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QUALITIVE AND QUANTITATIVE STUDY OF THE MICROBIAL FLORA IN VETERINARY CLINICAL INCUBATORS

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Abstract

Veterinary incubators play a critical role in modern veterinary medicine; however, they can also serve as reservoirs for microbial contamination. The present study aims to assess the presence and the diversity of microorganisms in veterinary incubators used in different veterinary clinics. Swab samples were collected from various inner surfaces across multiple incubators; the Koch method was applied for airborne infections control as well as liquid samples obtained from the air filtration components. The resulting CFU was compared to the references provided by the Romanian Health Ministry Order 961/2016. Microbial identification was performed by inoculating selective growth media, biochemical techniques, and identification of characteristics of microorganisms macroscopically and microscopically. A questionnaire was provided to the employes to assess their knowledge on incubator hygiene. The qualitative methods of examination revealed a diverse range of bacterial and fungal species. Obvious differences in microbial load were observed for both airborne and surface microflora based on incubator usage and sanitation frequency. These findings highlight the importance of updating cleaning and disinfection protocols and implementing routine microbiological monitoring to ensure the safety of veterinary patients.

Key words*: microorganisms, incubators, Koch, swab test, API, selective media.*

INTRODUCTION

Clinical incubators are essential devices in contemporary veterinary medicine. They improve treatment outcomes in various conditions, mainly in treating: hypothermia, neonates, postoperative recovery and patients in respiratory failure.

Patients in the aforementioned categories, that benefit the most from the use of incubators, also present an increased susceptibility to infections. Other factors that further amplify the risk of infections are the prolonged contact between the device and those in treatment, needed for optimal therapeutic results. Moreover, the incubators, with their inherently complex structure, require multiple steps in order to perform an effective disinfection.

According to the manufacturer's instructions the incubators surfaces must be disinfected after each patient, the air and humidity filtration components need to be removed and disinfected weekly and completely replaced every 6 months.

Research on neonatal incubators in human medicine reveal that infections can often occur, despite strict cleaning and disinfection protocols. Elevated CFU values have been reported in incubators with over 60% humidity, that present some colder spots (local internal temperature bellow 34°C). Taking those elements into consideration, we can identify high risk zones for potential microbial contamination by measuring local temperature variations within incubators (Lemmen et al., 2011). Other studies suggest that 24 h after disinfection, the bacterial load inside incubators was similar to that before decontamination (Mesquita et al., 2021). In another study of a unit with recurring *Enterobacter* infections in neonates, the epidemiological investigation identified neonatal incubators as the primary reservoir/source of infection. Using a new method of sampling airborne microflora: while the incubator was in use, led to identifying the source and replacing the devices thus preventing further infections. Said technic can aid us in monitoring airborne microflora more efficiently thus reducing the risk of nosocomial infections (Hernandez-Alonso et al., 2022).

Even though such problems are well documented in human medicine, we can remark

that in case of incubators used in veterinary clinics, our knowledge is incomplete or even nonexistent. The present study aims to evaluate the microflora of veterinary incubators through quantitative and qualitative microbiological analysis thus providing an original contribution in the field of infection control in veterinary medical settings.

Figure 1. Air filter in poor hygiene condition

MATERIALS AND METHODS

Samples were collected from six private veterinary clinics in Bucharest, over a period of eight months (November 2024 - May 2025). In total eight incubators were sampled, out of which three belong to the first clinic, the other specimens were sampled from one incubator per clinic. Laboratory analyses were carried out at the Faculty of Veterinary Medicine, University of Agronomic Sciences and Veterinary Medicine of Bucharest.

Air samples were obtained using the settling plates method (passive Koch's method) by exposing Petri dishes containing nutrient agar and Sabouraud agar supplemented with chloramphenicol inside the incubators for 5 minutes. Surface samples were collected by rubbing sterile swabs, moistened in saline solution, over 25 cm^2 areas that are most commonly in direct contact with animals. The swabs were then immersed in 0.5 ml sterile saline solution the resulting suspensions were inoculated directly or after serial dilutions (10^{-1}-10^{-2}) and inoculated onto solid culture media (Mitranescu, 2014). In some cases, liquid samples were also obtained by performing a lavage of the incubator's filtration components: air (sponges) and humidity filters.

Both solid and liquid cultures were incubated at 37°C for 24-72 h. After incubation the total count of aerobic mesophilic bacteria (TCAM) obtained on solid cultures from the swab tests was calculated as colony-forming units per cm^2 of surface, by using the standard formula. (Food Standards Scotland, nd) Airborne microbial load was expressed as CFU/m^3, following the plates' exposure and incubation computed by using the Omeliansky volumetric conversion formula. Results were compared with the permissible microbiological limits set by the Romanian Ministry of Health in decree Order no. 961/2016: ≤5 CFU/cm^2 for surfaces, ≤300 CFU/m^3 for airborne microorganisms, and with recommended literature thresholds for fungi respectively ≤150 CFU/m^3 (Pires, 2021) (Health Ministry ordinance 961/2016).

Solid and liquid selective media were used to enhance recovery and preliminary identification of microorganisms: Chapman broth and agar (for staphylococci), Mossel broth (for enterobacteria), Bile-Esculin broth and agar (for enterococci), MacConkey agar (for Gram-negative bacilli), Columbia blood agar (for fastidious bacteria), Sabouraud agar with antibiotics (for fungi) and Baird-Parker agar with egg yolk emulsion (for staphylococci).

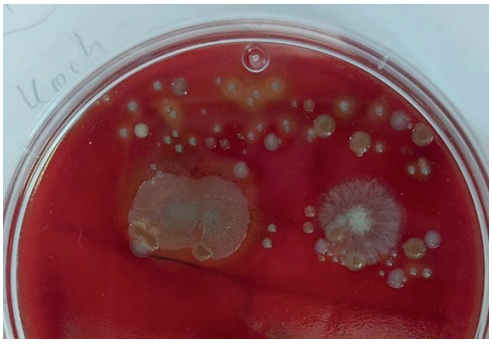

Figure 2. α-hemolytic, β-hemolytic, and non-hemolytic colonies on Colombia blood agar

Representative colonies were isolated for further investigations, including Gram staining and biochemical traits' testing. Staphylococci isolated from selective media were identified by using the API Staph system, while Gram-negative bacilli were tested using API 20E system. In total, multiple isolates obtained from Columbia blood agar and MacConkey agar

cultures were subjected to biochemical analysis. Microscopic and macroscopic identification of fungi was performed.

The present research was continued by conducting a survey. A short questionnaire was distributed to 20 veterinary professionals to assess the actual awareness levels about decontamination and maintenance practices across all six participating clinics as illustrated on Tabel 1.

Table 1. Participants' distribution

Veterinarians	11	1	2	0	0	1
Veterinary technicians	2	0	2	0	1	0

Table 2. Methods used for quantitative and qualitative assessment of incubators' microbial load, and number of samples collected

A	4	3	Bile-Esculin, Chapman, Mossel, MacConkey and Columbia (blood agar) Media	API 20E	3 air filters	2 Gram stain 2 fungi
B	4	3	Bile-Esculin, Chapman, Mossel, MacConkey and Columbia (blood agar) Media	API 20E	3 air filters 1 humidity filter	2 Gram stain 1 fungus
C	4	4	Bile-Esculin, Chapman, Mossel, MacConkey, Baird-Parker and Columbia (blood agar) Media	4 API Staph 2 API 20E	3 air filters	2 Gram stain
D	2	2	MacConkey medium	-	-	2 Gram stain
E	2	2	-	-	-	-
F	2	2	-	-	-	-
G	2	4	MacConkey and Columbia (blood agar) Media	API 20E	-	1 Gram stain
H	2	2	-	-	-	1 Gram stain

RESULTS AND DISCUSIONS

All the incubators evaluated presented an internal humidity of over 60% and a temperature lower than 34°C at the time of sample collection posing high contamination risks. (Lemmen et al., 2011).

Incubators A and B were disinfected using Virkon S and presented low levels of surface contamination. In contrast, incubator C, while belonging to the same clinic as the first two, and being disinfected with the same solution by the same staff, exhibited a considerably higher degree of contamination compared to the other two devices. Incubator D was most recently disinfected with ethanol two weeks before sample collection, right after its latest use, and the results indicated contamination levels close to the upper permissible limit. Incubator E was disinfected alternately with Virkon S or Dezicon solutions. While being in daily use, and disinfection being performed after each patient; the microbial load was consistent with this usage pattern. Incubator F, which yielded the most favourable results among all tested devices, was disinfected after each patient using an integrated ultraviolet (UV) lamp system. Incubator G, disinfected by using Innolin (an alcoholic surface disinfectant), yield a very particular result: despite presenting a very high level of contamination, cultures were almost pure, dominated by a sole bacterial species. Lastly, incubator H provided promising results despite a constant patient flow. It was disinfected alternately with Virkon S or Chloramine, demonstrating that appropriate implementation of chemical disinfection protocols can sometimes be effective.

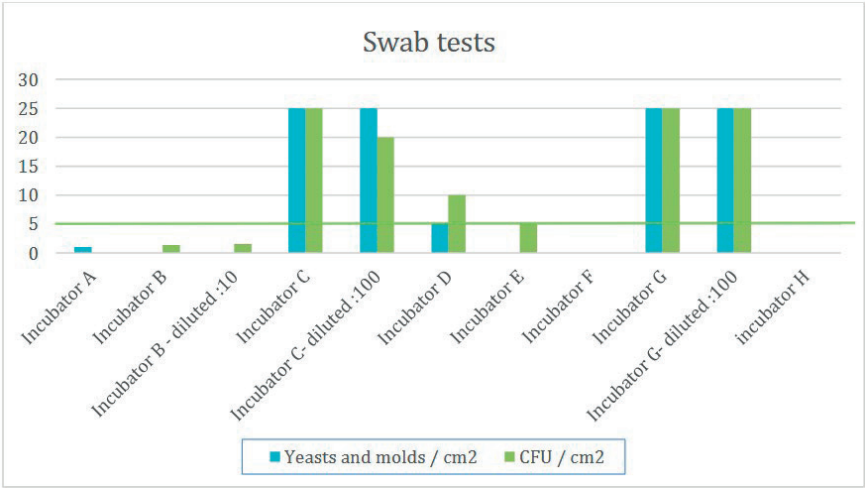

Figure 3. Swab test results of the total count of aerobic mesophilic bacteria and the total fungal count on the inner surfaces of the incubators, expressed per cm². Incubators' C, G, and G-100 CFU was too high to be numbered. The green horizontal line represents the legal threshold established by the Romanian Health Ministry

The air filters of incubators A, B, and C had been sanitized and replaced around the same time, at an unknown date but well beyond the recommended interval. Incubator A showed a low level of airborne contamination, likely due to a reduced flow of patients and limited contact time between patients and the device. Incubator B displayed a higher level of contamination, although still within permissible limits, the only difference compared to incubator A being the increased patient flow. Incubator C, by contrast, far exceeded the admissible microbial limits in airborne microflora, possibly as it was used for the treatment of animals with infectious diseases and due to the staff's laxity regarding proper cleaning and disinfection protocols. Incubator D also exceeded the acceptable threshold by a wide margin, due to the filters not being sanitized regularly-the date of their last replacement being unknown. Incubator E presented values close to the legal limits; while the filters were not sanitized, they were systematically replaced every six months by the supplier.

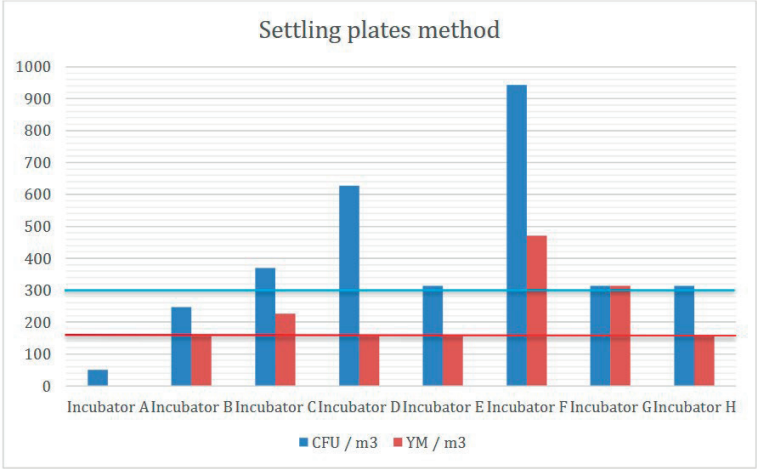

Figure 4. Koch method results for the total aerobic mesophilic bacteria count and the total fungal count in internal air flow, expressed per 1 m³ of air. The blue line indicates the legal threshold for the total bacterial count, while the admissible limit for the total fungal count

Incubator F presented the highest level of airborne contamination among all devices, as its filters had neither been sanitized nor replaced since the acquisition of the device (over two years ago).

The filters of incubator G were sanitized regularly by clinic staff, but the last replacement date was unknown. Despite a low patient flow, this incubator showed particularly high fungal contamination, while the total microbial count was close to the acceptable limit. Incubator H had bacterial as well as fungal flora close to the admissible thresholds. Data on its filter sanitation and replacement protocols were not available, although the incubator was subjected to a high flow of patients.

Microbial findings

***Rhodotorula* spp**. Identified on Sabouraud–Chloramphenicol agar inoculated by the settling plates method in incubator B, it was evaluated based on its cultural and microscopic characteristics. Common environmental yeast, it adheres to plastics, occasionally causes opportunistic infections (of the skin, wounds, ears, fungemia as well as lungs, spleen and liver infections in immunocompromised animals) (Wirth, 2012).

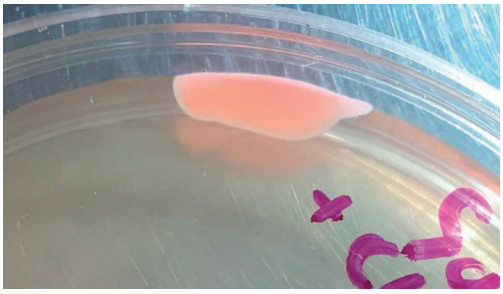

Figure 5. *Rhodotorula* spp. collected using the Koch method from incubator B

***Aspergillus* spp.** Isolated on Sabouraud-Cloramphenicol agar, inoculated with the liquid sample resulting from incubator A's swab test. It is a ubiquitous mold, mainly respiratory pathogen, that can disseminate in other tissues (Gull, 2023a).

***Penicillium* spp.** Isolated on Sabouraud-Cloramphenicol agar, inoculated with the sample from incubator A's swab test. Usually environmental contaminant; rarely causes opportunistic skin or respiratory infections (Gull, 2023a).

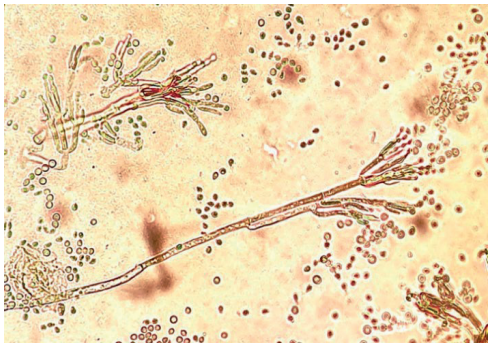

Figure 6. *Penicillium* spp., optic microscopy, hyphae and spherical spores

***Mucor* spp**. It developed on an agar plate inoculated with the liquid sample resulting from incubator C's swab test. It is an opportunistic fungus causing mucormycosis, potentially fatal if untreated (Gull, 2023b).

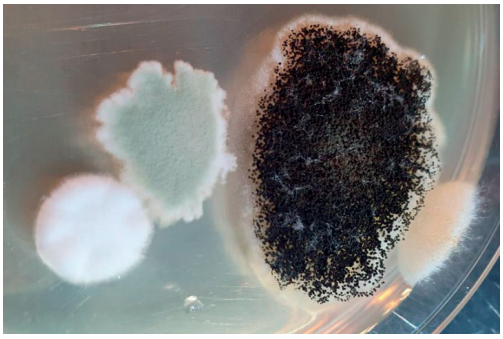

Figure 7. *Penicillium* spp., *Aspergillus* spp., *Mucor* spp., and other fungi obtained using the Koch sampling method from incubator A

Bacillus cereus Was isolated from samples obtained from the lavage of the air and humidity filters of the incubators A, B and C. It is an opportunistic pathogen; causing mostly foodborne intoxications (diarrheal, emetic forms) (Veterian Key, 2022).

***Enterococcus* spp.:** Was isolated from multiple samples: air filters and swab tests using selective culture medium. They are commensals with virulence factors (biofilm, enzymes); resistant to various antibiotics and difficult to eliminate (Sykes, 2016).

Escherichia coli: Isolated from incubator C's swab test using Mossel selective culture medium. It is an environmental and intestinal commensal bacterium; that can cause causes urinary, uterine, wound, neonatal, and GI

infections, if it migrates as well as sepsis. (Veterinar la domiciliu, n.d.)

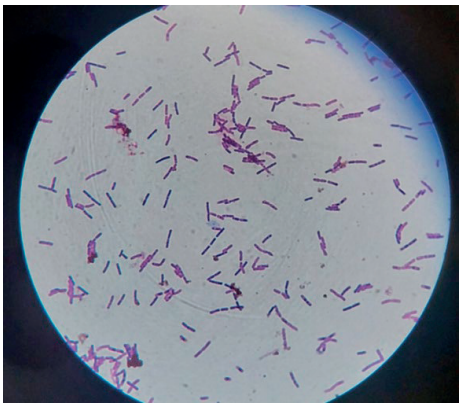

Figure 8. *Bacillus* spp., Gram staining

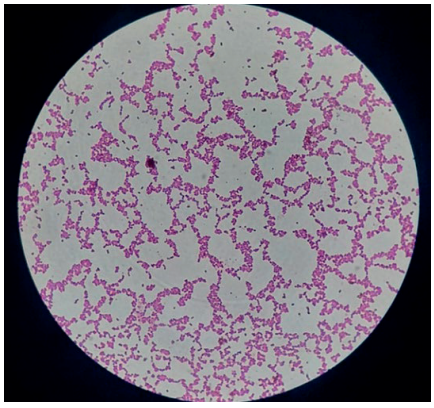

Figure 9. *Enterococcus* spp., Gram staining

Bacterium of the genus *Staphylococcus* were isolated on Chapman agar medium inoculated with the swab test sample from incubator C, and subsequently identified using API Staph biochemical tests.

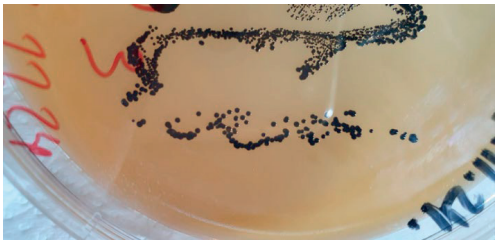

Figure 10. *Staphylococcus* spp., Braid-Parker agar

Staphylococcus auricularis. It is part of the normal microbial flora of the skin and ears, having a low virulence, and is rarely implicated in pathology. It is occasionally isolated in cases of otitis externa, more often as a secondary agent (Miszczak, 2023).

Staphylococcus epidermidis. Typically, a harmless symbiont, but it is also recognized as an opportunistic pathogen and among the most frequent causes of nosocomial infections, with incidence rates comparable to *Staphylococcus aureus*. It has been associated with wound infections, otitis, urinary tract infections, and device-related infections (catheters, orthopedic implants), and may progress to bacteremia or endocarditis (Lee & Anjum, 2023).

Staphylococcus capitis. Rarely reported in veterinary medicine, it has occasionally been isolated from skin and ear infections. Its clinical significance is often unclear, as it may represent contamination or colonization, though neonatal patients can be affected (Perianu, 2011; Miszczak, 2023).

Staphylococcus caprae: Although rarely reported in companion animals, it may act as an opportunistic pathogen in wound and device-related infections. More common in ruminants, it is considered an emerging concern in domestic animals, especially in bone, joint, and bloodstream infections in immunocompromised hosts (Perianu, 2011; Miszczak, 2023).

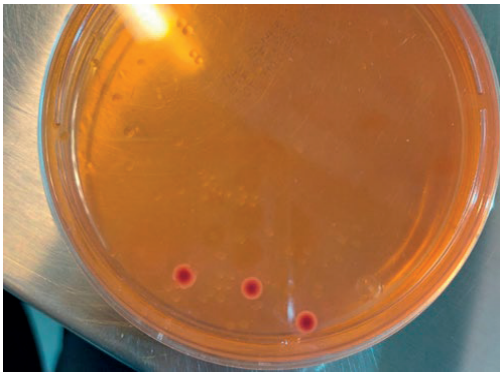

Figure 11. Lactose-fermenting colonies (pink): *Klebsiella pneumoniae*; non-lactose-fermenting colonies (transparent): *Burkholderia cepacia*

Burkholderia cepacia: The isolate was recovered on nutrient agar and Sabouraud–Chloramphenicol media from the sanitation test of incubator G, demonstrating intrinsic resistance to chloramphenicol. Identification was carried out using the API 20E system. A

major contaminant of pharmaceutical products and medical devices, due to its resistance to disinfectants and antimicrobial agents. It spreads through aerosolized particles, direct contact or contaminated surfaces (Tavares et al., 2020).

Burkholderia cepacia complex is considered an emerging opportunistic pathogen in companion animals, associated with deep pyoderma, urinary tract infections, respiratory disease, urinary tract infections, respiratory disease and sepsis in immunocompromised animals (Cain et al., 2018).

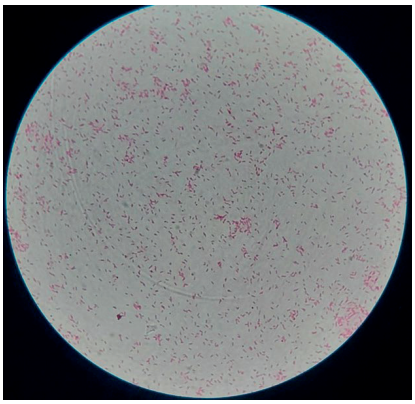

Figure 12. *Burkholderia cepacia*, Gram staining

Klebsiella pneumoniae: The isolate was recovered from the filter lavage sample on MacConkey agar. Environmental and commensal bacterium; that causes respiratory, urinary, enteric, reproductive, otic, dermal and systemic infections, with zoonotic potential (Ribeiro, 2022).

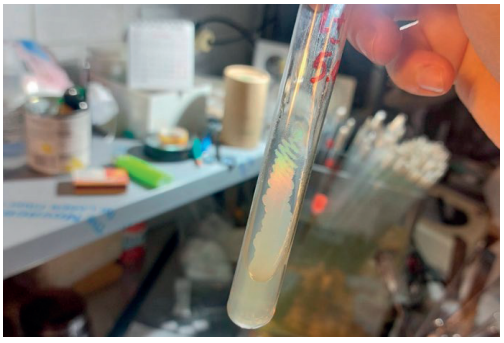

Figure 13. *Klebsiella pneumoniae*, agar slant culture

Some species couldn't be identified including: fungi, Gram positive bacilli, Gram negative coccobacilli, Gram positive cocci, α-haemolytic, β-haemolytic, non-haemolytic bacteria and more.

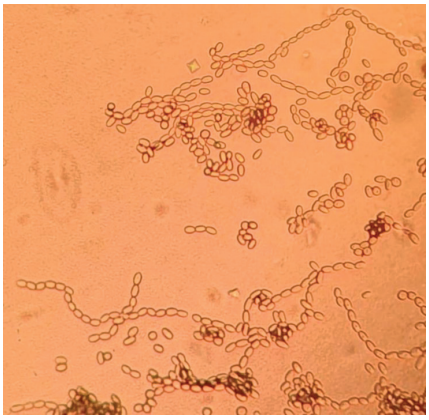

Figure 14. Unidentified fungus optic microscopy

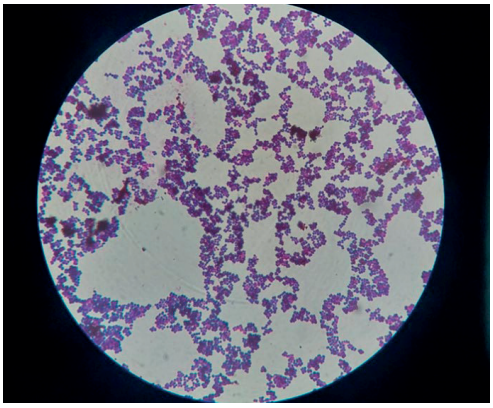

Figure 15. Gram positive cocci, Gram staining, Optic microscopy

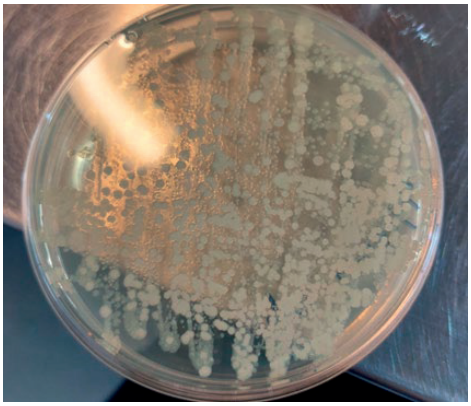

Figure 16. Solid agar culture inoculated with 0.1ml liquid collected from the humidity filter of incubator B

Results of the survey on the awareness of incubator decontamination and maintenance protocols.

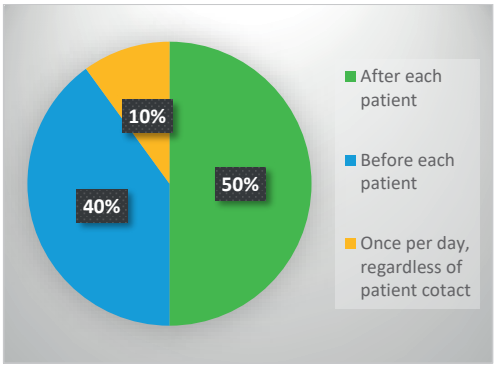

Figure 17. Veterinary clinical incubators' surfaces disinfection frequency awareness

Most participants were unaware that microbial flora can return to pre-disinfection levels within 24 h after chemical cleaning. Regular surface cleaning before admitting a new patient is recommended (Mesquita et al., 2021).

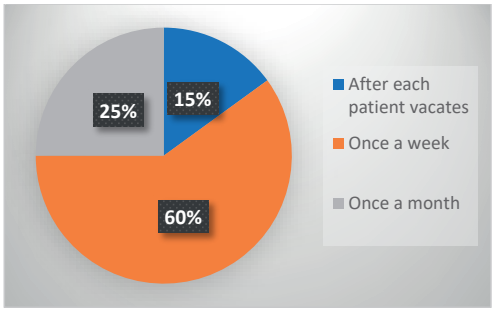

Figure 18. Veterinary clinical incubators' air and humidity filters disinfection frequency awareness

60% of participants knew that filters must be cleaned, but none of them knew when the last cleaning was performed, since there are no official records for every cleaning operation. According to manufacturer guidelines, cleaning should occur weekly (Rcom BROODER, n.d.).
Most staff believed assistants/technicians are responsible for the weekly disinfection of the filtration components. But they, themselves reported no prior training on methods also no materials were provided to said assistants from other staff or the manufacturing company.
Only one out of the six participating clinics had replacement protocols in place, under contractual terms with the device's supplier. No participants, out of all six clinics in question, were aware of the date the filters were last replaced. Manufacturer guidelines recommend changing filters every 6 months. About 35% of respondents admitted not knowing the correct answer (Rcom BROODER, n.d.).

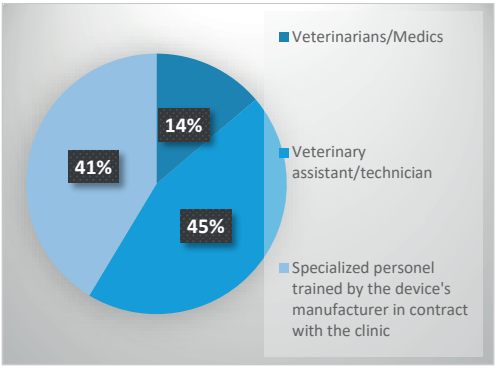

Figure 19. Delegation of the responsibility to regularly disinfect incubators' filtration components

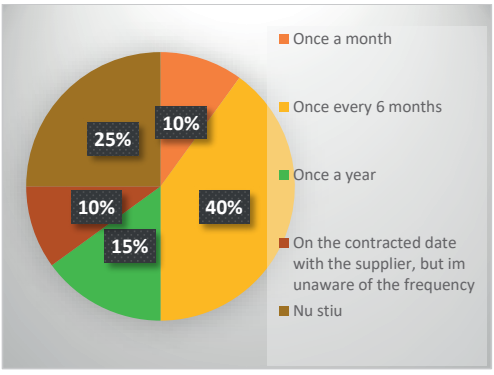

Figure 20. Awareness of filter replacement frequency/protocols

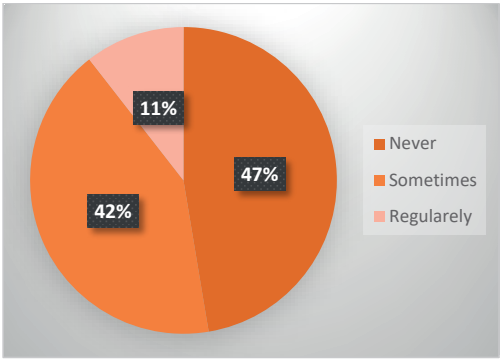

Figure 21. Personal involvement in filter disinfection frequency

Only 11% of staff across all clinics reported personally disinfecting incubators' filters regularly.

Figure 22. Air filters in use after surpassing their maximum filtration capacity

CONCLUSIONS

The most important conclusion of this study is the need for more detailed research on the microbial reservoirs represented by clinical incubators and the risk that they represent for veterinary patients as well as possible methods to reduce said risk, furthermore the need for improved disinfection methods possibly by combining two or more to obtain a synergic effect.

As well as identifying the specific points within the incubators with an elevated risk of microbial contamination in an effort of bettering the cleaning protocols as well as the design of future incubator models.

This study showcases the need for a better understanding of the correlation between incubators and nosocomial infections, by including the incubator's number in patients' medical records for an easier correlation in case of any possible future infections and thus limiting our response time in case of an outbreak.

It is important to implement routine microbiological testing of incubators surfaces, air, as well as relevant objects and personnel's hands to prevent possible contaminations, given the pathogenetic potential of some of the microorganisms identified in this study (ex. *Burkholderia cepacia).* Data collected from those tests can aid in researching the source and distribution pattern of microorganisms in clinical incubators' microclimate. Thus, finding an explanation for the surprising difference between airborne and surface microbial contamination levels in the same incubator's environment showcased in this study.

Lastly informing and properly training the members of staff that use these devices on the proper disinfecting protocols and maintenance that they require. This type of information that as proved by the questionnaire is not widespread therefore increasing the risk for patients and possibly masking design or protocol flaws that should be addressed.

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