Alfaxalone (3α-hydroxy-5α-pregnane-11,20-dione) is a neurosteroidal anesthetic agent that is an agonist of the γ-aminobutyric acid type A receptor, which causes a reduction in arousal and awareness. This anesthetic was first approved for use in dogs and cats by the US FDA in 2012. The previous formulation, alfaxalone-alfadalone, was associated with histamine release and anaphylaxis, which resulted in its withdrawal from the veterinary market. The alfaxalone-alfadalone agent was reformulated (the castor oil surfactant was replaced with 2-hydroxpropyl-β-cyclodextrin), which induced aqueous solubility and expanded the routes of administration to include IV, IM, intraperitoneal, and immersion. This enhanced the innate drug attributes that included rapid onset of action without drug accumulation, muscle relaxation, rapid anesthetic recovery, and minimal cardiorespiratory depression.

Medical care of wild animals in zoological collections or exotic animals kept as household pets commonly requires chemical immobilization to facilitate even the simplest diagnostic procedures (eg, physical examination, venipuncture, or radiography). Reasons to consider use of sedation or anesthesia include patient size, accessibility, safety, lack of effective manual restraint, or minimization of patient stress. Therefore, use of a safe and effective anesthetic such as alfaxalone that can be administered IM could prove beneficial. The efficacy of alfaxalone has been evaluated in a variety of species, including rabbits, ferrets, rodents, wallabies, birds, amphibians, reptiles, and fish. The small size of exotic or zoo animals often requires administration of small volumes of drugs to achieve the desired anesthetic effect. A vial of alfaxalone contains 10 mL.

**Objective**
To evaluate the microbial integrity of preservative-free cyclodextrin-based alfaxalone in a multiple-use system.

**Sample**
22 vials of preservative-free alfaxalone.

**Procedures**
2 storage conditions (room temperature, 22°C; refrigerated temperature, 4°C) and 3 handling techniques (closed system transfer device, nonclosed dispensing pin, and manufacturer-supplied vial stopper) comprised 6 treatment groups (3 replicates/group). An aliquot (0.5 mL) was withdrawn from each vial daily for 14 days. Samples were immediately inoculated into tryptic soy broth and incubated at 36°C for 24 hours; samples were subcultured onto 5% Columbia sheep blood agar and incubated for 48 hours. Isolated colonies were evaluated for identification.

**Results**
There was no evidence of microbial contamination of vials stored for 7 days in refrigeration and handled with a protected port (closed system transfer device or nonclosed dispensing pin).

**Conclusions and Clinical Relevance**
The US FDA prohibits the use of alfaxalone beyond 6 hours after the vial stopper is broached (punctured), as mandated for a preservative-free injectable medication. Findings for the study reported here supported the use of alfaxalone for 7 days when refrigerated and handled with a single puncture of the stopper by use of a protected port (closed system transfer device or nonclosed dispensing pin). This would appear to be a practical alternative for an injectable anesthetic. It would minimize drug waste and the subsequent environmental impact for disposal of unused drug and allow standardization of storage and handling protocols for alfaxalone use in veterinary practices across the United States. (Am J Vet Res 2018;79:704–710)
thus, the small volumes of alfaxalone administered to
exotic or zoo animals will often result in drug wast-
age and a subsequent environmental impact associat-
ed with the disposal of the unused portion of an FDA
class IV controlled substance.\textsuperscript{1} Drug wastage may
equate to a notable limitation for use of alfaxalone in
zoological medicine, despite its beneficial anesthetic
characteristics.

The Australian label of alfaxalone permits storage
of a broached vial at 4°C for up to 7 days, providing
contamination is avoided.\textsuperscript{27} However, US FDA prod-
uct labeling prohibits the use of alfaxalone beyond
6 hours after a vial is broached, and the USP chapter
797 mandatory standard for sterile compounding also
requires that this preservative-free injectable be dis-
carded 6 hours after opening.\textsuperscript{28,29} The potential for
bacterial colonization of propofol, alfaxalone, and
thiopental has been investigated, and it was conclud-
ed that alfaxalone is a less favorable medium for bac-
terial growth than is propofol.\textsuperscript{30} Although alfaxalone
does not have the bactericidal properties of thiopen-
tal, there was no evidence of bacterial colonization
within the vials of alfaxalone.\textsuperscript{30} This raises concerns
as to whether the 6-hour broaching claim for alfaxa-
lone is warranted.

To the authors’ knowledge, there have been no
published reports about the requirement for use of
alfaxalone within 6 hours after a vial is opened. The
objective of the study reported here was to evaluate
the microbial integrity of preservative-free cyclodex-
trin-based alfaxalone in a multiple-use system over
a 14-day period with 2 storage conditions (room
temperature and refrigerated) and 3 handling tech-
niques (CSTD, NCDP, and manufacturer-supplied vial
stopper). Our null hypothesis was that refrigeration
of preservative-free alfaxalone would result in no
microbial growth for at least 7 days, regardless of
the collection system that was used. Furthermore, if
microbial growth were identified, we predicted that
refrigeration or use of a CSTD (or both) would re-
sult in less microbial contamination, compared with
growth for storage at room temperature or the other
handling techniques.

**Materials and Methods**

**Sample**

Twenty-two vials of alfaxalone\textsuperscript{b} were obtained for
use in the study. Three handling techniques (CSTD,
NCDP, and manufacturer-supplied vial stopper) were
evaluated (Figure 1). The CSTD\textsuperscript{c} was a port designed
for veterinary safety during collection of chemothera-
pic agents, whereby there was only a single punc-
ture of the vial stopper during placement, and the
CSTD maintained a neutral, contained-pressure sys-
tem that eliminated the risk that the product would
aerosolize. The NCDP\textsuperscript{d} was designed for dispensing di-
luent or additive through a single puncture of a multi-
dose rubber-stoppered vial; it also was fitted with a
filtered vent to maintain neutral air pressure during

![Figure 1](image-url) — Photographs of 3 handling techniques (CSTD [A], NCDP [B], and manufacturer-supplied vial stopper [C]) and the attached 3-mL Luer-lock syringes used to obtain 0.5-mL samples for evaluation of the microbial integrity of preservative-free alfaxalone.
manipulation. The vial stopper handling technique involved use of only the original manufacturer's rubber stopper, and it required 1 puncture/sample withdrawal.

**Study design**

The study included 6 treatment groups (3 handling techniques, each of which was evaluated at 2 temperatures). All treatment groups were replicated in triplicate (3 vials/group). There were 2 negative control groups (1 for each storage temperature), each of which was replicated in duplicate (2 vials/group). Two storage conditions were used: room temperature (approx 22°C) and refrigerated (4°C).

**Medium preparation and storage**

Preparation of commercially available tryptic soy broth was conducted as per manufacturer instructions. Five milliliters of broth was aseptically pipetted into a sterile culture tube. The medium was incubated at 36°C for 24 hours prior to use to confirm it was free from contamination. Uninoculated tryptic soy broth was stored at 4°C until used; broth was used within the subsequent 14 days. Additionally, commercially available blood agar plates were refrigerated at 4°C until streak plating was performed, after which the plates were incubated at 36°C in accordance with the study protocol.

**Sample collection and evaluation**

Initial puncture of the rubber stopper for each alfaxalone vial (day 1) for the CSTD and NCDP groups was performed by North Carolina State University Veterinary Pharmacy staff in an International Organization for Standardization 5 clean room in compliance with all requirements in USP chapter 797 to ensure that microbial contamination was not introduced by access devices. The vials were maintained in light-protected, categorized drawer compartments (refrigerated and room temperature) in a controlled medication dispensing system for the duration of the study. Control vials were prepared similarly, but the rubber stopper of each control vial was not punctured until day 14 of the study. Sterile 70% isopropyl alcohol prep pads were used to swab each port or rubber stopper immediately before sample collection. An aliquot (0.5 mL) was withdrawn daily for 14 days from each vial by use of a 3-mL syringe attached directly to the port (CSTD and NCDP) or to a 22-gauge needle (manufacturer-supplied vial stopper). Within 30 minutes after collection, each sample was aseptically transferred by use of a new sterile 22-gauge needle into individual, labeled tubes containing tryptic soy broth. Tubes were incubated for 18 to 24 hours (ie, enrichment) in an incubator (36°C with atmospheric conditions) and then examined for visible growth. Positive growth was defined as overt turbidity of the broth, whereas negative growth was defined as a transparent light amber broth. Samples with a positive result for the enrichment incubation were subcultured onto a blood agar plate. In addition, at the discretion of the investigators, samples could be subcultured onto a blood agar plate on alternate days throughout the study, beginning on day 1. Tubes of tryptic soy broth were mixed on a vortex device, and samples then were streaked on plates for isolation. Plates were incubated (36°C) and evaluated for microbial growth at 24 and 48 hours. Additionally, when growth was identified on a blood agar plate, repeated subculture of the enrichment tryptic soy broth was performed, and tryptic soy broth from the day before and the day after the sample that yielded the microbial growth was subcultured to assess vial contamination versus sample handling error. The same investigators executed sample collection, evaluation, and microbial assessment for the duration of the study.

**Statistical analysis**

Descriptive statistics were used to evaluate categorical variables collected throughout this study. Samples were recorded as positive for microbial growth or negative for no microbial growth. Binary logistic regression was performed with commercially available statistical software to analyze relationships between categorical data. Values were considered significant at $P < 0.05$.

**Results**

Results indicated no evidence of microbial growth consistent with vial contamination for at least 7 days in vials with a protected port (CSTD or NCDP) that had been refrigerated. Overall, there was negative growth for the study period, with a few sporadic positive results for microbial growth on blood agar plates. Positive growth was evident as a single bacterial colony or a few bacterial colonies per plate. Handling technique, storage condition, and sample day had no significant effects on microbial growth in tryptic soy broth.

Six of the 22 samples yielded bacterial growth, and none of these 6 samples had repeated instances of bacterial isolates on subsequent days of evaluation. On day 3, 1 manufacturer-supplied vial stopper stored at room temperature yielded growth in the enrichment tryptic soy broth; we isolated *Bacillus* spp on a blood agar plate. Five samples yielded negative results for enrichment tryptic soy broth but yielded bacterial organisms on blood agar plates. This included 2 vials on day 7 (1 manufacturer-supplied vial stopper at room temperature and 1 refrigerated CSTD) and 3 vials on day 13 (1 manufacturer-supplied vial stopper at room temperature, 1 refrigerated manufacturer-supplied vial stopper, and 1 refrigerated NCDP). All observed bacterial isolates (positive growth) on blood agar plates subsequently yielded negative results when repeated plate inoculation was performed with a sample from the same tube. In total, 5 of 6 bacterial isolates were identified on day 7 or later, and 4 of the 6 were in the manufacturer-supplied vial stop-
per groups. The control vials all had negative results for microbial growth at day 14 for both broth and the blood agar medium. The number of vials that yielded microbial growth did not differ significantly among the 6 treatment groups or the control group and also did not differ significantly across period of storage.

Discussion

Results of the study reported here indicated that there was no microbial growth in preservative-free cyclodextrin-based alfaxalone for vials with a protected port system (CSTD or NCDP) and refrigerated for up to 7 days. Only 1 sample yielded evidence of microbial contamination in the tryptic soy broth enrichment tube (turbidity) and also yielded recovery of a bacterial isolate from the blood agar plate. Because this vial had negative results for microbial contamination of all subsequent samples and test media, and the organism identified was a common environmental contaminant, it was likely not an important finding in the study. It may be possible that the Bacillus organism isolated at that collection point died before subsequent collections; however, molecular methods were not used to confirm the absence of this organism.

To our knowledge, the study reported here was the first in which the microbial integrity of preservative-free alfaxalone has been evaluated, and we did not find significant outcomes of microbial contamination on the basis of period of storage, temperature of storage, or port handling techniques. The present study was an extended sterility study, which currently is allowable (according to USP chapter 797) to extend the beyond-use time for sterile preparations.28,29 The Australian label for alfaxalone states that use is preferred within 24 hours after a vial is broached; however, if necessary, storage of broached vials is permitted at 4°C for up to 7 days, providing contamination is avoided, which implies chemical stability for 7 days in a refrigerator.27 The Australian label also states that the solution must remain clear, colorless, and free from particulate matter.27 Hypotheses for the present study were delineated on the basis that aseptically collected alfaxalone should remain free from contaminants for at least 7 days if stored at 4°C, in accordance with the Australian label. Thus, the study reported here was conducted to evaluate microbial integrity with various storage and handling techniques in an attempt to pursue standardization of a multiple-use protocol for alfaxalone in the United States.

Staphylococcus spp, Streptococcus spp, Pseudomonas spp, and Escherichia coli were considered to be target bacteria on the basis that they would be the most likely environmental contaminants. The study design involved a stepwise culture technique, with an initial qualitative assessment that was followed by a blind subculture for microbial isolation and identification, when necessary. Tryptic soy broth was selected as a general-purpose medium for cultivation of fastidious and nonfastidious microorganisms, including common aerobic, facultative anaerobic, and anaerobic bacteria and fungi.31 Columbia sheep blood agar was used owing to an ideal general performance for culture of fastidious organisms with rapid growth, clearly defined zones of hemolysis, and good colonial differentiation.32 Furthermore, microbial isolation and identification were not required during the study because of the lack of growth indicative of vial contamination, which negated the need for this assessment.

Three handling techniques were selected to represent 3 tiers of sterility, as determined on the basis of the clinical experience of the authors. The use of a CSTD was not required as a biohazard safety for the investigators because this is unnecessary for alfaxalone; however, the CSTD could potentially have blocked the transfer of contaminants into the system and maintained sterility of the medication. In a 2016 study,33 it was determined that use of a manufactured CSTD extended sterility of a chemotherapeutic agent and could minimize drug shortages and medication waste and result in cost savings. The duration of the present study was 14 days, which dictated that 0.5-mL aliquots be withdrawn from each 10-mL vial on a daily basis, while accounting for expected dispensing losses.

The present study did not include positive-control vials because of the results of a previous study30 conducted to evaluate microbial colonization of vials containing propofol, alfaxalone, and thiopental injectable solutions. Results of that study30 indicated that growth of Staphylococcus aureus is not supported by alfaxalone when direct inoculation is performed, as evident by the fact that constant numbers of bacteria were initially maintained but numbers proceeded to decrease thereafter. Conversely, E. coli had exponential growth in alfaxalone, which was similar to results for the propofol vial, although there was a 24-hour lag phase for bacterial adaptation required.30 Alfaxalone does not possess bactericidal properties, contrary to results for thiopental.30 Ultimately, one would expect consistent and reproducible growth of a single bacterial agent or a mixed colony if vial contamination occurred during a study.34

Although sporadic growth of bacteria was observed in the study reported here, it could be explained on the basis of laboratory error in the sample handling stages. Microbial assessments were not performed in laboratories designed for sterility testing; they were conducted in an active microbiology laboratory. A single bacterial colony or a few bacterial colonies were identified on the 5 plates with positive growth, whereas the respective enrichment broth remained transparent, and repeated plating yielded no bacterial growth. There was 1 manufacturer-supplied vial stopper stored at room temperature that yielded bacterial growth on day 3 (turbidity of the tryptic soy broth and marked bacterial growth on the blood agar plate), which we believe indicated that there was an error in aseptic technique at the point of sample transfer into the enrichment broth after the sample was collected from the vial. Because subsequent sam-
ples from that vial yielded no growth, this supported the contention that the vial contents remained sterile, although definitive conclusions cannot be made without molecular diagnostic testing, which was not within the scope of the present study.

For the other samples that had positive growth, contamination likely occurred during streaking on the blood agar plates, as indicated by transparent enrichment broth and negative results for samples of tryptic soy broth obtained from the day before and the day after the sample that yielded the microbial growth. Results of the study reported here suggested that drug collection performed in accordance with aseptic techniques alone may be sufficient to prevent vial contamination for at least 14 days. Furthermore, we cannot comment as to whether the cyclodextrin vehicle may have also created a less favorable environment for microbial colonization. To minimize study variability, the same investigators performed the sample collection, sample transfer, and plate streaking, and microbial growth was evaluated by a single microbiologist.

The microbial integrity of multidose vials has been investigated with regard to withdrawal rate, and it was concluded that this is a separate factor for vial contamination. Although that conclusion may be specific for multidose vials that contain preservatives, it may also translate to alfaxalone if aseptic technique is maintained. Additionally, experiments conducted to evaluate multidose vials for maintenance of sterility reveal that bacterial contamination is not a significant hazard when low-level contamination is induced. However, lethal septic shock of a dog has been reported after IV administration of contaminated propofol; the contamination was associated with the fact that the single-use recommendation had not been followed.

The USP currently defines a multidose vial as a liquid medication intended for parenteral administration that contains more than 1 dose of medication and typically contains an antimicrobial preservative to help prevent the growth of bacteria. The beyond-use-date of 28 days refers to the time after which an opened or accessed multidose vial should be discarded, unless otherwise defined by the manufacturer. Conversely, a single-dose or single-use vial is defined as a liquid medication intended for parenteral administration to a single patient for a single case or procedure per injection and typically lacks an antimicrobial preservative. A single-dose vial is to be discarded according to the time specified by the manufacturer, at the end of the procedure, or no later than 6 hours after initial puncture if maintained within an International Organization of Standardization 5 environment. The beyond-use date for a sterile preparation is based on microbial integrity as well as chemical stability over time. Chemical stability testing was deemed cost prohibitive, and chemical stability was not evaluated in the present study. However, anecdotal clinical use of alfaxalone over a 14-day period after initial puncture of a vial has not indicated a change in the quality of the intended anesthetic effects. Furthermore, the Australian label for alfaxalone allows for use of vials stored for up to 7 days in a refrigerator, which indicates the manufacturer’s confidence in chemical drug stability for a minimum of 7 days.

We recognize that the alfaxalone vials did not contain preservatives to prevent microbial contamination; despite this fact, however, testing of 14 aliquots/vial over a 14-day period did not result in conclusive vial contamination in any of the 18 vials in the 6 treatment groups. This conclusion was reached despite the positive growth for a broth sample on day 3 (manufacturer-supplied vial stopper stored at room temperature) and on blood agar plates for sporadic samples (both storage conditions) after day 7. In accordance with a chemical stability of 7 days and the risk of contamination at >7 days, although considered minimal, we believe the results of the present study advocate for extending the use of alfaxalone for up to 7 days after a vial is broached. Because the tryptic soy broth sample with bacterial growth on day 3 was stored at room temperature, refrigeration at 4°C is the recommended storage condition. Although results of the study reported here suggested that alfaxalone maintained microbial integrity for 14 days, there is concern that there could be transient vial contamination, which could lead to devastating clinical consequences. Therefore, additional evaluation of alfaxalone chemical stability and further study of the extension of alfaxalone use after a vial is opened are warranted because a larger sample size may indisputably confirm bacterial contamination attributable to underlying laboratory error versus true vial contamination.

The 4 negative-control vials were not punctured until the conclusion of the study. They yielded no bacterial growth, which indicated that bacteria did not cross the rubber stopper of the vial. Other factors that may have affected vial contamination include appropriate use of aseptic technique by personnel, ability of the rubber stopper to maintain integrity after multiple punctures, and injection of environmental air into the vial during sample collection. The investigators used appropriate aseptic technique for withdrawal of each sample; the port or rubber stopper was initially swabbed with a 70% isopropyl alcohol pad, and sterile syringes and needles were used for sample collection. The collection of samples was performed within the North Carolina State University Veterinary Teaching Hospital; this confirmed that aseptic technique can be successfully executed in an active veterinary hospital. No comments can be made with regard to the ability of the rubber stopper to maintain integrity after multiple punctures or the potential impact of the injection of environmental air.

The extended sterility of alfaxalone detected in the present study could have a beneficial impact on the field of zoological medicine, particularly exotic pet medicine. Alfaxalone provides adequate chemical restraint for the performance of routine diagnostic tests or minimally invasive procedures in a vari-
ety of small exotic pets, such as rabbits, ferrets, rodents, and reptiles. From a clinical perspective, a common IM dose for rabbits is 2 mg/kg. This equates to 0.4 mL of drug for a 2-kg rabbit and 9.6 mL of unused drug remaining in a 10-mL vial, which represents massive drug wastage. Fortunately, results of the study reported here supported the fact that small volumes of drug can be collected from the same vial over a 7-day period before it would be necessary to discard the vial because of concerns about microbial contamination. Therefore, results of this study will likely encourage the use of alfaxalone and subsequently minimize the drug waste for this FDA class IV controlled substance.

To our knowledge, the microbial integrity of alfaxalone under various handling and storage conditions has not previously been evaluated. Results of the present study indicated that alfaxalone may be appropriate for multiple-use protocols for up to 7 days after the vial is initially punctured provided the vial is maintained in refrigerated storage and the drug volume is collected by use of aseptic techniques with a single puncture of the stopper with a CSTD or NCDP. These findings provided support for the use of alfaxalone in small exotic animals by minimizing drug waste and the subsequent environmental impact for disposal of unused drug after administration of small volumes. Additionally, results of this study could serve to aid in standardizing storage and handling protocols for alfaxalone use in veterinary practices across the United States. Additional studies on the microbial integrity of preservative-free medications and their use in veterinary practice are warranted.

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Footnotes
d. Mini-Spike dispensing pin, B. Braun Medical Inc, Bethlehem, Pa.
e. BD Bacto tryptic soy broth, Thermo Fisher Scientific, Waltham, Mass.
f. Disposable culture tubes, 16 X 150 mm, Thermo Fisher Scientific, Waltham, Mass.
g. Trypticase soy agar with 5% Columbia sheep blood, Remel, Lenexa, Kan.
h. Omnicell supply cabinet, Omnicell Inc, Mountain View, Calif.
j. Monoject 3-mL Luer-lock syringes, Coviden LLC, Mansfield, Mass.
k. Monoject 22-gauge needle, Coviden LLC, Mansfield, Mass.
l. SPSS Statistics, version 24, IBM, Armonk, NY.

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