact that initially MRSA was a causative agent of human disease, many reports today report isolation of MRSA from sick animals, mainly from companion animals (Milton et al., 2015; Habibullah et al., 2017; Rusdi et

al., 2018). Therefore, pets may be a source of transmission of resistance genes to their owners, and vice-versa: from owners to pets (Van Duijkeren et al., 2004; Leonard et al., 2006). The reports suggest that MRSA isolated from companion animals (dogs, cats) is impossible to distinguish from MRSA isolated from various biotopes of ill people (Baptiste et al., 2005; Loeffler et al., 2005; Goyal et al., 2013). In veterinary clinics, increase in the number of MRSA-caused infections in companion animals is related to post-surgical infections and wounds (Leonard et al., 2006; Mustapha et al., 2014; Taniguchi et al., 2020), as well as constantly used equipment and suture materials, where pathogens form biofilms (Leonard et al., 2006; Milton et al., 2015). Moreover, the main way MRSA transmits in veterinary clinics is via hands of medical personnel (Leonard et al., 2006; Goyal et al., 2013; Corò et al., 2018). Therefore, veterinary staff is exposed to high risk of MRSA colonization, and also spread the infection among other people (Anderson et al., 2008; van Duijkeren et al., 2010; Jordan et al., 2011).

Also, the following causative agents of nosocomial infections in hospitalized dogs and cats were identified as *Escherichia* spp., *Klebsiella* spp., *Salmonella* spp., *Serratia marcescens*, *Clostridium difficile* and *Acinetobacter baumannii* (Weese et al., 2010; Zordan et al., 2011; Milton et al., 2015). Especially important are Enterobacteriaceae, particularly *E. coli* that produces ESBL and AmpC, which are associated with a number of clinical diseases in canines, such as infections of urinary ducts, neonatal sepsis and wound infections (Pitout, 2010; Shaheen et al., 2011; Ewers et al., 2014). At the same time, numerous studies revealed that hospitalized dogs are exposed to the risk of being infected by *E. coli* with multidrug resistance, and thus animals can become rectal carriers of this bacterium (Gibson et al., 2011; Hamilton et al., 2013). Over the recent decades,

*Acinetobacter baumannii* has become a cause of hospital-acquired infection, and its pathogenicity is mainly due to multidrug resistance and formation of biofilms (Kempf & Rolain, 2012). *Acinetobacter baumannii* were isolated from clinical samples from companion animals (Francey et al., 2000; Zordan et al., 2011). Therefore, companion animals can be reservoirs for bacteria that are resistant to antimicrobial drugs, and therefore the role of pets in spread of antimicrobial resistance is obvious, especially after procedures in veterinary clinics.

An important part of the system of prophylaxis of spread of nosocomial infections in veterinary clinics is surveillance of bioaerosols in hospital rooms and particularly the boxes where sick animals are kept (Harper et al., 2013; Sitkowska et al., 2015; Darwich et al., 2021). Hospital-acquired pathogens contaminate instruments, implants, equipment, ambient objects, veterinary personnel, and therefore airborne transmission of infection is significant and must be considered (Harper et al., 2013). Taking into account this fact, the study of air microflora may provide data on sources and rates of release and spread of airborne pathogens in the environment of veterinary clinics (Harper et al., 2013; Shrivastava et al., 2013).

Thus, the main pathogens of nosocomial infection of small animals are known and certain general recommendations for prophylaxis and control of hospital-acquired pathogens have been developed. Study of microflora of various objects in veterinary clinics, where animals have the most social contact, would give a deeper evaluation of infection sources, ways of transmission, and improve the preventive measures against the spread of nosocomial pathogens, both among animals and people.

The objective of the study was evaluation of microbiological composition of surfaces of boxes and air where sick animals are kept in veterinary clinics and determining the effects of utilized sanitary measures on quantitative and qualitative composition of microflora.

## Materials and methods

The research was conducted in 2021 in veterinary clinics in the cities of Chernivtsi and Kolomyia (Ukraine). In those vet clinics, two types of boxes for hospitalization of sick animals are used, the first type of boxes is made of plastic (Fig. 1a), and second type is made of stainless steel (Fig. 1b). Doors in both types of boxes are made of transparent plastic. Over the year, 152 sick animals were in those boxes. In general, we studied 111 rinses from the surfaces of the boxes, 37 prior to sanitation, 37 after cleaning and 37 after disinfection and 72 air samples from the boxes and 30 air samples from the rooms. Samples for the study were taken once every two months. We studied film-formation properties in 127 bacterial cultures. In the experiment, we used disinfecting agent with active substances; N-(3-aminopropyl)-N-dodecylamine-1,3-diamine and N,N-didecyl-N,N-dimethylammonium chloride (France).

Rinses from the inner surfaces of the boxes, the area of around 100 cm<sup>2</sup> on average, were collected using disposable industrially-made sterile tampons. After collecting rinses, the tampon was put into transport tube with *Amies medium* and delivered to microbiological laboratory for the study. Air samples were selected from the boxes and the rooms using sedimentation method. For this purpose, open Petri dishes with meat-peptone agar (MPA) and Saburo were put in the box for 30 min, and in the rooms, the dishes were put according to the envelope method (four samples in angles, and fifth in the center), at the distance of 0.5 m from the wall and at the height of 1.6 m for 30 min, having windows and doors in the boxes and the room closed. After 30 min of exposition, the dishes were closed, placed in a refrigerator bag and transported to the laboratory in 2 h.

Account of the results of determining microbial number in the air and rinses from boxes' surfaces. Inoculated Petri dishes were placed into a thermostat at the incubation temperature of  $+37 \pm 1$  °C for 48 h. Then, we calculated the mean number of colonies and determined concentration of bacteria per m<sup>3</sup> of air according to the Omeliansky's formula (Sitkowska et al., 2015). Microbial number of mesophilous microorganisms in rinses from surfaces of the boxes for keeping animals was determined according to the generally accepted method. We made ten-fold dilutions of rinses, inoculated the dilutions to Petri dishes, 1 mL in each, submerged them by 15 mL of MPA, and after cooling, the inoculated dishes were put into a thermostat at  $30 \pm 1$  °C and incubated for 72 h. Then, we counted the

colonies and determined the mean number in 1 mL of the rinse. To isolate microorganisms from the rinses, we performed inoculations on the media. In particular, *Staphylococcus* and *Micrococcus* were isolated on blood agar containing 5% sodium chloride, *Enterococcus* on Bile Esculin Azide Agar medium, *Streptococcus* and *Corynebacterium* on *Streptococcus* Selective Agar (HiMedia, India) and blood agar. Bacteria of *Bacillus* genus were identified by inoculating the rinses and their dilution on MPA with following incubation in the temperature of 30 °C for 72 h. The samples were previously held for 10 min in a water bath, in the temperature of 85 °C. Fungi were inoculated on the Saburo medium (Farmaktyv, Ukraine). Enterobacteriaceae (*Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and others) were cultivated on Endo, Ploskiriev's and Levin's media (Farmaktyv, Ukraine). Isolation of *Pseudomonas* was performed on an acetamide-containing medium, and other non-fermentative bacteria (*Acinetobacter* spp. and *Alcaligenes* spp.) on MPA, incubated for 7 days in 10 °C.



**Fig. 1.** Boxes for holding veterinary patients: a – plastic, b – stainless steel

To isolate mesophilous microorganisms, the inoculations were incubated in the thermostat at the temperature of  $37 \pm 1$  °C for 24–48 h, and fungi in  $+28 \pm 1$  °C for 5 days. The isolated cultures were identified according to morphologic, tinctural, cultural, biochemical properties and features of pathogenicity, as described in the “Bergey's Manual of Systematic Bacteriology” (Vos et al., 2011).

We added 5 cm<sup>3</sup> of meat-peptone broth and 1 cm<sup>3</sup> of 24 h test culture of microorganisms in the concentration of 10<sup>5</sup> CFU/cm<sup>3</sup> to sterile dispo-

sable plastic Petri dishes, and incubated them in the temperature of 37 °C for 24 h. After the incubation, the dishes were rinsed from plankton (non-attached) microorganisms by phosphate buffer three times, and the formed biofilms were dried and fixed using 96% ethyl alcohol for 10 min. Then, we stained them with 0.1% solution of crystalline violet for 10 min. To the Petri dishes, we added 3.0 cm<sup>3</sup> of 96% ethyl alcohol and left it for 20–30 min, occasionally shaking them. The optical density of the alcohol solution was measured spectrophotometrically at the wavelength of 570 nm (Kukhtyn et al., 2017).

Counting bacteria in biofilms after the action of the disinfecting agent was conducted on 24 h microbial biofilms, cultivated in plastic Petri dishes. After 24 h incubation of the bacteria, the dishes were three times rinsed from plankton microorganisms using sterile phosphate buffer and 5 cm<sup>3</sup> of fresh-made operating solution of disinfectant was introduced. After the exposure, the disinfectant was removed, the dishes were rinsed using sterile phosphate buffer, and 5 cm<sup>3</sup> of sterile 0.9% of solution of sodium chloride was introduced, and the microbial biofilm were accurately rinsed off the walls and bottom of the dishes using a sterile tampon. From the dishes, we selected 1.0 cm<sup>3</sup> of the suspension, prepared a number of ten-fold dilutions, inoculated 1.0 cm<sup>3</sup> of each dilution in Petri dish, and filled the dishes with the MPA. To determine the number of bacteria, we performed the incubation at the temperature of 37 °C for 24–48 h.

Statistical analysis was performed using dispersion analysis with Fisher's criteria (ANOVA). The data are presented as  $\bar{x} \pm SD$  (mean  $\pm$  standard error). Significance of the obtained data was evaluated according to F-criterion with the significance levels of  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$  (taking into account Bonferroni correction).

## Results

According to the compositions of microflora of the surfaces of plastic boxes prior to morning sanitation, after mechanical cleaning and after disinfection by wiping (Table 1), the following genera of Gram-positive bacteria were found in 100% of cases: *Staphylococcus* spp., *Streptococcus* spp., *Micrococcus* spp. and *Corynebacterium* spp. Somewhat rarer, there occurred *Enterococcus* spp., in 62.1% of cases, and spore-forming bacilli *Bacillus* spp., in 75.7% of the examined samples. Among identified Gram-negative microflora, from the surfaces, we most often isolated *Escherichia* spp., *Acinetobacter* spp., in 89.1% of the examined samples, and *Enterobacter* spp., in 81.0%. Such Gram-negative bacteria as *Klebsiella* spp., *Citrobacter* spp. and *Alcaligenes* spp. were found on the surfaces of boxes in about 56.7–62.1% of the samples. The lowest number of identified Gram-negative bacteria from the studied samples was for *Pseudomonas* spp. – 18.9%.

**Table 1**

Microflora of the surfaces of plastic boxes for maintaining small sick animals in vet clinics (n = 37)

Microorganisms	Frequency of isolation of microorganisms from the objects, % of samples			
	prior to sanitation	after mechani- cal cleaning	after disinfection	
Gram- positive	<i>Staphylococcus</i> spp.	100.0	67.5 <sup>*</sup>	5.4 <sup>###</sup>
	<i>Streptococcus</i> spp.	100.0	43.2 <sup>*</sup>	0.0 <sup>###</sup>
	<i>Micrococcus</i> spp.	100.0	67.5 <sup>*</sup>	8.1 <sup>###</sup>
	<i>Corynebacterium</i> spp.	100.0	59.4 <sup>*</sup>	0.0 <sup>###</sup>
	<i>Enterococcus</i> spp.	62.1	40.5 <sup>*</sup>	5.4 <sup>###</sup>
	<i>Bacillus</i> spp.	75.7	35.1 <sup>*</sup>	2.7 <sup>###</sup>
Gram- negative	<i>Escherichia</i> spp.	89.1	43.2 <sup>*</sup>	0.0 <sup>###</sup>
	<i>Citrobacter</i> spp.	59.4	27.0 <sup>***</sup>	0.0 <sup>###</sup>
	<i>Enterobacter</i> spp.	81.0	37.8 <sup>**</sup>	2.7 <sup>###</sup>
	<i>Klebsiella</i> spp.	62.1	18.9 <sup>***</sup>	0.0 <sup>###</sup>
	<i>Pseudomonas</i> spp.	18.9	5.4 <sup>***</sup>	0.0 <sup>###</sup>
	<i>Acinetobacter</i> spp.	89.2	43.2 <sup>**</sup>	0.0 <sup>###</sup>
	<i>Alcaligenes</i> spp.	56.7	35.1 <sup>*</sup>	0.0 <sup>###</sup>
Others were not identified	8.1	5.4	0.0 <sup>###</sup>	

Note: \* –  $P < 0.05$ ; \*\* –  $P < 0.01$ ; \*\*\* –  $P < 0.001$  compared with samples prior to the sanitation; <sup>###</sup> –  $P < 0.001$  compared with samples after mechanical cleaning according to F-criterion.

Cleaning and washing of the boxes decreased the quantity of microflora from the surfaces. In particular, among Gram-positive bacteria, we

most often isolated such bacteria as *Staphylococcus* spp., *Micrococcus* spp. and *Corynebacterium* spp., in 67.5% and 59.4% of the samples respectively. Bacteria of *Streptococcus* spp. and *Enterococcus* spp. were isolated from surfaces of the boxes in 40% of the samples on average. That is, the frequency of isolation of Gram-positive cocci from the surfaces of plastic boxes after sanitation decreased by 1.5–2.5 times, compared with samples prior to the cleaning. Gram-negative bacteria were also found in the studied samples to a lower degree. Therefore, the frequency of isolation of bacteria *Escherichia* spp., *Acinetobacter* spp. and *Enterobacter* spp. accounted for 43.2% and 37.8% respectively, which was 2.0 times lower on average than prior to cleaning. Frequency of isolation of bacterial species of *Citrobacter* spp. and *Klebsiella* spp. from the surfaces equaled 27.0% and 18.9% of the cases, respectively, i.e. 2.2 and 3.2 times lower compared with the samples prior to cleaning. Bacteria of *Pseudomonas* spp. were to a lower degree isolated from the surfaces of plastic boxes in 5.4% of cases, which was 3.2 times less than before cleaning.

Wet disinfection by wiping the surfaces of plastic boxes using disinfecting agent significantly decreased microbial load. In particular, regarding Gram-positive bacteria, we isolated only bacteria of *Staphylococcus* spp., *Enterococcus* spp. in 5.4% of samples, *Micrococcus* spp. in 8.1% and *Bacillus* spp. in 2.7%. Practically no Gram-negative species of bacteria were isolated from surfaces of the boxes, species of *Enterobacter* spp. alone were found in 2.7% of samples.

Studies of frequency of isolation of microorganisms (Table 2) revealed that the main dominant microorganisms isolated from surfaces of the stainless-steel boxes were of the same genera of bacteria as from surfaces of the plastic boxes. In particular, in 100% of cases, from the samples, we isolated the following Gram-positive bacteria: *Staphylococcus* spp., *Micrococcus* spp. and *Corynebacterium* spp. On average, the samples were observed to contain 1.5 times lower numbers (64.8–62.1%) of cocci species of *Streptococcus* spp., *Enterococcus* spp. and bacilli *Bacillus* spp. From surfaces of the boxes of stainless steel, we most often isolated Gram-negative species of *Escherichia* spp., *Acinetobacter* spp. and *Enterobacter* spp., which were present in 83.8% and 75.7% of the samples. Species of *Citrobacter* spp. and *Klebsiella* spp. were identified in 64.4 and 56.7% of the examined samples. Almost 50% of the samples contained bacteria of *Alcaligenes* spp., and most rarely, we isolated *Pseudomonas* spp., in 24.3%.

**Table 2**

Microflora of surfaces of the stainless-steel boxes for holding small ill animals in vet clinics (n = 37)

Microorganisms	Frequency of isolation of microorganisms from objects, % of samples			
	prior to sanitation	after mechanic cleaning	after disinfection	
Gram- positive	<i>Staphylococcus</i> spp.	100	59.4 <sup>***</sup>	5.4 <sup>###</sup>
	<i>Streptococcus</i> spp.	64.8	27.0 <sup>**</sup>	0 <sup>###</sup>
	<i>Micrococcus</i> spp.	100	45.9 <sup>**</sup>	2.7 <sup>###</sup>
	<i>Corynebacterium</i> spp.	100	45.9 <sup>**</sup>	0 <sup>###</sup>
	<i>Enterococcus</i> spp.	64.8	35.1 <sup>*</sup>	0 <sup>###</sup>
	<i>Bacillus</i> spp.	62.1	32.4 <sup>**</sup>	0 <sup>###</sup>
Gram- negative	<i>Escherichia</i> spp.	81.0	35.1 <sup>***</sup>	0 <sup>###</sup>
	<i>Citrobacter</i> spp.	64.4	18.9 <sup>***</sup>	0 <sup>###</sup>
	<i>Enterobacter</i> spp.	75.7	32.4 <sup>***</sup>	0 <sup>###</sup>
	<i>Klebsiella</i> spp.	56.7	13.5 <sup>***</sup>	0 <sup>###</sup>
	<i>Pseudomonas</i> spp.	24.3	8.1 <sup>***</sup>	2.7 <sup>###</sup>
	<i>Acinetobacter</i> spp.	83.8	35.1 <sup>***</sup>	0 <sup>###</sup>
	<i>Alcaligenes</i> spp.	48.6	21.6 <sup>***</sup>	0 <sup>###</sup>
Other were not identified		5.4	2.7	0 <sup>###</sup>

Note: see Table 1.

After cleaning and washing the boxes, the frequency of isolation of microorganisms from the surfaces significantly decreased, similarly to the samples from plastic boxes. At the same time, most often found Gram-positive bacteria were *Staphylococcus* spp., in 59.4% of the samples, and *Micrococcus* spp. and *Corynebacterium* spp. were found in 45.9% of cases. Bacteria of *Enterococcus* spp. and *Bacillus* spp. were found in 35% of the samples on average, and the rarest Gram-positive bacterium identified after washing the surfaces of stainless-steel boxes was *Streptococcus* spp., found in 27.0%. In general, after preliminary sanitation of the boxes,



the frequency of isolation of Gram-positive bacteria decreased 1.6–2.4-fold, compared with the samples prior to morning cleaning. At the same time, the lowest number of bacteria rinsed off the surfaces of the boxes was observed for species of *Staphylococcus* spp., and the species observed most often were those of *Streptococcus* spp. Gram-negative bacteria were also often rinsed from the surfaces of steel boxes after preliminary sanitation, because frequency of their isolation decreased 2.2–3.4-fold, compared with prior to the treatment. However, such bacteria as *Escherichia* spp., *Acinetobacter* spp. and *Enterobacter* spp. were still found in significant amounts, in 35% of the samples on average.

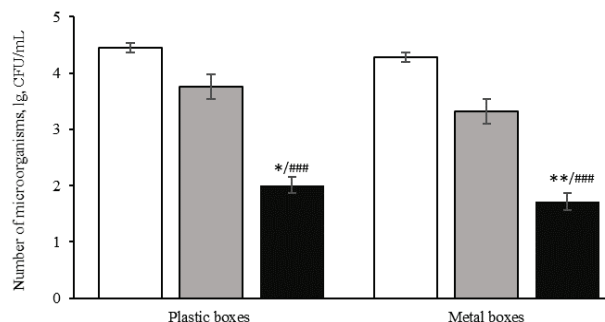
After disinfection, from the surfaces of stainless-steel boxes, we isolated only species of *Staphylococcus* spp. and *Micrococcus* spp., from 5.4% and 2.7% of samples, respectively. The only Gram-negative bacteria were species of *Pseudomonas* spp., found in 2.7% of samples.

Analyses of quantitative compositions of mesophilous microorganisms from surfaces of the boxes prior to cleaning and after sanitation (Fig. 2) revealed that the number of microorganisms on the surfaces of two types of boxes tended to decrease after sanitation. Furthermore, we found a 1.5 times higher number of microorganisms on the surfaces of plastic boxes prior to cleaning, compared with the stainless-steel boxes ( $4.45 \pm 3.18$  and  $4.28 \pm 295$  lg CFU/mL of rinse). After preliminary cleaning (washing with detergent), the overall number of mesophilous bacteria decreased to  $3.76 \pm 2.38$  lg CFU on plastic boxes and to  $3.32 \pm 2.19$  lg CFU on stainless-steel boxes. Therefore, the number of microorganisms on the surfaces of stainless steel boxes was 2.4 times lower. After disinfection, mesophilous microorganisms were found in 8.1% of the samples collected from plastic boxes and in 5.4% of the samples from metal boxes. At the same time, the overall number of microorganisms in those samples accounted for  $2.01 \pm 0.89$  and  $1.71 \pm 0.63$  lg CFU/mL of rinse, i.e. the concentration of bacteria on surfaces of stainless-steel boxes was 2.0 lower on average than on plastic surfaces.

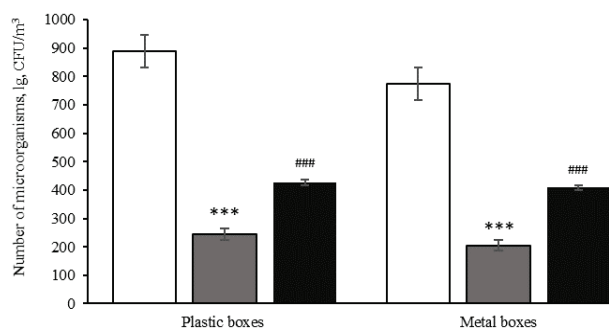
Change in the overall number of microorganisms in the air in the boxes and the room where they are located (Fig. 3) revealed that microbial number in the air in two types of boxes significantly decreased 3.6 and 3.8-fold after wet disinfection of surfaces. In the room where the boxes are kept, microbial load on the air was on average 2.0-fold lower than in the boxes before disinfection, though 1.7–2.0 times higher than in the boxes after disinfection. At the same time, no significant differences between microbial numbers in the air in the plastic boxes and the stainless-steel boxes were found.

Identification of microorganisms isolated from the air of two types of boxes (Table 3) revealed that according to frequency of isolation of microorganisms from air samples, no significant changes were determined, both prior and after applying disinfectant to wall surfaces. In general, air microflora before the sanitation was represented by genera of bacteria

which were isolated from the boxes' surfaces. At the same time, after wet disinfection, we isolated no Gram-positive species of *Enterococcus* spp. from the air of plastic boxes, and no *Enterococcus* spp. and *Bacillus* spp. from the air of steel boxes. Gram-negative bacteria were isolated from air samples much more rarely than Gram-negative. Moreover, after disinfection of the surfaces, from air samples of two types of boxes, we isolated no bacteria of *Escherichia* spp., *Citrobacter* spp., *Klebsiella* spp. and *Pseudomonas* spp., whereas species of *Enterobacter* spp., *Acinetobacter* spp. and *Alcaligenes* spp. were present in no more than 11.1% of the samples.



**Fig. 2.** Number of mesophilous aerobic microorganisms on surfaces of boxes: prior to sanitation (white column), after sanitation (grey column) and after disinfection (black column),  $x \pm SD$ ,  $n = 111$ ; \* – in 8.1% of samples; \*\* – in 5.4% of samples; ### –  $P < 0.001$  compared to samples after mechanical cleaning



**Fig. 3.** Number of mesophilous aerobic microorganisms in the air prior to disinfection in the boxes (white column), after disinfection (grey column) and in the room (black column),  $x \pm SD$ ,  $n = 111$ ; \*\*\* –  $P < 0.001$  compared with samples prior to disinfection; #### –  $P < 0.05$  compared with samples after disinfection

**Table 3**  
Composition of microflora of air in boxes for keeping ill animals

Microorganisms	Frequency of isolation of microorganisms from air, % of samples			
	plastic boxes		stainless-steel boxes	
	prior to sanitation, n = 18	after disinfection, n = 18	prior to sanitation, n = 18	after disinfection of walls, n = 18
Gram-positive	<i>Staphylococcus</i> spp.	77.8	33.3*	66.7
	<i>Streptococcus</i> spp.	66.7	11.1***	22.2***
	<i>Micrococcus</i> spp.	83.3	44.4**	61.1
	<i>Corynebacterium</i> spp.	83.3	88.9	38.9***
	<i>Enterococcus</i> spp.	61.1	33.3**	66.7
	<i>Bacillus</i> spp.	22.2	0.0***	22.2***
	<i>Escherichia</i> spp.	61.1	0.0***	0.0***
Gram-negative:	<i>Citrobacter</i> spp.	16.7	5.5***	50.5
	<i>Enterobacter</i> spp.	11.1	0.0***	22.2
	<i>Klebsiella</i> spp.	22.2	5.5***	0.0***
	<i>Pseudomonas</i> spp.	5.5	0.0***	5.5
	<i>Acinetobacter</i> spp.	11.1	0.0***	11.1***
	<i>Alcaligenes</i> spp.	33.3	11.1***	0.0***
		33.3	27.7	5.5***
Fungi		27.7	33.3	11.1***

Note: see Table 1.

As is known, among many natural mechanisms that help microorganisms to survive under the impact of unfavourable environmental factors, formation of biofilms by bacteria on various surfaces is considered one of the main mechanisms protecting target cells from the action of biocides.

We determined the density of biofilms of bacteria isolated from the surfaces of two types of boxes (Table 4).

We determined (Table 4) that among the bacteria isolated from plastic and metal boxes, there were no cultures that formed low-density bio-

films. At the same time, most Gram-positive species of bacteria formed highly dense and very highly dense biofilms. Particularly, 100% of cultures of *Staphylococcus* spp. species and 90% of *Micrococcus* spp. species formed very-high-density biofilms. Bacteria of *Enterococcus* spp. and *Bacillus* spp. formed biofilms of very high density in 71.4% to 77.8% of cases and of high density in up to 30% of cases. *Corynebacterium* cultures formed high-density biofilms in 50% of cases and very-high-density biofilms in another 50%. The lowest optical density of biofilm dilutions among Gram-positive bacteria was in species of *Streptococcus* spp., 57.1% of those cultures formed biofilms of average density and 42.8% formed biofilms of high density.

At the same time, isolated Gram-negative bacteria formed biofilms of lower density than the Gram-positive. Only very-high-density biofilms were formed by *Pseudomonas* spp., and bacteria of *Escherichia* spp. in 91.7% of cases formed biofilms of highest density. From 20 to 30% of species of *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp. formed average-density biofilms, 30–40% of those bacteria made biofilms of high density and 30–50% formed very-high-density biofilms. Isolated non-fermenting species of *Acinetobacter* spp. and *Alcaligenes* spp. formed biofilms of average, high and very high density in almost the same numbers.

Taking into account the fact that after wet disinfection of surfaces of two types of boxes, still some species of bacteria were isolated, we per-

formed studies on determining the number of microbial cells in biofilms of various densities prior and after the action of disinfectant (Table 5).

**Table 4**

Formation of biofilms by bacteria isolated from boxes for keeping ill animals (%; n = 127)

Microorganisms	Number of cultures, n	Number of cultures that formed biofilms with respective density			
		low	average	high	very high
<i>Staphylococcus</i> spp.	12	–	–	–	12 (100)
<i>Streptococcus</i> spp.	7	–	4 (57.1)	3 (42.8)	–
Gram-positive <i>Micrococcus</i> spp.	10	–	–	1 (10.0)	9 (90.0)
<i>Corynebacterium</i> spp.	8	–	–	4 (50.0)	4 (50.0)
<i>Enterococcus</i> spp.	9	–	–	2 (22.2)	7 (77.8)
<i>Bacillus</i> spp.	7	–	–	2 (28.6)	5 (71.4)
<i>Escherichia</i> spp.	12	–	–	1 (8.3)	11 (91.7)
<i>Citrobacter</i> spp.	10	–	2 (20.0)	3 (30.0)	5 (50.0)
Gram-negative <i>Enterobacter</i> spp.	10	–	3 (30.0)	3 (30.0)	4 (40.0)
<i>Klebsiella</i> spp.	10	–	3 (30.0)	4 (40.0)	3 (30.0)
<i>Pseudomonas</i> spp.	8	–	–	–	8 (100)
<i>Acinetobacter</i> spp.	12	–	4 (33.3)	4 (33.3)	4 (33.3)
<i>Alcaligenes</i> spp.	12	–	5 (41.7)	3 (25.0)	4 (33.3)

Note: optical density of dilution of biofilm to 0.50 U – low-density biofilm; from 0.51 to 1.00 – average density; from 1.01 to 1.50 – high; 1.51 < very high.

**Table 5**

Effect of disinfectant on microbial biofilms of various density (lg CFU/cm<sup>2</sup>, x ± SD, n = 57)

Microorganisms	Number of cells in average-density biofilm		Number of cells in high-density biofilm		Number of cells in very-high-density biofilm	
	prior to the action of biocide	after the action of biocide	prior to the action of biocide	after the action of biocide	prior to the action of biocide	after the action of biocide
<i>Staphylococcus</i> spp., n = 5	–	–	–	–	6.81 ± 4.13	3.13 ± 1.37***
<i>Streptococcus</i> spp., n = 5	5.87 ± 3.92	0***	6.15 ± 3.98	0***	–	–
<i>Micrococcus</i> spp., n = 5	–	–	6.23 ± 4.08	2.55 ± 1.24***	6.70 ± 4.11	3.05 ± 1.44***
<i>Enterococcus</i> spp., n = 5	–	–	6.26 ± 4.05	2.43 ± 1.22***	6.68 ± 4.15	2.88 ± 1.33***
<i>Bacillus</i> spp., n = 5	–	–	6.14 ± 4.01	2.66 ± 1.48***	6.29 ± 4.07	3.11 ± 1.52***
<i>Enterobacter</i> spp., n = 9	5.92 ± 3.88	0***	5.98 ± 3.95	2.51 ± 1.34***	6.14 ± 4.07	2.95 ± 1.33***
<i>Pseudomonas</i> spp., n = 5	–	–	–	–	6.67 ± 4.14	3.75 ± 2.02***
<i>Acinetobacter</i> spp., n = 9	5.86 ± 3.75	0***	5.96 ± 3.84	0***	6.20 ± 3.96	2.71 ± 1.27***
<i>Alcaligenes</i> spp., n = 9	5.79 ± 3.70	0***	5.91 ± 3.86	0***	6.27 ± 4.02	2.77 ± 1.30***

Note: see Table 1.

We determined (Table 5) that the survival of microbial cells in biofilms after the action of disinfectant depended on its density, i.e. after the action of biocide, biofilms of high and very high density were observed to have more viable bacteria, and after the action of disinfectant on biofilms of average density, we isolated no live bacteria from biofilm matrix. In particular, in biofilm of high density, the most protected species were those of *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp. and *Pseudomonas* spp., for after the action of the biocide, viable cells were isolated from the matrix in quantity higher than 3.0 lg CFU/cm<sup>2</sup> of the area of the biofilm. After the effect of disinfectant on biofilm of very high density, the number of isolated viable cells of species of *Acinetobacter* spp. and *Alcaligenes* spp. accounted for 2.71 ± 1.27 and 2.77 ± 1.30 lg CFU/cm<sup>2</sup> of the area of biofilm.

During the evaluation of the action of disinfectant toward high-density biofilms, we found completely inactivated bacterial cells of species of *Streptococcus* spp., *Acinetobacter* spp. and *Alcaligenes* spp. At the same time, biofilms of high density of such bacteria as *Micrococcus* spp., *Enterococcus* spp., *Bacillus* spp. and *Enterobacter* spp. helped the microbial cells to survive the action of disinfectant, since we isolated viable cells in the amounts of 2.43 ± 1.22 to 2.66 ± 1.48 lg CFU/cm<sup>2</sup> of the area of biofilm.

## Discussion

Nosocomial infection is well known in the sphere of human medicine, since it causes significant economic losses due to complications of treatment of the main disease, promotes development and spread of antibiotic-resistant strains of microorganisms (Zazharskyi et al., 2019, 2020), and sometimes leads to patients' death. Therefore, medical institutions have developed systemic measures for control and prevention of spread of

pathogens inside hospitals. In veterinary clinics, especially in developing countries, measures against nosocomial infection are at the stage of development and partial introduction (Kisera et al., 2021). Nonetheless, nosocomial outbreaks of various etiologies were recorded in many veterinary clinics in developed countries, and practical measures against the infection have been taken (Weese et al., 2006; Dallap Schaer et al., 2010; Steneroden et al., 2010).

In our study, by comparing frequencies of isolation of microorganisms from two types of boxes (made of plastic and stainless steel) for keeping hospitalized animals in veterinary clinics, we determined that Gram-positive cocci bacteria of *Staphylococcus* spp., *Streptococcus* spp., *Micrococcus* spp., *Corynebacterium* spp. and *Enterococcus* spp. comprised the basis of the microflora of the surfaces, having isolated them from 64.8–100.0% of the examined morning samples prior to sanitary measures. At the same time, in those conditions, we found no significant difference between the frequencies of isolation of microorganisms from plastic and steel boxes. This gives reason to think that bacteria species we found are common inhabitants of the mucous membranes, skin of animals, and are easily released into the environment and form microflora of surfaces. There are reports (Morley et al., 2005; Hardy et al., 2006; D'Agata et al., 2012) suggesting that sources of nosocomial infections are usually the patient's own microflora, medical personnel, instruments and equipment in the hospital. Gram-negative bacteria of Enterobacteriaceae family, genera *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter* were found in 56.7–89.1% of the analyzed samples, which is obvious since the rinses had been collected prior to morning cleaning. Microorganisms representing the environmental microflora and animal skin surface, particularly *Acinetobacter* spp., were isolated from 83.8–89.2% of the samples. The studies Giannouli et al. (2013) report that *Acinetobacter* spp. has increased resistance to drying because of the ability to produce biofilms

and is quite often found in veterinary clinics, and companion animals can be carriers of those bacteria (Belmonte et al., 2014). Furthermore, there are reports about the spread of antibiotic-resistant species of *Acinetobacter* spp. among pets (cats, dogs) which can cause nosocomial infection in patients, in both medical and veterinary clinics (Kempf & Rolain, 2012; Giannouli et al., 2013; Zhang et al., 2013). Therefore, we agree with the scientists (Francey et al., 2000; Zordan et al., 2011) that companion animals can be a reservoir for antibiotic-resistant *Acinetobacter* spp. and source of nosocomial infection. Bacteria of species of *Pseudomonas* spp. were isolated from surfaces of both types of boxes prior to sanitation in about 20% of cases. Obviously, their source was animals with infected wounds or traumas, since in our studies, we did not take into account the compositions of surface microflora depending on type of pathology of animals that were in the boxes.

Decrease equaling 1.5–3.5 times in finding microorganisms from surfaces of boxes after morning cleaning by washing using a detergent indicates the efficiency of this procedure. However, Gram-positive cocci were also found in 40.5–67.5% of the cases, representatives of Enterobacteriaceae family in 13.5–43.2%, and non-fermenting in 35.1–43.2% of the cases, compared with samples prior to cleaning. Thus, cleaning boxes for hospitalization of animals using detergents significantly decreases microbial load, and at the same time needs the use of disinfectants, since scientists indicate that increase in the risk of transmission and spread of nosocomial infection in veterinary clinics among animals due to non-compliance to hygiene and sanitation during their stay (Morley et al., 2005; D'Agata et al., 2012). Studies of the rinses from surfaces of the boxes show the significant effect the disinfection had on microbiological composition of isolated microflora, since from surfaces of plastic boxes, Gram-positive microflora comprised only species of *Staphylococcus* spp. and *Enterococcus* spp. in 5.4% of samples, *Micrococcus* spp. in 8.1% and *Bacillus* spp. in 2.7%. Regarding Gram-negative bacteria, we found only *Enterobacter* spp., in 2.7% of samples. Boxes made of stainless steel underwent disinfection better, since after biocide application, we found only species of *Staphylococcus* spp. and *Pseudomonas* spp., in 2.7% of the samples. Plastic boxes may be harder to disinfect due to presence of microscratches on the surfaces, where bacteria form biofilms so disinfectant cannot reach the target cells. Role of relief of surface in decrease in bactericidal action of disinfectants was reported (Kukhtyn et al., 2017; Horiuk et al., 2019). Those studies found that biocides did not penetrate depressions of scratches and microorganisms were not inactivated. The overall number of microorganisms was also 2.0–2.4 times lower in rinses from steel boxes compared with plastic boxes after sanitary measures, respectively equaling  $2.01 \pm 0.89$  lg CFU/mL of rinse from plastic surfaces and  $1.71 \pm 0.63$  lg CFU/mL of rinse from stainless-steel surfaces. Therefore, we think that relief of surfaces of the boxes, their condition, frequency of disinfection and efficacy of applied disinfectants in veterinary clinics are significant for preventing spread of nosocomial infection via boxes.

As of now, there are no regulations on the number of microorganisms in the air of veterinary clinics, which could indicate heightened risk of transmission of an airborne infection. This is despite the fact that researchers reported (Harper et al., 2013; Milton et al., 2015; Sitkowska et al., 2015) that bacteria inhabiting the mucous membranes of nasal and oral cavities, including dangerous MRSA, can easily transmit on skin surface by air. In our study, we found that the number of mesophilous bacteria in two types of boxes before cleaning was within 775.0–889.3 CFU/m<sup>3</sup> of air, being 2.0-fold lower on average in the room. This gives reasons to think that there is little air exchange when animals are kept in boxes of such types, resulting in accumulation of microorganisms from animal biotopes over the day. At the same time, wet disinfection of box surfaces reduced the number of microbes in the air inside the boxes by 3.7 times on average, compared with such prior to disinfection. Our data are coherent with the study (Harper et al., 2013) reporting the highest number of microbes in air in rooms of vet clinics, where animals are kept around-the-clock, in the morning prior to cleaning. From the air of two types of boxes, after disinfection of surfaces, we most often isolated Gram-positive species *Micrococcus* spp. in 38.9–44.4% of samples and *Corynebacterium* spp. and *Staphylococcus* spp. in 22.2–33.3%. As with Gram-negative bacteria, we found *Enterobacter* spp., *Acinetobacter* spp. and *Alcaligenes*

spp. in 5.5–11.1% of samples. The studies Morley et al. (2005), Harper et al. (2013), Sitkowska et al. (2015) also suggest that in rooms of vet clinics (procedure room, surgical, diagnostic room and room for keeping sick animals), there develop conditions for transmission of many airborne bacteria, including microorganisms that are conditionally pathogenic for people and animals. Therefore, we emphasize the need to use safe methods of sanitation of air of boxes (and if needed in the room in general) where hospitalized animals are kept. This is especially relevant in the case of keeping animals with infectious pathology of the airways or skin. In general, monitoring the risk of airborne spread of nosocomial infections in veterinary clinics would help prevent transmission of bacteria from animals to veterinary personnel.

One of the goals of our study was determining the density of formed biofilms in bacteria isolated from surfaces of the boxes. Many authors (Horiuk et al., 2019) indicate that while in biofilms, bacteria are more resistant to the action of biocides. We thought that frequent use of disinfectants during sanitation of boxes contributes to formation of dense biofilms by bacteria. We found that in 100% of cultures of species of *Staphylococcus* spp., *Pseudomonas* spp. and 90% of *Micrococcus* spp. species formed biofilms of very high density. Bacteria of *Corynebacterium* spp., *Enterococcus* spp., *Bacillus* spp. and *Escherichia* spp. species formed biofilms of high and very high density. At the same time, *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., *Acinetobacter* spp. and *Alcaligenes* spp. species formed biofilms of average density in 20–40% of the cultures. Therefore, most of the bacteria isolated from the boxes for holding animals in veterinary clinics belonged to the highly biofilm-forming types. At the same time, after the action of disinfectant toward the formed biofilms, the highest number of live cells was isolated from of high-density and very-high-density biofilms ( $2.43\text{--}3.75$  lg CFU/cm<sup>2</sup> of biofilm area). At the same time, after the disinfectant's action toward biofilms of average density, there was complete degradation, and no live bacterial cells were found. Thus, the obtained results are coherent with the data of the researchers (Milton et al., 2015; Kukhtyn et al., 2017) about the role of biofilm-forming bacteria in the spread of microbial cells on surfaces of various objects, including veterinary clinics. In biofilms formed on surfaces of instruments, intravenous, urethral catheter, endotracheal tubes, various implants, bacteria can survive after disinfection and be a cause of spread of antibiotic resistance and even mortality (Milton et al., 2015).

Therefore, we can summarize that development and introduction of a system of surveillance of nosocomial pathogens in veterinary clinics in Ukraine should add to the national strategy of fighting antibiotic-resistant microorganisms. First, this would decrease animals' morbidity with resistant strains, second, decrease the use of broad-range antibiotics, third, prevent the spread of antibiotic-resistant bacteria in the environment of veterinary clinics among animals, between animals and clinic personnel, between personnel and other people and animals.

## Conclusion

Most often, from surfaces of boxes (made of plastic and stainless steel) for holding hospitalized animals in veterinary clinics, we isolated Gram-positive cocci and bacilli-like microorganisms of *Staphylococcus* spp., *Streptococcus* spp., *Micrococcus* spp., *Corynebacterium* spp., *Enterococcus* spp. and *Bacillus* spp. Gram-negative species we detected belonged to *Escherichia* spp., *Acinetobacter* spp. and *Enterobacter* spp.

After wet cleaning and disinfection of plastic boxes, we found species of *Staphylococcus* spp. and *Enterococcus* spp. in 5.4% of samples, *Micrococcus* spp. in 8.1% and *Bacillus* spp. in 2.7%. Regarding Gram-negative bacteria, we found only *Enterobacter* spp., in 2.7% of samples. Boxes made of stainless steel were easier to disinfect, since after biocide application, we found only species of *Staphylococcus* spp. and *Pseudomonas* spp., in 2.7% samples. Therefore, those species of bacteria may be a potential source of nosocomial infection in clinics. At the same time, the number of microorganisms in samples where we found bacteria on surfaces of boxes of stainless steel after disinfection was two times lower than on surfaces of plastic boxes.

After wet disinfection of surfaces of boxes, the microbial number in the air of boxes was on average 3.7 times lower compared with prior to disinfection. The main air microflora after disinfection was represented by

species of *Micrococcus* spp., *Corynebacterium* spp. and *Staphylococcus* spp. Bacteria isolated from the boxes after disinfection (*Micrococcus* spp., *Staphylococcus* spp.) formed mostly very-high and high-density biofilms. We determined that formation of biofilms of high density on surfaces of the boxes helps microbial cells to survive sanitary treatment and disinfection.

The authors declare no conflict of interest.

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