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Evaluating ATP Bioluminescence Testing for Microbial Surveillance of Materials and Solutions Used in the Routine Cleaning of Cranial Implant Chambers in Rhesus Macaques (*Macaca mulatta*)

Kristen Bagley, DVM,[1,*] Charlotte Armstrong, BS, LAT,[2] Benjamin Risk, MS, PhD,[3]
Fawn Connor-Stroud, DVM, DACLAM,[4] Denyse Levesque, DVM,[4] and Adriana Galvan, PhD[2]

Nonhuman primates with long-term cranial implant chambers require regular chamber cleaning with antiseptic solutions. The purpose of this study was to determine if ATP bioluminescence testing can be used to identify microbial contamination of fomites and environmental samples in the context of cranial implant chamber cleaning procedures. ATP bioluminescent swab samples were compared with traditional bacterial culture swab samples from the same sources collected during the scheduled chamber maintenance procedures for Rhesus macaques (*Macaca mulatta*) that are part of studies related to the modulation of brain circuits in parkinsonism. Over the course of 17 d, samples were collected from the chamber rims, forceps (pre- and postcleaning), povidone-iodine bottle, quaternary ammonium and alcohol-based disinfectant solution containers, and cotton ball jar (ATP swab: n = 10 per environmental source; bacterial culture swab: n = 6 per environmental source). Chamber rims yielded the highest ATP relative light unit values compared with the other environmental sample groups and heavy growth on bacterial culture. A total of 16/36 (44%) swab samples from environmental sources yielded growth on bacterial culture, and clinically relevant bacterial species were identified in samples from the chamber rims, cotton ball jar, povidone-iodine bottle, and forceps. Although high ATP RLU levels and positive bacterial growth were identified for these environmental samples, there was a poor correlation between the ATP RLU values with the semiquantitative bacterial culture scores. Based on the results of this study, a high ATP RLU cutoff threshold would be needed to maximize the accuracy of using this method instead of bacterial culture to identify potential sources of microbial contamination. This study represents the first published microbial surveillance investigation of environmental samples from materials and solutions used in cranial implant chamber maintenance.

Abbreviations and Acronyms: ATP, adenosine triphosphate; ENPRC, Emory National Primate Research Center; PVP-I, povidone-iodine; QAC-IPA, quaternary ammonium chloride-isopropyl alcohol; ROC, receiver operator characteristic; RODAC, replicate organism detection and counting; RLU, relative light unit

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Introduction

Nonhuman primates serve as the most translationally relevant species for neuroscience research, given their anatomic, physiologic, and behavioral similarities to humans.[1–3] Neuroscience studies with nonhuman primates have led to breakthroughs in our understanding of Parkinson's disease, epilepsy, stroke, cognition, as well as neurodevelopmental conditions and neuropsychiatric illnesses.[3] An ad hoc committee of the National Academies of Sciences, Engineering, and Medicine recently evaluated the anticipated future needs for nonhuman primates in NIH-funded research and concluded that neuroscience and neurodegenerative disorders will be a priority, as the burden of these diseases will increase with an aging population.[3] The study of nonhuman primate models is critical to develop new methods of prevention, diagnosis, and treatment.[2]

Some of the studies that investigate neural circuits underlying neurologic and psychiatric diseases in nonhuman primates rely on the ability to chronically access brain tissue to conduct electrophysiological recordings or other procedures that monitor and modulate neuronal activity. This is frequently achieved by placing cranial implant chambers into the animals.[4,5] Cranial implant chambers may vary in their design depending on scientific needs. One design (used in this laboratory) involves the surgical placement of covered chambers overlaying an exposed dura mater. Other designs surgically place chambers onto intact bone, with a subsequent procedure to create small burr holes for electrode insertion.[6] In addition, a third design involves the removal and replacement of the dura with an artificial substitute before chamber placement.[6] Cranial implant chambers allow access to targeted brain regions.[7–9] Procedural refinements to postsurgical chamber care and maintenance have occurred over time to improve animal welfare.[4,10–13] However, the current landscape has few universal practices for long-term chamber

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[1]Department of Comparative Medicine, The University of Texas MD Anderson Cancer Center, Michale E. Keeling Center for Comparative Medicine and Research, Bastrop, Texas; [2]Division of Neuropharmacology and Neurologic Diseases, Emory National Primate Research Center, Atlanta, Georgia; [3]Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, Georgia; and [4]Division of Animal Resources, Emory National Primate Research Center, Atlanta, Georgia
**Corresponding author. Email: kbagley@mdanderson.org*
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care and maintenance.[13] With any chronically implanted device, there is a risk for clinical complications.[13–15] These complications may include infection of the chambers, which can be treated with either topical or systemic antimicrobials. Timely and appropriate treatment of chamber infections is necessary to prevent more serious complications, such as meningoencephalitis.[13] Given the increasing global prevalence of antimicrobial resistance,[16–21] prevention of infection is essential to minimize the use of systemic antibiotics and preserve the future efficacy of these drugs.

Regular maintenance cleaning of chamber implants is required to prevent infection. The Association of Primate Veterinarians (APV) has published guidelines for Cranial Implant Care. Neuroscience laboratories and veterinary collaborators have established internal procedures, based on the APV guidelines, that aim to prevent complications of cranial implant chambers, including infections.[4,7–9,13] In our laboratory, all animals with a cranial implant have their chambers cleaned at least 3 times weekly. The cleaning process involves the use of sterile saline, povidone-iodine antiseptic solution and exchanging the chamber lid for a disinfected replacement. Disinfected forceps are used to manipulate autoclaved cotton balls to facilitate lavages. Biocidal disinfectant and antiseptic solutions are preferred for maintenance cleanings, as they have a wider spectrum of activity against microbes than topical or systemic antibiotic medications.[4,22–24]

Still, certain species of microorganisms, including *Mycobacterium* and the spore-forming *Clostridium* species, are inherently resistant to specific topical antiseptics and surface disinfectants, and other microbial species are acquiring resistance to these compounds.[23,25–29] In both veterinary and physician-based medical contexts, microorganisms have been identified inside bottles of disinfectant solutions and have led to case reports of hospital-acquired infections.[30–45]

Microbial surveillance involving environmental sampling is essential to identify trends in resistance to these antiseptics and surface disinfectants. Our institution currently relies on 3 main methods to validate our environmental sanitation practices: replicate organism detection and counting (RODAC) plates, bacterial cultures, and ATP bioluminescence assays.[46–48] Although sensitive and specific, RODAC plates and bacterial cultures are laborious, expensive, and take several days to yield results, which could delay the implementation of measures to eliminate bacterial contaminations.

ATP bioluminescence assays for monitoring environmental sanitation have been of increasing interest to the laboratory animal science community.[49–55] ATP bioluminescence assays have several practical advantages over bacterial culture and RODAC plates. This method is considerably less expensive and can provide real-time results, making it a highly pragmatic tool for a sanitation validation program. However, the results of ATP bioluminescence assays must be interpreted carefully. The presence of ATP correlates with the presence of any organic matter; it does not necessarily indicate live, replicating, pathogenic bacteria.[55–68] Several publications[55,56,60,63,65–68] have cautioned against relying on ATP swab luminescence results in nonvalidated contexts.

The purpose of this study was to determine if ATP bioluminescence testing can be used to identify microbial contamination of fomites and environmental samples in the context of cranial implant chamber cleaning procedures. ATP bioluminescent swab samples were compared with traditional bacterial culture swab samples from the same environmental and fomite sources. We hypothesized that ATP swabs of these sources would yield positive relative light unit (RLU) data that would correlate with the number of cfu obtained from bacterial culture. In addition, we hypothesized that samples with a higher RLU would have a greater chance of predicting growth on bacterial culture.

Methods

Animals. This study was performed at the Emory National Primate Research Center (Atlanta, GA). The facilities and Division of Animal Resources are fully AAALAC-accredited. Care and handling of animals was conducted in a humane manner consistent with the USDA Animal Welfare Act and Regulations, the *Guide for the Care and Use of Laboratory Animals*, and institutional policies. All studies involving nonhuman primates were previously approved by Emory's University IACUC. Environmental samples for this study were collected opportunistically from the instruments and solution containers used in the routine chamber cleaning process of 2 adult female rhesus macaques, designated as M1 and M2. These animals were born and reared in the ENPRC breeding colony and during this study were part of investigations related to monitoring and modulation of brain circuits in parkinsonism. Both animals are tested semiannually to exclude *Macacine herpesvirus* 1 and *Mycobacterium tuberculosis*. These animals were pair-housed with conspecifics (not participating in the present study) in cages appropriate for their size and weight, under controlled conditions (room temperature: 68 to 76 °F [20 to 24 °C]; relative humidity: 30% to 70%; 12:12-h light:dark cycle; 10 to 15 room air changes per hour). They were fed a standard commercial primate diet (Monkey Diet Jumbo 5037; Lab Diet, Inc., Richmond, IN) that was supplemented daily with a quarter of fresh orange and varying enrichment items including rice cakes, peanut butter, yogurt, and fresh vegetables in accordance with the ENPRC standard operating procedure.

The animals had undergone thorough desensitization and were first acclimated to the researchers and trained to sit on a primate chair during cleaning procedures using positive reinforcement techniques.[12] At least 2 mo before this aim of the study, they each underwent surgery to place a methyl methacrylate cap containing 2 cranial implant chambers. The surgery was conducted under 1% to 2% isoflurane anesthesia, and the animals received perioperative antibiotics and analgesics. Two craniotomies (one on each side of the brain) were performed, exposing the dura. Stainless steel chambers (19-mm inner diameter) were placed on the trephines and covered with stainless steel caps which were closed with set screws. The chambers were anchored to the cranium with stainless steel screws. The screws, chambers, and a head bolt were embedded in the acrylic. Three days after the surgery, the animals received regular chamber cleanings at least 3 times weekly as part of the standard chamber care protocol. The same researcher (CA) performed the chamber cleaning procedure throughout the duration of the surveillance period.

Chamber cleaning procedure. Routine chamber cleanings for the 2 animals in this study are performed in a small, dedicated procedure room. Animals are transported from the vivarium to the procedure room via a primate chair designated for each animal. Figure 1 shows the materials and solutions prepared for the chamber cleaning procedure. Each animal has separate, designated forceps and containers for disinfectant solutions, which are not shared with other animals. The chamber cleaning procedure is conducted as follows: Two forceps are placed in a glass bead sterilizer (B1215; VWR International, Radnor, PA) at least 4 cm deep for 30 s then each is placed in a container of undiluted, commercially available 0.31% quaternary ammonium chloride and 21% isopropyl alcohol (QAC-IPA) disinfectant (Opti-cide 3; Microscientific, Inc., Gurnee, IL) solution for 3 min.

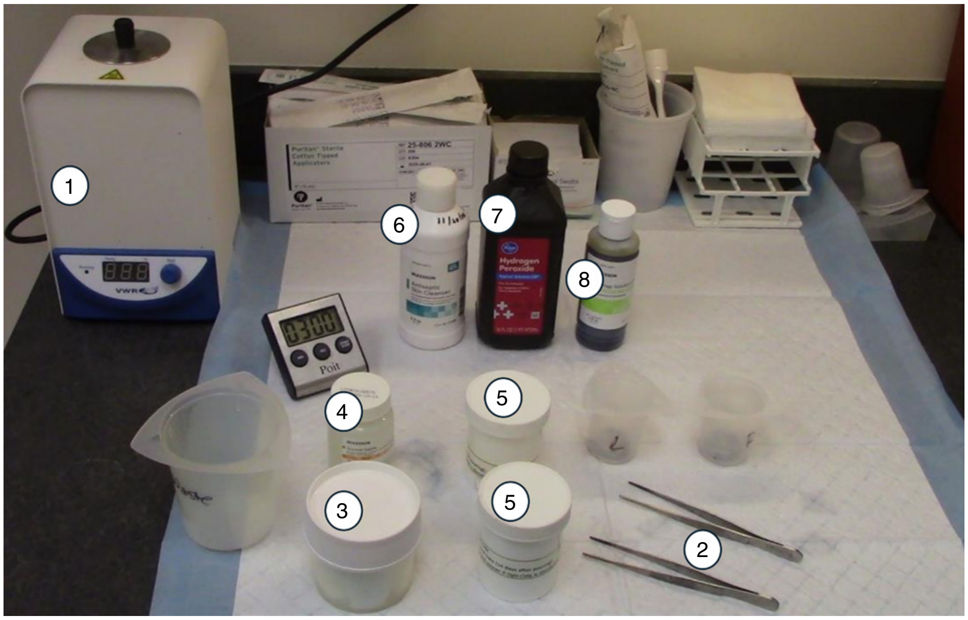

Figure 1. Instruments and solutions required for cranial implant chamber maintenance. (1) Glass bead sterilizer. (2) Separate forceps dedicated to the left and right chambers. (3) Cotton ball jar. (4) 0.9% NaCl solution. (5) Containers of QAC-IPA, each dedicated to the left and right chambers. (6) Chlorhexidine gluconate solution. (7) Hydrogen peroxide. (8) PVP-I bottle.

Manufacturer instructions for the QAC-IPA solution specify that a 2-min contact time is sufficient to kill pathogenic organisms such as *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecium*, and *Mycobacterium tuberculosis*. The acrylic cap and the external surfaces of the chambers are cleaned with 4% chlorhexidine gluconate scrub solution (Antiseptic Skin Cleanser; McKesson, Irving, TX). A gauze sponge saturated in 0.9% NaCl irrigation solution (Normal Saline–Isotonic Irrigation Solution; McKesson, Irving, TX) is placed around the margin of the acrylic cap and skin to prevent liquid from the chambers from getting into the margins. A hex wrench is used to loosen the screws of the chamber lids for each chamber. The lids are removed and, together with the screws, they are placed in 3% hydrogen peroxide solution for the duration of chamber cleaning and then cleaned with sterile cotton balls and placed in a separate container of the QAC-IPA solution and will remain there for at least 24 h for thorough disinfection. The fluid inside each chamber is inspected for changes in color, odor, transparency, or consistency that could indicate a possible infection. Separate forceps and QAC-IPA disinfectant containers are dedicated to each chamber to prevent cross-contamination. Sterilized forceps are used to grasp a sterilized cotton ball and gently place it into the chamber to absorb the fluid inside. Care is taken to avoid contact with the cotton ball with the rims of the chambers during placement. More than one cotton ball may be placed and removed in sequence until the volume of fluid has been sufficiently removed. When not in use, the forceps are placed in the QAC-IPA disinfectant container to maintain sterility. Next, a sterile 0.9% NaCl solution is poured into each chamber for irrigation. Cotton balls are inserted in the chamber to absorb and remove the 0.9% NaCl solution. The outer surface of the chamber is cleaned with cotton-tipped applicators soaked in hydrogen peroxide solution. Povidone-iodine 10% solution, equivalent to 1% available iodine (PVP Prep Solution; McKesson, Irving, TX), is poured to fill each chamber and remains in contact for 3 min. Then, cotton balls are used to absorb the povidone-iodine (PVP-I) solution, followed by rinsing with sterile 0.9% NaCl to remove all residue of the PVP-I solution; in between rinses, the saline solution is absorbed using sterile cotton balls. After the final rinse, a small amount of saline is left inside each chamber, enough to cover the tissue. Each chamber is closed with a disinfected lid, which has been soaked in QAC-IPA solution for at least 24 h, thoroughly rinsed with sterile saline, and then dried with sterile cotton balls. The lids are secured into place with screws using a hex wrench, and the outside of the chambers and acrylic cap is rinsed with 0.9% NaCl solution.

Each animal has a dedicated pair of forceps and separate containers of QAC-IPA and 3% hydrogen peroxide solutions that are not shared across animals. Forceps for the same animal may be used for multiple chamber cleaning sessions. After each chamber cleaning session, the forceps are scrubbed with soap and a soft nylon brush, rinsed with water, and let air dry. This treatment removes the accumulation of organic matter and chemical residues. The forceps are placed in the glass bead sterilizer before being used in the chamber cleaning procedure. The disinfection procedure performed on the forceps was developed based on lab-specific protocols.

Personnel performing chamber cleaning procedures undergo training and competency assessment to ensure they can consistently adhere to procedure steps, strictly maintain sanitation practices, and monitor the health of the chambers and the well-being of the animals. The individual performing chamber cleanings wears dedicated facility scrubs, a long-sleeve scrub jacket, para-aramid synthetic fiber sleeves and gloves, water-resistant gloves, face mask, face shield, and bouffant cap. During the chamber cleaning procedure, animals are monitored for signs of discomfort, and the chambers are monitored for inflammation and infection. Clinical abnormalities within the chamber (such as those noted above) are reported to the veterinarian for further evaluation and treatment. For suspected chamber infections, diagnostic samples may be collected for gram staining and/or bacterial culture and susceptibility testing. Treatment may include topical or systemic antimicrobials, as well as increased frequency of chamber cleanings. If needed, animals also receive analgesic treatment.

Cotton balls are packaged into separate containers and are autoclaved in-house before use in chamber maintenance cleanings. The screw-top lids are left slightly loose to allow for adequate

steam penetration. Successful autoclave cycles are confirmed via color-change indicator tape. Autoclaved cotton balls are stored within these closed containers until they are ready to be used for chamber cleanings. Any cotton balls unused after a chamber cleaning may be repackaged into a new container, which must be autoclaved before use for future chamber cleanings. To ensure the safety and sterility of the cotton balls and solutions before animal contact, swab samples are collected for bacterial culture for quarterly validation testing. Swab samples are collected from the center of an autoclaved cotton ball container. A small amount of PVP-I is poured into a sterile container, simulating the process of pouring the PVP-I into the chambers, and a swab of the solution is collected. A swab is passed along the inner rim and along the bottom inside the bottle of 0.9% NaCl solution. Regular testing has confirmed the sterility of all products and procedures. In contrast to the purpose of previous validation procedures, the scope of this study entails the evaluation of a novel method for identifying environmental contamination. Thus, sources for environmental samples were selected to identify the most likely areas of bacterial contamination and at time points during the cleaning process that could potentially allow for seeding of the environment with organisms from the animals' microflora.

Study design. The data collection for this study took place over a period of 17 d (Table 1). New bottles of QAC-IPA and PVP-I were opened on day 1 of the study. Replicate swab samples for ATP RLU quantification and bacterial culture were collected from 6 different environmental sources throughout the study period to determine if ATP bioluminescence testing could be used to identify microbial contamination of potential fomites in the context of cranial implant chamber cleaning procedures (Table 2). Limited opportunities for randomization were available to incorporate into the study design. Therefore, specific procedures were defined to ensure consistency between each chamber cleaning time point and sample collection to minimize subjective bias and the potential for confounding factors. Tables 1 and 2 describe the sample collection schedule, the environmental group sources, and the standardized sample collection procedures. The same researcher (KB) collected all swab samples throughout the study duration.

An a priori power analysis was conducted using G*Power 3.1 software to determine the number of samples required to achieve statistical significance for ATP RLU analysis.[69,70] Assuming a moderate-to-large effect size ($f = 0.5$), an α error probability of 5%, and 80% power, a total of 60 swab samples would be needed for statistical significance, assuming that a 1-way ANOVA would be an appropriate statistical test. A total of 10 swab samples were collected from each environmental source for ATP RLU quantification, and a total of 6 swab samples were collected from each environmental source for bacterial culture.

Just before the initiation of this study, 1 animal (M1) developed signs consisting of inflammation and granulation tissue overgrowth within the cranial chambers. Initial treatment measures included anti-inflammatory medications, removal of granulation tissue, and increasing the frequency of chamber cleanings. However, the previously reported clinical signs persisted, and new changes in fluid consistency developed within the cranial chambers, suggesting an infection. Bacterial culture and susceptibility testing of the internal chamber fluid was performed, yielding growth of *S. aureus*, and the animal was started on treatment with ceftriaxone. Thus, animal M1

Table 1. Sample collection timeline.

Day 1	Day 2	Day 5	Day 9	Day 12	Day 17
ATP swab	ATP swab	ATP swab only	ATP swab	ATP swab only	ATP swab
Bacterial culture	Bacterial culture	M1 and M2	Bacterial culture	M1 and M2	Bacterial culture
M1 only	M2 only		M1 and M2		M1 and M2

M1 and M2 indicate the aliases of the animals involved in the study.

Table 2. Environmental groups and swab collection procedures.

Environmental sample source groups	Swab collection procedure
1) Tips of forceps prior to chamber maintenance	Use one swab for both sets of forceps. Run the swab over both prongs' internal and external surfaces 2 cm away from the working tips 3 times.
2, 3) Rims of the left and right chambers after lids removed, prior to maintenance	Use separate swabs for left chamber rim and right chamber rim. Perform 3 circumferential swabs in order. Carefully swab uppermost surface of one chamber rim 2 mm from the top of the rim along the inside surface, then swab one complete circumference on the top surface of the chamber rim, then pass swab one circumference along the outside surface, extending 2 mm from the top of the rim. Repeat with new swab for second chamber.
4) PVP-I bottle rim and lid after chamber maintenance	Swab bottle rim circumference 3 times (first pass 2 mm along the inside surface, second pass along the top of the bottle rim, third pass 2 mm along the outside surface of the rim). Then pass swab around inside walls of the lid 3 times. Swab the inside surface of the middle of the lid in a grid pattern.
5) Rims and lids of QAC-IPA containers after chamber maintenance	Use one swab for both containers. Swab container rim circumference 3 times (first pass 2 mm along the inside surface, second pass along the top of the container rim, third pass 2 mm along the outside surface of the rim). Then pass swab around inside walls of the lid 3 times. Swab middle inside surface of the lid in a grid pattern.
6) Rims and lids of cotton ball containers after chamber maintenance	Use one swab for both containers. Swab container rim circumference 3 times (first pass 2 mm along the inside surface, second pass along the top of the container rim, third pass 2 mm along the outside surface of the rim). Then pass swab around inside walls of the lid 3 times. Swab middle inside surface of the lid in a grid pattern.
7) Tips of forceps after chamber maintenance	Same procedure described previously for the tips of the forceps prior to chamber maintenance.

For each environmental group, a total of 10 ATP swab samples and 6 bacterial culture swab samples were collected.

was receiving a systemic antimicrobial during the last day of ATP swab and bacterial culture sample collections (day 17). This animal responded well to a full course of antibiotic treatment, and the infection within the chambers resolved.

Sample collection. Swab samples for bacterial culture (Remel Bactiswab; Thermo Scientific, Waltham, MA) were collected before ATP swab samples (PocketSwab Plus ATP Swab; Charm Sciences, Lawrence, MA) for each environmental group. The ATP swabs were premoistened with a mild detergent, allowing for the disruption of biofilms. Therefore, the order of swab collection could not be randomized; bacterial culture swabs were collected before ATP swab samples to ensure the viability of potential microorganisms. On days where only ATP swab samples were collected, culture swabs were still performed to simulate any potential mechanical cleaning of the swabs. These mock culture swabs were subsequently discarded. Separate culture and ATP swab sets were used to collect from each chamber rim to prevent cross-contamination. A single swab for bacterial culture and a single ATP swab were used for both sets of forceps as well as the rims and lids of both QAC-IPA containers. A total of 42 and 70 bacterial swabs and ATP swabs were collected, respectively. The ATP swab samples collected from the left and the right chamber rims were processed individually, and separate ATP RLU values were recorded. The bacterial swab samples from the left and right chamber rims were pooled to process as a single culture. Thus, a total of 36 bacterial cultures were performed, and a total of 70 ATP RLU values were obtained.

Sample processing. ATP swabs were stored in the dark at room temperature until they were able to be processed and read via luminometer (novaLUM II-X system; Charm Biosciences, Lawrence, MA). Before processing samples from environmental groups, a positive control ATP swab and a negative control ATP swab were analyzed using the luminometer to ensure the swabs and device were functioning properly. The negative control ATP swab was activated and processed without collecting a sample, and an RLU value of 0 was obtained for every batch. ATP swab samples collected from the bottom of a shoe served as the positive control; each batch yielded a nonzero RLU. ATP swabs were activated according to the manufacturer instructions; swabs were twisted to introduce the sample to the reagents, gently shaken for 20 s to mix thoroughly, and immediately read by the Luminex. Raw ATP RLU values were recorded for further analysis ($n = 70$).

Bacterial swabs were stored in the dark at room temperature until able to be submitted to the ENPRC Microbiology Laboratory for culture. Bacterial cultures were performed in-house at the ENPRC Microbiology Laboratory. Certified medical laboratory technologists performed the plating, incubation, colony isolation, and bacterial species identification for the culture samples yielding unique colony morphology and growth characteristics. Swabs were labeled with environmental groups and animal codes. The technologists processing bacterial culture samples were blinded to the quantitative ATP RLU values, and they did not observe the chamber cleaning procedures or sample collection. Swabs for bacterial cultures were plated onto blood agar, chocolate agar, PEA agar, MacConkey agar, CDC anaerobic blood agar, and CDC anaerobic PEA agar media, as well as inoculated in thioglycollate broth.[71] Swabs collected from the left and right chamber rims were pooled for bacterial culture; all other environmental group swabs were plated and cultured individually. Bacterial colony growth data was designated according to the following: no growth: 0 colonies; rare growth: 1 to 10 colonies; light growth: 11 to 20 colonies; moderate growth: 21 to 40 colonies; and heavy growth greater than 40 colonies. Bacterial species identification was performed for selected samples using a VITEK 2 Compact system (BioMérieux) and BactiStaph Latex Agglutination Test (Thermo Scientific) for *S. aureus*.

Statistical analysis. Raw ATP RLU data and bacterial culture data were recorded and organized in Microsoft Excel for summarization and preparation for statistical analysis. ATP RLU values were transferred to GraphPad Prism (Dotmatics) for statistical analysis and visualization. For data visualization on the logarithmic scale, a constant of 1 was added to the ATP RLU values for each sample. Due to the nonnormality of data, a Kruskal-Wallis test with the Dunn multiple comparisons posttest was used to elucidate environmental group differences. For the bacterial culture data, semiquantitative scores for each environmental sample were assigned as follows: for each bacterial species cultured under aerobic or anaerobic conditions: rare growth = 1, light growth = 2, moderate growth = 3, and heavy growth = 4. A value of 1 was added to the score for the presence of bacterial growth in the broth. Samples yielding no growth on culture plates or broth were assigned a score of 0. Environmental samples that grew multiple bacterial species had aerobic, anaerobic, and broth scores summated for a total semiquantitative score. Semiquantitative culture scores were plotted with Microsoft Excel. The correlation between the ATP RLU data and the semiquantitative bacterial culture scores was assessed using the Spearman rho. The raw ATP RLU values for the left and right chambers were added together to generate a single value corresponding with the bacterial culture score resulting from the pooled swab samples. Since the chamber rim samples yielded much higher ATP RLU values and bacterial scores relative to the other environmental sample groups, we also wanted to investigate the relationship between ATP RLU and bacterial culture scores for the "abiotic" samples alone. Therefore, an additional Spearman correlation analysis was repeated excluding the chamber rim environmental group to evaluate the relationship between ATP RLU and semiquantitative culture scores for the other environmental groups. To calculate the sensitivity, specificity, positive predictive value, negative predictive value, accuracy, positive likelihood ratio, and negative likelihood ratio at increasing ATP RLU cutoff thresholds (0, 500, 1,000, 2,000, 4,000, 5,000, 6,000, and 8,000), we constructed 2 by 2 contingency tables (Excel; Microsoft). We used the culture results as the "gold standard" and the ATP RLU results as the evaluated test. True positive values were defined as samples yielding positive growth on bacterial culture with an ATP RLU above the cutoff threshold. False positive values were defined as samples yielding no growth on bacterial culture but ATP RLUs above the cutoff threshold. True negative values were defined as samples yielding no growth on culture with an ATP RLU below the cutoff threshold. False negative samples were defined as yielding positive growth on bacterial culture, with an ATP RLU below the cutoff threshold. Standard equations were input into Excel to calculate the parameters above, using the values within the contingency tables.[72] A receiver operator characteristic (ROC) curve was generated in GraphPad Prism to determine if ATP RLU values are predictors of growth in bacterial culture. A curve was generated with the raw ATP RLU values for each environmental sample and the corresponding binary bacterial culture result, where any bacterial growth on culture = 1 and no growth = 0. A simple logistic regression was fit to the data set to generate the curve equation and calculate the predicted probability of a positive bacterial culture for a given ATP RLU. The best-fit curve equation was $\ln(\text{odds}) = -0.5365 + 1.677\text{e-}007*(X)$, where X is the ATP RLU value. The ROC curve was generated by plotting the predicted probabilities

calculated from the logistic fit equation for all possible ATP RLU cutoffs. The AUC was calculated from the plotted data. The ROC curve analysis was repeated using the same procedure described above, but excluding the M1 chamber rim ATP RLU and bacterial culture data on the last day of sample collection (day 17), due to the animal receiving a systemic antimicrobial at that time. The resultant best-fit curve equation from the repeated analysis was ln(odds) = −0.6604 + 6.018e-005*(X). Due to the negligible difference in the AUC between both data sets, the ROC curve analysis performed with the inclusive data set is visually presented in the Results section. The same researcher (KB) who collected the swab samples performed the data analysis.

Literature search criteria for bacterial species. Bacterial species identified from environmental samples are presented in Supplementary Table S1. A literature review was performed to determine (1) whether the bacterial species have been previously cultured from cranial implant chambers in rhesus macaques; (2) if they have been previously cultured from other sites in rhesus macaques and are considered "normal flora"; (3) if the bacterial species has been associated with disease in rhesus macaques, humans, or other animal species; (4) if the bacterial species has been identified in disinfectant bottles or other sterile solutions; and (5) if nosocomial transmission of the bacterial species has been reported and/or if the species has been identified in the environments of a human or veterinary hospital. On October 6 to 19, 2024, the researcher (KB) conducted literature searches in PubMed (coverage 1966 to present day) and Web of Science (coverage from 1900 to present day). A "snowball" search was performed to identify additional relevant publications by searching the reference lists of applicable articles and using Google Scholar to identify studies citing them. The search terms for the databases include the [Bacterial Species name] with each of the following, (1) rhesus macaque, (2) cranial implant chambers, (3) infection, (4) veterinary, (5) nosocomial, (6) environment, and (7) disinfectant. Relevant articles cited original research publications, human and veterinary case reports, and systematic reviews. In vitro studies of the bacterial species were not applicable to the research questions.

Results

ATP bioluminescence swab testing. Kruskal-Wallis with the Dunn multiple comparisons testing revealed significant differences in the ATP RLU values between environmental groups (Figure 2). Both the left and right chamber rims yielded a greater ATP RLU than the QAC-IPA containers ($P < 0.0001$), PVP-I bottles ($P < 0.0001$), and cotton ball containers ($P < 0.0005$). The 2 chambers were not significantly different from each other. The tips of the forceps before the chamber maintenance procedure and after the chamber maintenance procedure were not significantly different from any other environmental group. Each sample taken from the PVP-I bottle rim and lid produced an ATP RLU value of 0.

Bacterial culture. Growth on bacterial culture was identified for 16 out of 36 total samples taken (Figure 3A). All samples taken from the rim and lid of the cotton ball jars were found to have bacterial growth. Semiquantitative culture scores for each sample by environmental group are shown in Figure 3B–F. Samples taken from the chamber rims yielded the highest semiquantitative culture scores. Diverse bacterial species were cultured from the rim and lid of the cotton ball jar after the chamber maintenance procedure was completed. Species identification was pursued for select environmental

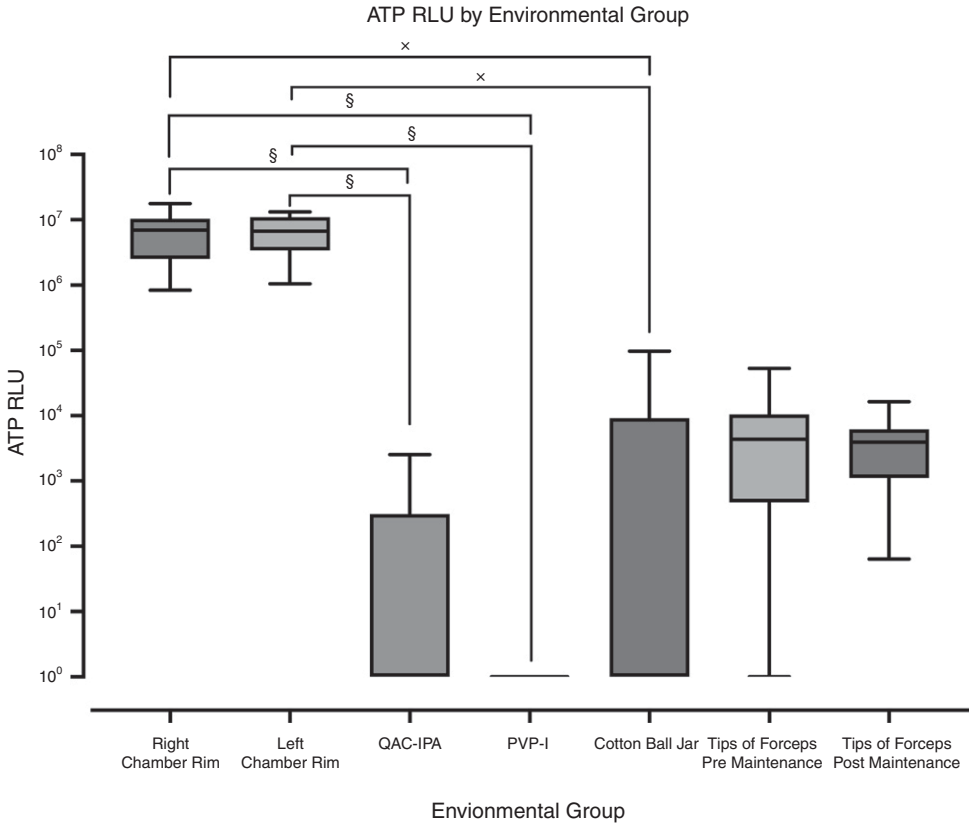

Figure 2. Box and whisker plot of ATP RLU value by environmental group. Transformed ATP RLU values used for graphical representation and analyses (ATP RLU +1). Environmental samples from both animals were pooled together. For each group $n = 10$, for a total of 70 samples. For each box, the line inside indicates the median, the top and bottom of the boxes represent the 75th and 25th percentiles respectively, and the whiskers extend from the 10th to the 90th percentile. ×, $P < 0.0005$; §, $P < 0.0001$.

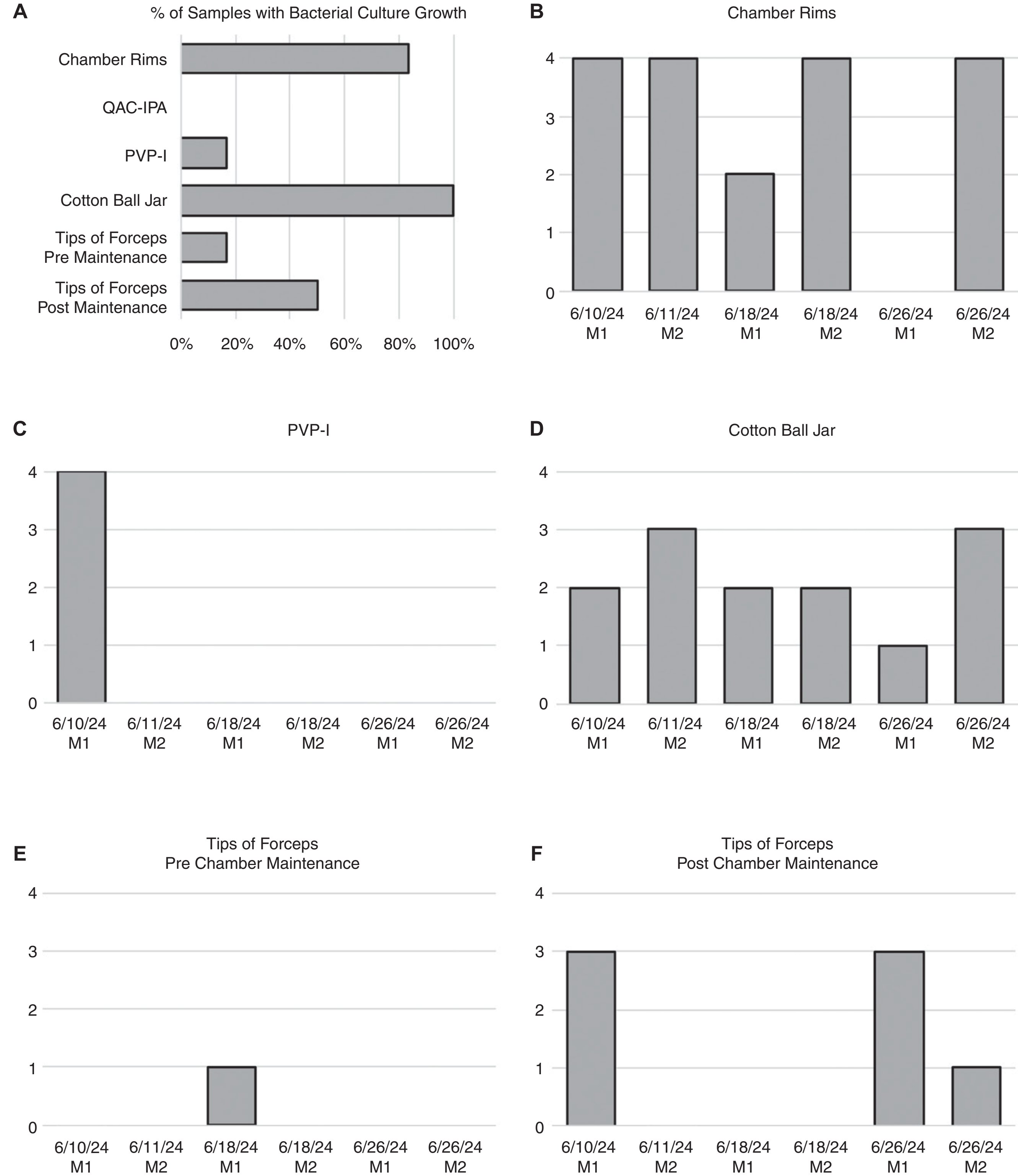

Figure 3. Bacterial culture results. (A) Percentage of samples yielding bacterial growth per environmental group, samples for both animals were pooled together. (B–F) Semiquantitative culture scores depicted for each sample by environmental group and animal; $n = 36$.

samples; the results are presented in Supplementary Table S1. A literature search was conducted to determine the clinical relevance of these identified species. Eight of the 13 bacterial species identified have been reported as primary or opportunistic pathogens in rhesus macaques. All other bacterial species identified have been reported as primary or opportunistic pathogens in humans or other animals. The disease manifestations vary widely, but several notable examples include cranial implant chamber infections (*S. aureus*, *E. coli*), opportunistic CNS infections (*Staphylococcus warneri*, *Staphylococcus auricularis*, *Corynebacterium simulans*), infections associated with orthopedic implants (*Staphylococcus epidermidis*, *Staphylococcus capitis*, *C. simulans*, *E. coli*), and indwelling intravenous catheters (*S. aureus*, *Staphylococcus saprophyticus*). Three bacterial species have been reported as contaminants in disinfectant or sterile solutions, while 11 bacterial species have been reported as

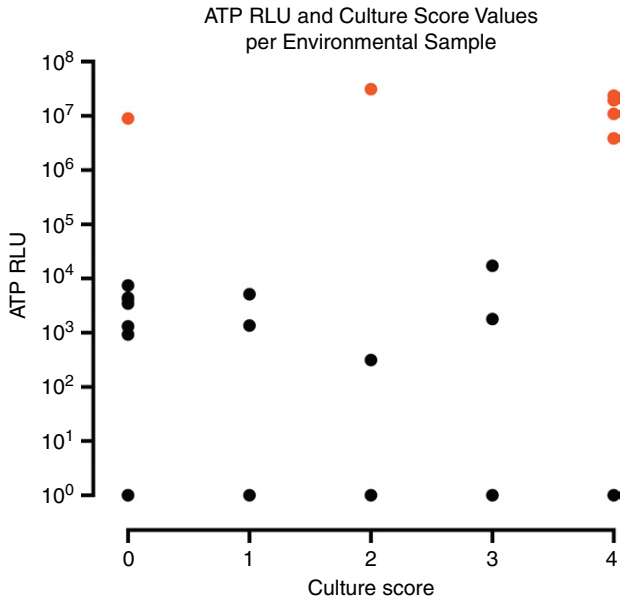

Figure 4. Scatterplot of ATP RLU by semiquantitative culture scores for environmental samples. Red points indicate values obtained from chamber rim samples. Black points represent all other environmental groups. Transformed ATP RLU values used for graphical representation and analyses (ATP RLU +1). For all environmental samples (n = 36), Spearman ρ = 0.3553; 95% CI = 0.02016 to 0.6186 ($P < 0.05$). Excluding the chamber rim samples (n = 30), Spearman ρ = 0.03965; 95% CI = −0.3352 to 0.4037 (P = 0.8352).

nosocomial agents and/or have been identified via environmental surveillance programs in human or veterinary hospital settings.

Correlation between ATP and semiquantitative bacterial scores. To determine if ATP bioluminescence testing could be used as a proxy for bacterial culture surveillance sampling in the context of cranial implant chamber maintenance, the following analysis was conducted to explore the relationship between these 2 parameters. Figure 4 depicts a scatterplot of the ATP RLU values against the semiquantitative culture scores for all environmental samples. The red points represent the values obtained from the chamber rim samples, and the black points represent all other environmental groups. Spearman correlation testing of all samples (n = 36) revealed a rho of 0.3552, indicating a weak, positive correlation (95% CI, 0.02016 to 0.6186; P = 0.0335). Since the chamber rim samples yielded much higher ATP RLU values and bacterial scores than the other environmental sample groups, we also wanted to investigate the relationship between ATP RLU and bacterial culture scores for the "abiotic" samples alone. An additional Spearman correlation test was conducted excluding the data from the chamber rim samples (n = 30) and yielded a rho of 0.03965 (95% CI = −0.3352 to 0.4037; P value = 0.8352). Thus, no association could be identified between ATP RLU and semiquantitative bacterial culture scores when evaluating the potential fomite and environmental sources of bacterial contamination alone.

We next evaluated the predictive abilities of the ATP bioluminescence testing for identifying any positive bacterial growth on culture. Treating the bacteria culture results as the "gold standard" for identifying bacterial contamination, the sensitivity, specificity, positive predictive value, negative predictive value, accuracy, positive likelihood ratio, and negative likelihood ratio were calculated using increasing ATP RLU cutoff values, and the results are shown in Table 3. For example, an ATP RLU cutoff threshold of 0 has the highest sensitivity at 63%, but the lowest negative likelihood ratio. This would indicate that, at this cutoff, an ATP RLU > 0 can correctly identify 63% of the samples that have bacterial contamination but will miss 37% of samples with bacterial contamination. An ATP RLU value equal to 0 only slightly reduces the probability that the sample does not have bacterial contamination. In contrast, an ATP RLU cutoff threshold of 8,000 maximizes specificity, positive predictive value, accuracy, and positive likelihood ratio but reduces sensitivity. This higher cutoff threshold minimizes false positives. A sample with an ATP RLU greater than 8,000 would also exhibit bacterial growth 86% of the time, assuming the prevalence of bacterial contamination identified in this study population is representative of the general prevalence. At this cutoff, a sample with an ATP RLU above 8,000 has a 7.5 times greater probability of having bacterial contamination than not having bacterial contamination. An ATP RLU value of 8,000 is high compared with published ATP RLU thresholds established for other healthcare applications. ATP RLU cutoff thresholds in hospital and laboratory animal contexts range from 10 to 500 RLU.[51,73–78] To assess the ability of ATP RLU values to predict growth on bacterial culture, a ROC curve was generated (Figure 5). The area under the ROC curve was 0.6594, an SE of 0.09437, with a 95% CI of 0.4744 to 0.8443 (P value = 0.1045). A repeated ROC curve analysis was performed excluding the data from animal M1's chamber rim samples collected on day 17, when this animal was receiving a systemic antimicrobial. The area under the ROC curve was 0.6809, with a SE of 0.09381 and a 95% CI of 0.4971 to 0.8648 (P value = 0.0686). To have value as a predictive test, the lower end of the CI must be greater than 0.5. Therefore, the use of ATP RLU to predict growth on bacterial culture is not statistically significant.

Discussion

The purpose of this study was to determine if ATP bioluminescence testing can be used to identify microbial contamination of fomites and environmental samples in the context of cranial

Table 3. Sensitivity, specificity, positive predictive value, negative predictive value, accuracy, positive likelihood ratio, and negative likelihood ratio for different ATP RLU cutoff thresholds.

ATP RLU cutoff	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy	Positive likelihood ratio	Negative likelihood ratio
0	63%	60%	56%	67%	61%	1.56	0.63
500	56%	60%	53%	63%	58%	1.41	0.73
1,000	56%	65%	56%	65%	61%	1.61	0.67
2,000	44%	70%	54%	61%	58%	1.46	0.80
4,000	44%	75%	58%	63%	61%	1.75	0.75
5,000	44%	85%	70%	65%	67%	2.92	0.66
6,000	38%	85%	67%	63%	64%	2.5	0.74
8,000	38%	95%	86%	66%	69%	7.5	0.66

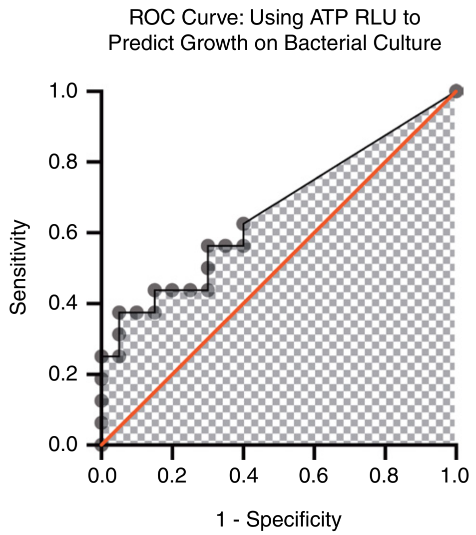

Figure 5. Receiver operator characteristic (ROC) curve using ATP RLU to predict growth on bacterial culture. Points on the scatterplot correspond to the sensitivity and 1-specificity for different ATP RLU cutoff thresholds. The area under the ROC curve is depicted by the shaded portion (AUC = 0.6594; 95% CI = 0.4744 to 0.8443; $P = 0.1045$). The red line denotes the 0.5 AUC, corresponding to the 50/50 chance for a sample yielding a positive or negative result. Since the 95% CI extends below 0.5, the use of ATP RLU as a test to predict bacterial culture growth is not significantly significant.

implant cleaning procedures. This environmental surveillance study was designed to sample potential sources with minimal interruptions to current practices, with the goal of reflecting real-life conditions. Samples were strategically collected at time points distributed across the lifespan of the working bottles of disinfectant solutions. The environmental samples were collected after the maintenance cleaning procedures were completed to identify potential routes of transmission from the animal to the environment, except for the premaintenance cleaning procedure swab of the forceps and the swab of the chamber rims collected during the maintenance cleaning procedure. A total of 16/36 (44%) bacterial cultures from selected environmental groups yielded growth, and many of the species identified were clinically relevant. Significantly higher ATP RLU values were consistently obtained from the rims of the chambers compared with the other environmental sample groups. Although high ATP RLU levels and positive bacterial growth were identified for these environmental samples, there was a poor correlation between the ATP RLU values with the semiquantitative bacterial culture scores. When the chamber rim values were excluded from analysis, no association between ATP RLU and semiquantitative bacterial culture scores could be identified for the potential fomites and environmental sources of contamination. The results of the ROC curve indicate that, in this context, ATP RLU is a poor test to predict growth on bacterial culture since the lower end of the 95% CI for the AUC is below 0.5. Based on the results of this study, a high ATP RLU cutoff threshold would be needed to maximize the accuracy of using this method instead of bacterial culture to identify potential sources of microbial contamination.

The most practical advantage of ATP bioluminescence testing is its ability to produce rapid results, allowing for prompt identification of potential contamination, as compared with a multiday turnaround time for bacterial culture. Several studies[57,66,73,75,77,79–81] have shown that ATP testing and rapid feedback can be useful in assessing user-dependent cleaning and sanitization methods. ATP RLU values that exceed established thresholds will prompt individuals to resanitize environmental surfaces or instruments dedicated to clinical procedures. Recent publications[82] describe the successful application of ATP bioluminescence detection to identify the sources of environmental contamination in acute outbreaks of hospital-acquired infections. The rapid identification of bacterial contamination can lead to immediate correction and reduced transmission, which improves patient outcomes. While there was no outbreak scenario to prompt the present study, these ATP results help to establish a baseline threshold for comparison, which increases our preparedness to investigate infections should it ever be necessary in the future. Future studies may be able to better define the ATP threshold by investigating increased numbers of environmental samples and extending the scope to additional laboratories with different staff members performing chamber cleanings.

The inherent caveats to ATP bioluminescence testing require context-specific validation.[55,66,67] The weak correlation between ATP RLU and culture scores in this study is not surprising given the mixed findings from previous studies[57,61,63,83–89] evaluating the correlation between ATP RLU and bacterial culture cfu. Any organic matter can result in elevations in ATP RLU values.[61,90] The significantly higher ATP RLU values from the chamber rims likely are due to both the known microbial colonizers as well as the organic material secreted from the animal. In this study, the necessary order of bacterial swab collection before ATP swab collection could have reduced the amount of ATP present on the environmental surfaces, through slight mechanical cleaning. On the days that only ATP RLUs were analyzed, bacterial swabbing procedures were still performed to minimize the potential impact of this mechanical cleaning on the overall RLU results. In addition, prior studies have shown that disinfectant solutions can interfere with ATP analysis.[91] If any residual solution was left on the rim or lid of the bottles, then the ATP RLUs for these samples could have been artificially reduced.[91,92] For comparison, the bacterial culture samples were treated as the gold standard method for determining the presence of microbes. However, potential false positives on bacterial culture could occur by contamination during either the sample collection process or the plating and incubation period. When interpreting the results for clinical purposes, the potential for unintended contamination during collection is considered before final diagnosis and treatment decisions, especially for bacterial cultures yielding uncommon species.

The identification of an unusual, nonendogenous bacterial species from clinical samples might prompt further investigation into potential environmental sources. The decision to interpret a positive culture sample as either a true result or a false positive due to contamination depends on multiple factors. If the same species can be repeatedly cultured from sequential samples, the confidence in the diagnosis is increased. Certain bacterial species should be considered at higher risk for potential environmental contamination, such as *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and *Proteus* spp that have inherent resistance to chlorhexidine and quaternary ammonium compounds.[38] Other species have been identified in case reports as intrinsic or extrinsic bacterial contaminants within antiseptics and other medical solutions,[39,93–95] and *Staphylococcus* spp, *Enteroccocus* spp, and *E. coli*, which have been culprits of hospital-acquired infections that have been identified on environmental sources.[96–98] If any of these species are identified from a patient's bacterial culture of chamber fluid, an investigation into the potential environmental and fomite sources could help identify and eliminate a nidus,

helping prevent any potential cross-contamination between other animals, or reinfection of the index case after treatment.

Two specific samples producing discordant ATP RLU and bacterial culture results are notable exceptions in this study. On the first day of sample collection, the PVP-I bottle rim and lid yielded heavy growth of *S. aureus*, while the ATP RLU was 0. The same PVP-I bottle was swabbed the following day but did not produce growth on the bacterial culture. The original PVP-I bottle was discarded upon receipt of culture results (3 d after the first sample was taken) and was replaced with a new bottle as a precaution. We hypothesize that the initial growth of bacteria was due to contamination of the rim by the physical removal of the thin, protective film overlying the opening of the bottle. The cultures taken on the second day were likely negative after the PVP-I was able to coat and disinfect the inside surface of the lid and rim upon bottle inversion when the PVP-I is poured into the chambers during the cleaning procedure. Upon discovery of these results, the standard procedure to remove the protective film now involves the use of clean forceps instead of gloved fingers, to prevent accidental contamination of the PVP-I solution. On the last day of sample collection, the chamber rims of animal M1 produced high ATP RLU values but yielded no bacterial growth on culture. This animal was receiving a systemic antimicrobial at the time these samples were collected due to clinical signs of an active chamber infection. The presence of ATP from the animal's own secretions coupled with residual ATP from killed microorganisms likely produced the high RLUs, while the presence of antimicrobials likely prevented bacterial culture growth.

The consistent bacterial contamination identified from the rim and lid of the cotton ball jar was an unexpected but valuable finding from this study. During the chamber cleaning process, the cotton ball jar is opened for a greater time period while it is actively in use. Forceps are repeatedly moved from the cotton ball jar to the animals' chambers. It is possible that the increased exposure to air currents generated from the back-and-forth movement between the jar to the animal made it more likely for bacteria to fall onto the rims and/or inside the lid of the jar during the chamber maintenance procedure. In contrast, the lids of the QAC-IPA and PVP-I containers are only opened for a short duration. Given this, the lab has modified the chamber cleaning routine to reduce the possibility of using cotton balls that may have become contaminated during the cleaning process. After the rinse with PVP-I, a new sterile cotton ball jar is opened for the subsequent 0.9% saline rinses. Interestingly, despite consistent bacterial growth on culture, the rim and lid of the cotton ball jar yielded overall lower ATP RLU values (for 5 out of 6 samples with both ATP and bacterial culture analysis, the ATP RLU was 0, while the corresponding culture was positive for bacterial growth). The shape or composition of the jar and lid may have facilitated the removal of any contamination during the bacteria swab collection procedure, with the consequence that no ATP was recovered by the second swabbing procedure.

The scope of this study was limited to evaluating the ATP RLU values and the presence of bacterial growth on culture, although we pursued bacterial species identification for selected samples to obtain a qualitative understanding. Further studies could evaluate the antimicrobial and biocide resistance patterns of bacterial species cultured from environmental sources. Understanding population-level bacterial resistance trends can potentially help veterinarians predict the likely susceptibilities of patients acquiring new infections and improve antimicrobial stewardship practices. In addition, genetic analyses can reveal strain similarities, which would provide definitive evidence of identical bacteria present in different environmental samples, supporting potential routes of transmission.

Previous studies have investigated the impact of cranial implant recording chambers on the physiology and microbiome within the chambers of the implanted animal.[99–101] To our knowledge, this study represents the first published microbial surveillance investigation of environmental samples related to the chamber maintenance cleaning process. Documentation of these procedures and results can help identify and establish universal best practices for cranial implant recording chamber maintenance. Ultimately, the aim of these environmental surveillance procedures is to prevent infections. Improved health and welfare in animals with cranial implant recording chambers enable the collection of high-quality longitudinal data for neuroscience studies.

Supplementary Materials

Supplemental Table S1. Bacterial species identified from environmental samples with clinical relevance literature search summary. References are indicated by superscript numbers.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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