

## CHARACTERIZATION OF AIRBORNE BACTERIAL COMMUNITIES AND EVALUATION OF THE DISINFECTION EFFICACY OF 5% HYDROGEN PEROXIDE IN ANIMAL HOUSING ROOMS AT THE LABORATORY ANIMAL DEPARTMENT

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**Abstract:** The composition of microorganisms in the air directly affects the quality of laboratory animals, staff, and determines the cleanliness level of the animal facility as well as the value of experiments. In order to eliminate pathogenic factors, 5% hydrogen peroxide was used at the Experimental Animals Department to disinfect animal housing rooms and auxiliary areas. The study results showed that the identified bacterial composition was mainly non-pathogenic Gram-positive bacteria under normal conditions, with staphylococci accounting for 72–92%, and other bacteria including *Kocuria rhizophila*, *Enterococcus gallinarum*, *Bacillus atrophaeus*, and *Bacillus pumilus* accounting for 8–28%. The average bacterial density in 12 sampled rooms mostly ranged was generally between 100–500 CFU/m<sup>3</sup> of air, with no room exceeding 2000 CFU/m<sup>3</sup>. After disinfection, bacterial loads decreased by 4 to 30 times. Four weeks post-disinfection, all rooms maintained concentrations below 500 CFU/m<sup>3</sup>.

**Keywords:** *Airborne microorganisms, hydrogen peroxide, laboratory animals, disinfection.*

### 1. Introduction

The quality of air within animal housing facilities significantly influences both the well-being of laboratory animals and the health of personnel handling them. According to Neelam Rani et al. (2018), environmental hygiene conditions can be reflected through the microbiological quality of the air, which is also correlated with disease occurrence. Therefore, it is essential to monitor air quality in animal housing facilities [1].

Based on the findings of a study evaluating the disinfectant efficacy of hydrogen peroxide, Chloramine B, and

Benkocid against selected microbial agents, the Laboratory Animals Department selected 5% hydrogen peroxide for disinfection of animal housing rooms and heat-sensitive equipment [2].

Given that the composition of airborne bacteria varies across different areas, specific sanitation and monitoring protocols are required for breeding facilities of guinea pigs, breeding mice, and experimental animals within the department.

To establish a protocol for monitoring and assessing the microbiological quality of air to ensure the quality of laboratory animals, we conducted the study titled: “Determination of airborne bacterial composition in animal housing facilities

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and evaluation of the disinfection efficacy of 5% hydrogen peroxide in the Laboratory Animals Department”.

## 2. Materials and Methods

### 2.1. Study Subjects

- Study subjects: The composition and density of airborne bacteria in laboratory animal housing facilities, as well as the disinfection efficacy of H<sub>2</sub>O<sub>2</sub> concentration of 5%.

### 2.2. Study Site and Duration

- Study site: Laboratory Animals Department, National Institute for Control of Vaccines and Biologicals.

- Study duration: From May 2024 to December 2024.

### 2.3. Materials and Equipment

#### 2.3.1. Test Samples

- Air samples were collected from animal housing facilities using a MiniCapt 100M air sampler, both before and after disinfection with Hydrogen peroxide concentration of 5%.

#### 2.3.2. Instruments, Equipment, and Reagents

- Equipment: MiniCapt 100M microbial air sampler (code: BS03-MT); Phileas disinfectant sprayer, Devea; Class II biosafety cabinet (code: LH27-TN); Microscope (code: MS17-TN); Incubator (code: IC33-TN); Sample storage cabinet (code: CI26-TN); BD Phoenix bacterial identification system (code: BS01-TN); various types of micropipettes, calibrated and maintained annually in accordance with ISO/IEC 17025 standards.

- Media and reagents: DHL agar, blood agar, BD Phoenix identification panels for Gram-positive bacteria (448614), BD Phoenix identification panels for Gram-negative bacteria (449027), and Gram staining kit.

### 2.4. Methods and Study Design

- Research method: Experimental descriptive study.

- Study design:

<p>Determination of the composition and density of airborne bacteria.</p>	<p>- A MiniCapt 100M air sampler was used to collect 150 liters of air from three areas: the laboratory animal care unit, the mouse breeding unit, and the guinea pig breeding unit, with four rooms in each area, onto blood agar medium. Sampling was performed three times at the beginning of each week, prior to sanitation and disinfection.</p> <p>- All bacterial colonies were counted, and pure cultures of each bacterial type were sub-cultured on blood agar for Gram-positive bacteria and on DHL agar for Gram-negative bacteria.</p> <p>- Bacterial identification was performed using the BD Phoenix™ M50 Automated Microbiology System.</p> <p>- The experiment was repeated three times, and the mean values were calculated.</p>
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Evaluation of the disinfection efficacy of H <sub>2</sub> O <sub>2</sub> concentration of 5% after spraying in animal rooms.	- Air samples were collected in the selected animal rooms 4 hours after disinfection using the Phileas device, with samples collected once per week for one month. The experiment was repeated three times, and the mean values were calculated.
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## 2.5. Content

- Determination of the composition and density of bacteria in 1 m<sup>3</sup> of air in laboratory animal rooms.

- Evaluation of the disinfection efficacy of Hydrogen peroxide concentration of 5% after spraying in animal rooms at the Laboratory Animals Department by determining the composition and density of bacteria in 1 m<sup>3</sup> of air following disinfection, with sampling collected once per week for one month.

## 2.6. Data Analysis Method

- Statistical analysis and data processing were performed using Microsoft Excel software.

## 3. Results

### 3.1. Determination of the Composition and Density of Airborne Bacteria in Animal rooms.

All bacteria detected and identified were Gram-positive. The bacterial composition, which varied across different housing areas, is summarized in Tables 1, 2, and 3.

**Table 1. Bacterial composition in the experimental animal area (percentage, %)**

Room	<i>Sta. equorum</i>	<i>Sta. xylosus</i>	<i>Sta. hemolyticus</i>	<i>Sta. aureus</i>	<i>Ko. rhizophila</i>	<i>E.gallinarum</i>	<i>Bacillus</i>
101	45.50	20.61	5.21	3.32	14.93	7.58	2.84
103	44.66	23.37	5.98	1.90	12.23	10.33	1.54
104	45.07	26.71	4.10	1.01	13.39	7.63	2.09
109	54.28	19.12	2.14	0.80	14.04	7.62	2.01
Gram Stain	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive

The composition of airborne microorganisms in the experimental animal care unit consisted of the following species: *Staphylococcus equorum*, *Staphylococcus xylosus*, *Staphylococcus hemolyticus*, *Staphylococcus aureus*, *Kocuria rhizophila*, *Enterococcus gallinarum*,

*Bacillus atrophaeus*, *Bacillus pumilus*. *Staphylococcus* accounted for 74.64 – 76.89%, *Kocuria rhizophila* for 12.23 – 14.93%, *Enterococcus gallinarum* for 7.58 – 10.33%, *Bacillus* for 1.54 – 2.84%.

**Table 2. Bacterial composition in the mouse breeding area (percentage, %)**

Room	<i>Sta. equorum</i>	<i>Sta. xylosus</i>	<i>Sta. hemolyticus</i>	<i>Sta. aureus</i>	<i>Kocuria rhizophila</i>	<i>E. gallinarum</i>	<i>Bacillus</i>	Other components
201	45.17	23.91	3.86	0.72	8.46	9.42	2.66	5.80
202	53.00	19.46	3.31	0.83	10.97	10.15	1.24	1.03
203	48.97	20.00	2.99	0.92	15.40	9.43	0.69	1.61
204	47.85	24.67	2.82	0.74	15.16	6.24	1.49	1.04
Gram Stain	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive	

The composition of airborne microorganisms in the mouse breeding area included the following species: *Staphylococcus equorum*, *Staphylococcus xylosus*, *Staphylococcus hemolyticus*, *Staphylococcus aureus*, *Kocuria rhizophila*, *Enterococcus gallinarum*, *Bacillus pumilus*. Including, *Staphylococcus* for 72,88 – 76,6%, *Kocuria rhizophila* for 8,46 – 15,40%, *Enterococcus gallinarum* for 6,24 – 10,15%, *Bacillus* for 0,69 – 2,66%, other bacterial components accounted for 1.03–5.8%.

**Table 3. Bacterial composition in the guinea pig breeding area (percentage, %)**

Room	<i>Sta. equorum</i>	<i>Sta. xylosus</i>	<i>Sta. hemolyticus</i>	<i>Sta. aureus</i>	<i>E. gallinarum</i>	<i>Bacillus</i>	Other components
301	65.50	16.96	4.13	1.43	3.64	7.48	0.86
302	62.96	23.51	4.25	0.71	3.47	4.46	0.64
303	66.62	20.65	2.73	0.72	4.39	4.10	0.79
304	73.18	17.17	1.69	0.38	4.06	2.84	0.69
Gram Stain	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive	

The composition of airborne microorganisms in the guinea pig breeding area included the following species: *Staphylococcus equorum*, *Staphylococcus xylosus*, *Staphylococcus hemolyticus*, *Staphylococcus aureus*, *Enterococcus gallinarum*, *Bacillus atrophaeus*, *Bacillus pumilus*. Including, *Staphylococcus* for 88,03 – 92,41%, *Enterococcus gallinarum* for 3,47 – 4,39%, *Bacillus* for 2,84 – 7,48%, other bacterial components accounted for 0,64 – 0,86%.

The average bacterial density in the air of animal room after three sampling repetitions is presented in Table 3.4.

**Table 4. Average bacterial density in the air of experimental animal rooms (CFU/m<sup>3</sup>)**

Room	Average/m <sup>3</sup>
101	312,59
103	817,78
104	1028,89
109	554,07

201	306,67
202	357,78
203	322,22
204	498,52
301	1039,26
302	1045,93
303	1029,63
304	966,67

The summarized results showed that:

- Among the 12 rooms sampled, 7 rooms exhibited an airborne bacterial density of  $>500$  CFU/m<sup>3</sup> in all three inspections, and 1 room showed an airborne bacterial density  $>500$  CFU/m<sup>3</sup> in one inspection.
- The rooms with high airborne bacterial density included the guinea pig breed-

ing rooms (301, 302, 303, 304), the experimental guinea pig room (104), the isolation room (109), and the general safety room for mice (103), whereas the mouse breeding rooms (201, 202, 203) and the mouse observation room prior to quality control transfer (101) all had average bacterial densities  $<500$  CFU/m<sup>3</sup>.

### 3.2. Evaluation of the efficacy of hydrogen peroxide concentration 5% after spraying for disinfection of animal rooms at the Laboratory Animals Department

The efficacy of hydrogen peroxide concentration 5% after spraying for disinfection of animal room is presented in Tables 3.5 and 3.6.

**Table 5. Disinfection efficacy of hydrogen peroxide concentration 5% in animal rooms (CFU/m<sup>3</sup>)**

Room	Before disinfection	After disinfection	Week 1	Week 2	Week 3	Week 4
101	312,59	31,11	97,78	140	175,56	251,11
103	817,78	26,67	164,44	200	253,33	297,78
104	1028,89	62,22	193,33	275,56	328,89	395,56
109	554,07	35,56	146,67	271,11	362,22	375,56
201	306,67	68,89	97,78	126,67	160	231,11
202	357,78	71,11	135,56	186,67	200	280
203	322,22	57,78	144,44	191,11	222,22	251,11
204	498,52	73,33	155,56	188,89	322,22	384,44
301	1039,26	142,22	233,33	322,22	337,78	448,89
302	1045,93	157,78	268,89	288,89	377,78	431,11
303	1029,63	133,33	266,67	322,22	435,56	486,67
304	966,67	82,22	280	371,11	355,56	462,22

- The results from Table 5 showed that, within the first 4 hours after spraying with 5% hydrogen peroxide, most rooms exhibited an airborne bacterial density of

$<100$  CFU/m<sup>3</sup>. The lowest bacterial density was recorded in room 103 (26.67 CFU/m<sup>3</sup>), while the highest was observed in room 302 (157.78 CFU/m<sup>3</sup>).

- After 4 weeks of monitoring, the airborne bacterial density in all rooms remained below 500 CFU/m<sup>3</sup>. The lowest density was observed in room 201 (231.11 CFU/m<sup>3</sup>), while the highest was recorded in room 303 (486.67 CFU/m<sup>3</sup>). *Staphylococcus aureus* is a bacterial agent that requires monitoring in laboratory animals due to its ability to cause abscesses and dermatitis; at high densities, it may lead to disease and thereby compromise research outcomes. Therefore, evaluation after disinfection is necessary. The immediate efficacy of H<sub>2</sub>O<sub>2</sub> concentration 5% spraying in animal room against *Sta. aureus* is expressed in Table 3.6.

**Table 6. Post-disinfection efficacy of hydrogen peroxide concentration 5% in animal housing facilities against *Staphylococcus aureus* (CFU/m<sup>3</sup>)**

Room	Before disinfection	Immediate after disinfection
101	3,32	0
103	1,90	0
104	1,01	0
109	0,80	0
201	0,72	0
202	0,83	0
203	0,92	0
204	0,74	0
301	1,43	0
302	0,71	0
303	0,72	0
304	0,38	0

- The above results demonstrated that immediately after disinfection, 100% of *Sta. aureus* bacteria were completely eliminated.  
 - Some images of bacterial culture results after one sampling round.



**Figure 1. Bacterial culture results from room 109 before disinfection**



**Figure 2. Bacterial culture results from room 109 after 4 hours of disinfection**



**Figure 3. Bacterial culture results from room 109 after 1 week of disinfection**



**Figure 4. Bacterial culture results from room 109 after 2 weeks of disinfection**



**Figure 5. Bacterial culture results from room 109 after 3 weeks of disinfection**



**Figure 6. Bacterial culture results from room 109 after 4 weeks of disinfection**

## 4. Discussion

### 4.1. Composition and density of airborne bacteria in laboratory animal rooms

The quality of air in animal housing facilities plays a crucial role in the health of both laboratory animals and the personnel handling them. Environmental hygiene conditions can be reflected through the microbiological quality of the air, which is also correlated with disease occurrence and directly affects the quality of laboratory animals [1]. Differences in geographical location, climate, and infrastructure of

laboratory animal facilities domestically and internationally result in variations in bacterial composition and density. Therefore, it is necessary to study the composition and density of bacteria in the housing rooms of the Laboratory Animals Department.

As the animal housing facilities of the Laboratory Animals Department use an HVAC system, all rooms and auxiliary areas are provided with similar environmental conditions. The experimental rooms are designed as closed systems, resulting

in no changes in the airborne bacterial composition across all inspections. All detected bacteria were Gram-positive, with no Gram-negative bacteria observed. The primary bacterial constituents in the airborne microbiota of the housing areas were *Staphylococcus* spp., accounting for 72–92%, while other bacteria including *Kocuria rhizophila*, *Enterococcus gallinarum*, *Bacillus atrophaeus*, *Bacillus pumilus*, and other components made up 8–28%. No significant bacteria that may occur in room air, such as *Pseudomonas aeruginosa* or Enterobacteriaceae, were detected. Most of these species are non-pathogenic under normal conditions. However, during some air sampling events, one agent listed among the monitored bacteria in the department, *Staphylococcus aureus*, was detected at a low proportion ranging from 0.38–3.32%. If present at high levels in the air, this agent could pose a risk of disease to laboratory animals; therefore, it needs to be controlled through hygiene and disinfection programs to reduce its abundance in the airborne microbiota. Monitoring results immediately after spraying with 5% H<sub>2</sub>O<sub>2</sub> showed that all *Sta. aureus* in the rooms were completely eliminated. This is consistent with previous laboratory studies, which demonstrated that 5% H<sub>2</sub>O<sub>2</sub> can eliminate 10<sup>5</sup> CFU/ml of *Sta. aureus* [2]. However, as *Staphylococcus aureus* is a commensal bacterium on the skin and mucosa of humans and animals, disinfectants have only a short-term effect. According to a 2019 study by Marian A. Esvelt et al., airborne microbiota analysis on the lids of ICR mouse cages using an air-supplied cage system still detected *Sta. aureus*, albeit in very low numbers [7].

The results of this study showed that air sampling from 12 rooms across three areas of the Laboratory Animals Department revealed no room with a bacterial density classified as “very high” (>2000 CFU/m<sup>3</sup> air). However, seven rooms were classified as having a “high” bacterial density (500–2000 CFU/m<sup>3</sup> air) according to the European Commission standards for non-industrial facilities [5]. These rooms included the guinea pig breeding units 301 (1039.26 CFU/m<sup>3</sup>), 302 (1045.93 CFU/m<sup>3</sup>), 303 (1029.63 CFU/m<sup>3</sup>), and 304 (966.67 CFU/m<sup>3</sup>), as well as the testing animal rooms 103 (817.78 CFU/m<sup>3</sup>), 104 (1028.89 CFU/m<sup>3</sup>), and 109 (554.07 CFU/m<sup>3</sup>). The primary reason is that these rooms housed guinea pigs and rabbits without bedding, which resulted in moist environments due to the accumulation of urine and feces, leading to high air humidity; alternatively, they were mouse housing rooms with animal densities relatively high compared to the room area. Rooms with bacterial densities <500 CFU/m<sup>3</sup> air were those belonging to the mouse breeding area and the monitoring room for mice prior to quality control testing. These rooms used rice husk bedding with good moisture absorption capacity, thereby maintaining relatively stable humidity. This finding is consistent with the study of Yujia Qiu et al. (2022), which reported that under nutrient-rich conditions at 26 °C, with humidity increasing from 50–70% and absence of ventilation, bacterial growth increased by 2.8-fold. Therefore, to limit bacterial growth, ventilation should be improved and humidity reduced [6].

The airborne bacterial density in laboratory animal housing facilities

observed in this study was relatively high compared with those reported in some countries such as India and Thailand. According to the National Laboratory Animal Center, Mahidol University, Thailand, the minimum airborne microbial density in animal rooms was reported to be below 15 CFU/ft<sup>3</sup>/min (equivalent to <500 CFU/m<sup>3</sup>) [3]. Similarly, a study conducted in 2016 at the Disease-Free Small Animal House (DFS AH), LUVAS, Hisar, India reported maximum airborne bacterial densities of 310 CFU/m<sup>3</sup> in guinea pig housing rooms and 347 CFU/m<sup>3</sup> in mouse housing rooms [1]. The difference may be attributed not only to facility infrastructure but also to the use of bedding materials with good moisture-absorbing capacity, which helped reduce humidity in the cages and thereby limited microbial growth [1].

#### ***4.2 Evaluation of the effectiveness of hydrogen peroxide concentration 5% disinfection after spraying in animal housing facilities at the Laboratory Animals Department***

After spraying the animal housing rooms with 5% hydrogen peroxide, air sampling results within the first 4 hours showed that the average airborne bacterial density decreased by 4–30 fold, with 9 out of 12 rooms achieving <100 CFU/m<sup>3</sup>, classified as “low” according to the European Commission standards for non-industrial facilities [5]. The 5% hydrogen peroxide solution completely eliminated *Staphylococcus aureus* in the air of the rooms at all sampling tests. This effect can be attributed to the fact that hydrogen peroxide is a strong oxidizing agent capable of oxidizing macromolecules that constitute

the structure and function of microorganisms, such as proteins, lipids, carbohydrates, and nucleic acids. Such effects accumulate over time during exposure, leading to structural and functional loss, and ultimately to the inactivation of microorganisms and their components (e.g., toxins). It is also possible that a cascade of free radicals (particularly hydroxyl radicals, -OH) and the reactions of microbial species that decompose peroxide into water and oxygen contribute to the overall bactericidal activity of hydrogen peroxide [2].

After 4 weeks of disinfection, airborne bacterial density was maintained below 500 CFU/m<sup>3</sup>, with the lowest recorded in room 201 (231.11 CFU/m<sup>3</sup>) and the highest in room 303 (486.67 CFU/m<sup>3</sup>). This variation may be attributable to differences in room size, temperature, humidity, animal density, and the presence or absence of environmental obstructions. Therefore, the disinfection effectiveness of 5% hydrogen peroxide was more evident in mouse housing rooms.

#### **5. Conclusion**

This study determined that the average bacterial density in 12 sampled housing rooms was classified as moderate (100–500 CFU/m<sup>3</sup>) to high (500–2000 CFU/m<sup>3</sup> of air), according to the European Commission microbiological standards for non-industrial facilities.

Due to variations in factors such as stocking density, temperature, humidity, air exchange frequency, and airflow rate vary, the composition and proportion of airborne bacteria differed among the housing areas. Among them, one agent

requiring surveillance was identified as *Staphylococcus aureus*, accounting for 0.38–3.32%.

Disinfection of animal rooms with hydrogen peroxide concentration 5% reduced the average airborne bacterial density by 4- to 30-fold. Four weeks after disinfection, the bacterial density in all tested rooms remained below 500 CFU/m<sup>3</sup> of air.

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