Comparative Medicine
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Vol 71, No 3
June 2021
Pages 210–214

Original Research

Antimicrobial Susceptibility of *Corynebacterium bovis* Isolates from Immunodeficient Rodents

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***Corynebacterium bovis*, the causative agent of hyperkeratotic dermatitis in immunodeficient mice, is a significant problem in preclinical oncology research. Infection results in lifelong skin colonization and a decrease in successful engraftment of patient-derived xenograft tumor models. The use of antimicrobial agents for *C. bovis* is controversial in light of reports of poor efficacy and the possibility of selection for resistant strains. The purpose of this study was to describe the antimicrobial susceptibilities of *C. bovis* isolates obtained exclusively from immunodeficient rodents in order to aid in antimicrobial dose determination. Between 1995 and 2018, 15 isolates were collected from 11 research institutions across the United States. Antimicrobial susceptibility testing was performed for 24 antimicrobials commonly used against gram-positive bacteria. Our results provide an updated understanding of the susceptibility profiles of rodent *C. bovis* isolates, indicating little variability between geographically and temporally distant isolates. These results will facilitate appropriate antimicrobial use to prevent and treat *C. bovis* infections in immunodeficient rodents.**

Abbreviations: MIC_{50}, minimum inhibitory concentration of antibiotic effective against 50% of the isolates; MIC_{90}, minimum inhibitory concentration of antibiotic effective against 90% of the isolates

DOI: 10.30802/AALAS-CM-20-000107

Corynebacterium bovis is a gram-positive, facultatively anaerobic pleomorphic bacillus that infrequently causes infections in humans[5] but is more clinically relevant in veterinary medicine. Veterinary interest in this bacterium originated in the dairy industry, where it causes subclinical mastitis in infected animals and is the most common *Corynebacterium* spp. isolated from infected udders. When present as a primary infection, *C. bovis* can cause decreases in milk quality with no significant decrease in milk yield.[9,12] Despite being considered a minor pathogen, the impact of *C. bovis* on milk quality remains economically important to the dairy industry.

C. bovis was first recognized in the mid1970s in athymic nude mice with hyperkeratotic dermatitis, a condition that would later be termed 'scaly skin disease.'[6] Once genetically characterized in the mid1990s and confirmed to have an association with clinical disease, *C. bovis* emerged as an important pathogen of immunodeficient mice in the laboratory animals.[7] Historically, *C. bovis* infections of research mice primarily occurred in athymic nude mice. However, as the number of transgenic immunodeficient strains has expanded, *C. bovis* is no longer considered an infection exclusively of athymic nude mice, as infections have been reported in immunodeficient and 'immune-vague' research rodents around the world.[3,10,11,15,21]

Antimicrobial susceptibility testing is used to identify the minimum inhibitory concentration (MIC) of specific antimicrobials that prevents the growth of an individual bacterial isolate in vitro. By including many isolates of the same organism into a test population, the MIC can be calculated that inhibits the growth of 50% (MIC_{50}) or 90% (MIC_{90}) of the isolates.[22] MIC have been published for *C. bovis* isolates obtained from dairy cows.[25] In the dairy industry, dry cow therapy (the administration of antibiotics at the end of lactation) is highly effective at eliminating subclinical mastitis caused by *Corynebacterium* spp.[1] However, elimination of *C. bovis* from immunodeficient mouse populations is much more challenging.[15,17] To date, the dose of amoxicillin used to treat *C. bovis*-infected immunodeficient mice has been informed by MIC data from dairy cows isolates[25] and in vivo pharmacokinetic data in the form of blood plasma concentrations of amoxicillin administered in the drinking water.[16] However, our group and others have demonstrated the reemergence of infection in immunodeficient mice after the discontinuation of antibiotic administration in a *C. bovis*-free environment. These findings suggest that the MIC for *C. bovis* isolates from mice may differ from that of cows.[2]

Recently, the genomes of *C. bovis* isolates obtained from humans, cows, mice, and rats were sequenced. Subsequent genomic comparisons assessing the average nucleotide identity between isolates identified sequence divergence obtained from humans and cows as compared with isolates from rodents.[4] In particular, the number of genomic islands and virulence factors

Received: 13 Nov 2020. Revision requested: 03 Dec 2020. Accepted: 02 Feb 2021.
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were significantly higher in the rodent isolates than in the human and cow isolates. However, whether phenotypic changes in antimicrobial susceptibility accompany this genetic divergence is unknown. Considering the prior observations and new developments in our understanding of *C. bovis* across multiple species, the purpose of this study is to describe antimicrobial susceptibility profiles of *C. bovis* isolates obtained exclusively from immunodeficient rats and mice.

Materials and Methods

Bacteria. *C. bovis* isolates (n = 15) from 11 US biomedical research facilities were collected between 1995 and 2018. Referral veterinary diagnostic laboratories Charles River Laboratories (Wilmington, MA) and IDEXX BioAnalytics (Columbia, MO) provided isolates of *C. bovis* originally obtained from either 1) infected immunodeficient rodents or 2) environmental samples within mouse housing areas. Isolates obtained from these companies were de-identified from the originating research institutions prior to being provided to the authors (ACF, JBD, CAM). Isolates were named according to the region or primary coast of the United States from which they were obtained, the 2-digit year in which the isolate was obtained and a letter when multiple isolates were obtained from the same region in the same year. In addition, 2 isolates were obtained from the University of Colorado Anschutz Medical Campus and 4 isolates were obtained from Memorial Sloan Kettering Cancer Center, spanning 4 and 8 y respectively. Lastly, the HAC (hyperkeratotic-associated coryneform) strain was obtained from Charles River Laboratory, representing the first *C. bovis* isolate from immunodeficient rodents characterized in publication.[6] In addition, *C. bovis* type strain ATCC 7715 was included for comparison (Table 1).

Isolate identification confirmation. Two methods were used to identify each isolate as *C. bovis*. Species-specific quantitative PCR primers that amplify the 16S rRNA gene were used to confirm the identification of each isolate, as previously described.[14] Subsequently, either MALDI-TOF mass spectroscopy or whole-genome sequencing was used as a confirmatory method.[4] MALDI-TOF mass spectroscopy was performed by IDEXX BioAnalytics as previously described,[19] or by Charles River Laboratories.

Antimicrobial susceptibility testing. Isolates were propagated on trypticase soy agar supplemented with 5% sheep blood (catalog no. 221239, Becton Dickenson, Franklin Lakes, NJ) for 36 to 48 h at 37 °C with 5% CO_2. Broth microdilution testing was performed in duplicate by using Sensititre COMPGP1F plates (Thermo Fisher Scientific, Waltham, MA), which were read after 48 h on a BIOMIC 2017 instrument (Giles Scientific, New York, New York) using Clinical and Laboratory Standards Institute methods.[7] To prepare each sample for testing, a swab of pure isolated colonies was used to inoculate sterile demineralized water to achieve a 0.5 McFarland standard (1.5×10^8 cfu/mL), confirmed by using a Sensititre Nephelometer that is calibrated daily by using a 0.5 McFarland standard. Once an approximate 0.5 McFarland standard was achieved,10 µL was transferred to 11 mL of Mueller Hinton Broth with Lysed Horse Blood (catalog no. CP114-10, Thermo Fisher Scientific) using a calibrated 10-µL loop. Two broth tubes were prepared for each isolate, and a Sensititre plate was prepared from each broth tube. All positive and negative controls passed on each plate tested. Owing to the lack of published interpretive breakpoints for *C. bovis* in rodents according to Clinical and Laboratory Standards Institute standards, MIC are provided (Table 1). When replicates differed between runs, the highest MIC value was reported.

Results

We obtained MIC in duplicate for 15 rodent-derived isolates and the *C. bovis* type culture ATCC 7715 (Table 2). Our study found no differences in MIC among any of the 15 rodent isolates and ATCC 7715 with the exception of cefovecin (0.5 to 1 µg/mL) and oxacillin (≤0.25 to >2 µg/mL). Two isolates (WC95 and HAC) showed discrepancies between MIC replicates, with 1- to 2-fold differences for some antibiotics. For these situations, the higher of the 2 MIC is shown. The MIC_{90} and MIC ranges for the isolates characterized in this study are described in Table 3. For this test population, the MIC_{50} was determined to be identical to the MIC_{90} and therefore was not presented separately.

In an attempt to replicate previously published HAC findings,[6] we tested the HAC isolate using our system. Of the 4 antibiotics used previously to test resistance of the HAC isolate,[6] only TMS was used in both our study and the previous report. In contrast to the earlier study,[6] we observed a reduction in growth of the HAC isolate to TMS at 2 µg/mL, but without the establishment of a TMS break point, we cannot conclude resistance to TMS.[6]

Discussion

Athymic nude mice and other immunodeficient rodents are frequently used in preclinical oncology research. However, results from preclinical trials can be confounded by *C. bovis* infection of these highly susceptible rodents.[24] Clinical signs in athymic nude mice affected by *C. bovis*-associated hyperkeratotic dermatitis include alopecia and scaling, especially on the dorsum. In haired immunodeficient strains, clinical signs can include generalized dermal hyperemia, conjunctivitis, alopecia, pruritus, increased water consumption, and loss of body condition. At the University of Colorado Anschutz Medical Campus, we found that prophylactic and metaphylactic administration of amoxicillin in drinking water can be useful in preventing infection.[18] As a result, we often use antibiotic in the drinking water to aid in the systematic elimination of *C. bovis* from immunodeficient rodent colonies. Similarly, Memorial Sloan Kettering Cancer Center has used 0.12% amoxicillin-impregnated feed (1200 ppm) as a means to ameliorate both acute and chronic *C. bovis* infections in immunodeficient rodent colonies.[2,13] Despite the experience of these institutions, a successful antibiotic treatment regimen for *C. bovis* isolated from immunodeficient rodents has not been validated. To begin the process of filling this void, we collected and performed antimicrobial susceptibility testing on 15 *C. bovis* isolates from across the United States, isolated over a span of 23 y (1995 to 2018). Our goal for this study is to better inform the laboratory animal veterinary community of antimicrobial susceptibility patterns for *C. bovis* isolates from rodents. These data can lay the foundation for clinical efficacy studies in rodents using varying doses, varying routes of administration, and consideration of skin penetration to reach the site of infection. Antimicrobial pharmacokinetic and pharmacodynamic studies will help inform susceptibility breakpoints that define clinically susceptible, intermediate, and resistant categories.[22,23] This information will more effectively guide antimicrobial therapeutic interventions for *C. bovis* infected rodents.

In the absence of these data to guide use, the administration of amoxicillin at 0.26 mg/mL or amoxicillin plus clavulanate at 0.35 mg/mL (amoxicillin trihydrate–clavulanate potassium, equivalent to 0.3 mg/mL amoxicillin) in nonchlorinated, nonacidified, sterile drinking water is an appropriate dose according to studies investigating prophylactic and

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Table 1. Names, descriptions, and origins of *C. bovis* isolates characterized in study

Name	Description	Year	Source	Species
ATCC 7715	ATCC strain 7715	1930	ATCC	Cow
HAC	Hyperkeratosis-associated coryneform	1995	CRL	Mouse
WC95	West Coast 1995	1995	IDEXX	Mouse
MSK-08-7894	MSK accession 08-7894	2008	MSK	Mouse
CUAMC1	CUAMC #1	2014	CUAMC	Mouse
EC15	East Coast 2015	2015	IDEXX	Mouse
MSK-16-1683	MSK accession 16-1683	2016	MSK	Mouse
MSK-16-3465	MSK accession 16-3465	2016	MSK	Mouse
MSK-16-2004	MSK accession 16-2004	2016	MSK	Rat
NE18a	North East 2018 (a)	2018	IDEXX	Mouse
NE18b	North East 2018 (b)	2018	IDEXX	Mouse
NE18c	North East 2018 (c)	2018	IDEXX	Mouse
NE18d	North East 2018 (d)	2018	CRL	Mouse
WC18	West Coast 2018	2018	CRL	Mouse
MA18	Mid Atlantic 2018	2018	CRL	Mouse
CUAMC3	CUAMC #3	2018	CUAMC	Mouse

CRL, Charles River Laboratory; CUAMC, University of Colorado Anschutz Medical Campus; IDEXX, IDEXX BioAnalytics; MSK, Memorial Sloan Kettering

Table 2. Description of MIC (μg/mL) for isolates characterized in this study

	Assay range	ATCC 7715	HAC	WC 95	MSK-08-7894	CUAMC 1	EC 15	MSK-16-1683	MSK-16-3465	MSK-16-2004	NE 18A	NE 18B	NE 18C	NE 18D	WC 18	MA 18	CUAMC 3
Amikacin	16–32	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16	≤16
Amoxicillin-clavulanate	0.25–8	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Ampicillin	0.25–8	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Cefazolin	2–4	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2
Cefovecin	0.06–8	0.5[a]	1[a]	1	1	0.5	1	1	1[a]	0.5	1	1[a]	1	1	0.5	1	1[a]
Cefpodoxime	2–8	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2
Cephalothin	2–4	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2
Chloramphenicol	8–32	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8	≤8
Clindamycin	0.5–4	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Doxycycline	0.12–0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Enrofloxacin	0.25–4	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Erythromycin	0.25–4	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Gentamicin	4–16	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4
Imipenem	1–4	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Marbofloxacin	1–4	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Minocycline	0.5–2	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Nitrofurantoin	16–64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Oxacillin	0.25–2	2	0.5	2	2	0.5	≤0.25	2	1	2[a]	>2[a]	>2[a]	2	2	1	2	1
Penicillin G	0.06–8	0.25	0.25	0.25*	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Pradofloxacin	0.25–2	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Rifampicin	1–2	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Tetracycline	0.25–1	0.5	0.5	0.5[a]	0.5[a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Trimethaprim–sulfamethoxazole	2–4	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2
Vancomycin	1–16	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1

[a]MIC varied between replicates, and the higher of the MIC was provided.
Each isolate was run in duplicate.

metaphylactic amoxicillin use.[18] Mice will drink an adequate volume of amoxicillin-treated water, and the plasma concentrations of amoxicillin achieve the MIC_{90} for *C. bovis* isolates obtained from infected immunodeficient rodents.[16] However, after the withdrawal of oral amoxicillin–clavulanic acid or amoxicillin administered for 7 and 8 wk to previously infected nude mice, 42% and 87%, respectively, returned to a culture-positive *C. bovis* status. When antibiotic administration is maintained, *C. bovis* is undetectable by culture and is intermittently detected by PCR analysis.[2,18] As a result, we

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Table 3. Summary table describing MIC_{90} and MIC range (μg/mL) for isolates characterized in this study

Antibiotic	MIC_{90}	Range[a]
Amikacin	≤16.0	≤16.0
Amoxicillin–clavulanate	≤0.25	≤0.25
Ampicillin	≤0.25	≤0.25
Cefazolin	≤2.0	≤2.0
Cefovecin	1	0.5 to 1
Cefpodoxime	≤2.0	≤2.0
Cephalothin	≤2.0	≤2.0
Chloramphenicol	≤8.0	≤8.0
Clindamycin	≤0.5	≤0.5
Doxycycline	0.25	0.25
Enrofloxacin	≤0.25	≤0.25
Erythromycin	≤0.25	≤0.25
Gentamicin	≤4.0	≤4.0
Imipenem	≤1.0	≤1.0
Marbofloxacin	≤1.0	≤1.0
Minocycline	≤0.5	≤0.5
Nitrofurantoin	>64.0	>64.0
Oxacillin	2	≤0.25 to >2.0
Penicillin G	0.25	0.25
Pradofloxacin	≤0.25	≤0.25
Rifampicin	≤1.0	≤1.0
Tetracycline	0.5	0.5
Trimethoprim–sulfamethoxazole	≤2.0	≤2.0
Vancomycin	≤1.0	≤1.0

[a]A single value is provided for the range when all isolates evaluated demonstrated the same MIC for the antimicrobial agent used.

continue to support the conclusion that antibiotics cannot be used reliably to eliminate *C. bovis* in mice with established infections.

In 1995, the first antimicrobial susceptibility test results were published for a *C. bovis* isolate (HAC) collected from infected athymic nude mice.[6] Using the qualitative antibiotic disk-diffusion method, this report concluded that the isolate was resistant to nafcillin, nalidixic acid, sulfamethoxazole–trimethoprim, and trisulapyrimidine. However, the break points used were not provided, and no clinical efficacy studies were performed to validate these findings. In an attempt to replicate these findings, we tested the HAC isolate in our system using the broth microdilution method. Of the 4 antibiotics to which the HAC isolate was previously determined as resistant, only TMS was used in both studies. In contrast to the 1995 study,[6] we did observe reduced growth of the HAC isolate in the presence of TMS, but without the establishment of a TMS break point, we are not able to conclude resistance to TMS. Although we cannot corroborate the prior findings, differences in the concentrations of antibiotics used and testing methodologies are confounding variables for direct comparison.[20] Data from the qualitative antibiotic disk-diffusion method for HAC led to the conclusion that it was resistant to nafcillin, nalidixic acid, sulfamethoxazole–trimethoprim, and trisulapyrimidine.[6] However, break points were not provided in that study, and clinical efficacy studies were not performed.

Despite the difficulty in comparing our results to those of studies that used the antibiotic disk-diffusion method, the Sensititre plate microdilution method allows a more accurate comparison of our present results and the previous results of others for isolates of *C. bovis* obtained from dairy cows with subclinical mastitis.[25] Of the 24 antibiotics we evaluated, 7 overlap with an earlier study.[25] We saw few differences in susceptibility patterns in these 2 data sets. However, a clear limitation to this comparison is the high level of sensitivity possible in the earlier study[25] due to the use of low concentrations of these antibiotics in their microdilution assay as compared with the concentrations available for the manufactured Sensititre plates. Two antibiotics for which the data provided by the Sensitire plate and the earlier study[25] directly overlap are oxacillin and tetracycline. For oxacillin, the MIC_{90} for the rodent isolates (2 μg/mL) are lower than those of the cow isolates (4 μg/mL). Conversely, for tetracycline, the MIC_{90} for the rodent isolates (0.5 μg/mL) are higher than those of the cow isolates (0.25 μg/mL). However, these differences are probably not clinically relevant.

Considerable interest has arisen in *C. bovis* subcultured colony phenotypes seen on semisolid agarose media, given relationships to both antimicrobial susceptibility and genetic variation.[2,4] These reports describe both large- and small-colony phenotypes. As compared with the *C. bovis* large-colony type, the small-colony type is suggested to be resistant to trimethoprim-sulfamethoxazole.[18] In addition, the small-colony type has less susceptibility than the large colony type to a variety of antimicrobials, including amoxicillin–clavulanic acid, ampicillin (an in vitro surrogate for amoxicillin), and enrofloxacin. However, we could not study the effect of colony size on antimicrobial susceptibility because we could not replicate this phenotypic difference by using trypticase soy agar supplemented with 5% sheep blood or Columbia agar with 5% sheep blood. We also made multiple attempts with isolates CUAMC1 and MSK-16-1683, which were recently reported to produce these phenotypes.[4] Even if we had been able to reproduce and isolate the different colony phenotypes previously described, the reported instability of the small colony type and the inability to confirm the observed phenotype in a liquid culture assay might have been challenging for MIC determination using a microdilution method. Moreover, due to our inability to obtain phenotypic difference in colony size, we cannot make any conclusions regarding the importance of colony phenotype in the development of recurring infections after the withdrawal of antibiotics. Additional investigation into colony phenotype will be necessary to determine its overall clinical relevance.

The primary limitation of the current study is the relatively small number of isolates that we could obtain and evaluate (n = 15). In general, this limitation is a direct reflection of the limited use of bacterial culture and isolation for the identification of *C. bovis* in rodent colonies as compared with molecular diagnostics. The primary strength of our data is due to our exclusive use of *C. bovis* isolated from immunodeficient rodents and the wide geographic and temporal distribution from which the isolates were obtained. In our 15 rodent isolates, no differences in MIC were observed, except for cefovecin and oxacillin. As a result, our data provide a baseline with which to compare future isolates. Future efforts should further characterize susceptibility patterns in *C. bovis* isolates obtained from immunodeficient rodent colonies and in other mammals clinically affected by *C. bovis*. Future work ideally will establish clinically relevant breakpoints for antimicrobial therapy and elucidate the biologic relevance and relationship between *C. bovis* colony size phenotype and its antibiotic resistance profiles.

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Acknowledgments

We thank our partners for providing isolates of *C. bovis* and Michael Russell (Colorado State University Veterinary Diagnostic Laboratory) for performing antimicrobial susceptibility testing. Marcus J Crim and Kenneth S Henderson are employees of IDEXX BioAnalytics and Charles River Laboratories, respectively. This project was supported by a grant from the American College of Laboratory Animal Medicine (ACLAM) Foundation (to CAM). Additional funding was provided by NIH/NCATS Colorado CTSA grant number TL1 TR002533 and NIH/NCI Cancer Center Support Grant P30 CA008748.

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