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Strategies to Prevent, Treat, and Provoke *Corynebacterium*-Associated Hyperkeratosis in Athymic Nude Mice

Holly N Burr,[1,2,*] Neil S Lipman,[1,2] Julie R White,[1,2] Junting Zheng,[3] and Felix R Wolf[1,2]

Athymic nude mice infected with *Corynebacterium bovis* typically exhibit transient hyperkeratotic dermatitis. Our vivarium experienced an increased incidence of disease characterized by persistent skin lesions and increased mortality, leading to this study. For detection of infection, skin and buccal swab methods showed comparable sensitivities in nude mice. Various prevention, treatment, and eradication strategies were evaluated through clinical assessment, microbiology, and histopathology. In experimentally naïve athymic nude mice, a 2-wk course of prophylactic amoxicillin-containing diet (1200 ppm amoxicillin; effective dose, 200 mg/kg) was ineffective at preventing infection or disease. There was also no significant difference in disease duration or severity in athymic nude mice that received amoxicillin diet or penicillin–streptomycin topical spray (penicillin, 2500 U/mL; streptomycin, 2500 µg/mL). Prolonged treatment with 4 or 8 wk of amoxicillin diet cleared only a small number of athymic nude mice that had subclinical *C. bovis* infections. Antibiotic sensitivity of *C. bovis* isolates demonstrated a small colony isolate with less susceptibility to all antibiotics compared with a large colony isolate. Resistance did not appear to develop after prolonged treatment with amoxicillin. Provocation testing by administration of cyclophosphamide (50 mg/kg IP every 48 to 72 h for 90 d) to subclinically infected athymic nude mice resulted in prolonged clinical disease that waxed and waned without progression to severe disease. Our findings suggest that antibiotic prophylaxis and treatment of clinical disease in experimentally naïve mice is unrewarding, eradication of bacterial infection is difficult, and severe disease associated with *C. bovis* is likely multifactorial.

Abbreviation: CAH, *Corynebacterium*-associated hyperkeratosis.

Corynebacterium-associated hyperkeratosis (CAH), commonly known as 'scaly skin disease,'[3,5,10,27] has anecdotally been reported as early as 1976 in athymic nude mice,[5] with subsequent global outbreaks described in the 1980s and 1990s.[5,11,25,27] The predominant clinical sign of the disease is yellow–white keratin flakes adherent to the skin,[5,27] with acanthosis, orthokeratotic hyperkeratosis, and a mononuclear cell infiltrate evident on histologic examination of the skin.[5,27] A similar disease has been reported in furred immunodeficient mice that includes alopecia and a lesser degree of adherent white keratin flakes on the skin.[28] In 1998, the causative agent was identified as *Corynebacterium bovis* by using 16S rRNA sequence analysis.[8,26]

C. bovis remains a commonly encountered pathogen in athymic nude mouse colonies at academic and industry vivaria, as determined through personal communication with affected institutions. Bacterial control and elimination from colonies in academic animal care programs can be quite challenging, because depopulation and restricted colony access are often not options. Literature regarding the management of *C. bovis* is sparse, including variable eradication success with colony depopulation and disinfection[27] and mention of a nondescript antibiotic treatment.[25] Strategies of antibiotic prophylaxis, various treatments to ameliorate disease, or prolonged antibiotic administration to eradicate subclinical infection have not been reported in the literature. Further, provocation of disease to better understand disease manifestation has not been reported.

We sought to better manage this disease as a result of a recent increased incidence of cases characterized by persistent skin lesions, loss of body condition, and increased mortality in athymic nude mice at our institution. Our study centered on 4 hypotheses: (1) antibiotic prophylaxis would provide the immune system time to mount an effective response and prevent clinical disease but not *C. bovis* infection; (2) various antibiotic treatments would affect clinical disease severity or duration; (3) prolonged antibiotic administration would eradicate *C. bovis* infection from athymic nude mice; and (4) provocation testing would elicit severe disease mimicking that previously seen in our vivarium. Our findings provide a foundation on which further *C. bovis* management strategies can be developed.

Materials and Methods

Animals. Male and female athymic NCr-*nu/nu* mice (age, 4 to 6 wk; National Cancer Institute, Frederick, MD, and Wilmington, MA) were used. Mice were specified by the supplier to be free of murine viruses, pathogenic bacteria including *C. bovis*, and endo- and ectoparasites. Other immunodeficient mice, both furred and strains carrying the nude mutation, and rats used in our study for prevalence sampling for *C. bovis* were included on other approved research protocols within the institution. Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*[17] in an AAALAC-accredited facility. All procedures outlined in the study were approved by the Memorial Sloan-Kettering Cancer Center IACUC.

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[1]Tri-Institutional Training Program in Laboratory Animal Medicine and Science, Memorial Sloan-Kettering Cancer Center, Weill Cornell Medical College, and The Rockefeller University, [2]Center of Comparative Medicine and Pathology, Memorial Sloan-Kettering Cancer Center and Weill Cornell Medical College, and [3]Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, New York.
[*]Corresponding author. Email: holly.burr@bms.com

Animals were housed in individually ventilated caging systems (model nos. 19-140-10-14-1-4-7TMAL and 9-140-10-14-1-4-7TMAL, Thoren Caging Systems, Hazleton, PA), whose effluent was exhausted directly into the building's HVAC system, on autoclaved aspen chip bedding (PJ Murphy Forest Products, Montville, NJ) and were provided a γ-irradiated commercial diet (PicoLab Rodent Diet 20, 5053 LabDiet, PMI Nutrition International, St Louis, MO), except where indicated, and acidified water (pH 2.5 to 2.8) ad libitum. Mice were housed at a population density that ranged from 1 to 5 mice per cage in an environment providing a temperature of 21.1 to 22.2 °C (70 to 72 °F), 30% to 70% humidity, 10 to 15 fresh air exchanges hourly, and a 12:12-h light:dark cycle (lights on, 0600 to 1800). Cage components were sanitized without use of chemicals in mechanical washers (Basil 6000 and 4602 Series, Steris, Mentor, OH) that provided a 180-°F water final rinse.

Mouse cages were changed weekly within a 5-ft. class II type A2 biosafety cabinet (NU602-500, Nuaire, Plymouth, MN). Personal protective equipment included dedicated scrubs, a disposable gown, face mask (TECNOL Procedure Mask, Kimberly-Clark, Roswell, GA), hair bonnet, shoe covers, and latex gloves. The biosafety cabinet was operational for a minimum of 5 min prior to use, and correct magnahelic gauge readings were confirmed. Prior to use, the interior of the cabinet was disinfected with chlorine dioxide solution [Clidox-S (1:18:1), Pharmacal Research Laboratories, Naugatuck, CT] and then lined with chlorine dioxide-soaked 'blue pads' (Dukal Corporation, Hauppauge, NY). Chlorine dioxide disinfectant has previously been shown effective against *C. bovis*.[27] According to the manufacturer's recommendations, bottles of the disinfectant were changed at a minimum of every 14 d to ensure solution efficacy.

Mice were transferred from soiled to clean cages by using 1:18:1 chlorine dioxide solution-soaked forceps; 2 forceps were alternated between cage changes to increase time in the disinfectant. Forceps soaked in the chlorine dioxide solution for approximately 3 min and remained wet with disinfectant when used to transfer mice. Chlorine dioxide solution used to disinfect the forceps was fresh at the start of each cage change to prevent the accumulation of organic debris. Gloves were disinfected with alcohol foam (Alcare Antiseptic Handrub, Steris) before handling each cage and were changed after each side of the ventilated cage rack had been changed (no more than 70 cages) or when obviously soiled. At a minimum, all experimental manipulations of mice were done within a biosafety cabinet by using 1:18:1 chlorine dioxide-soaked double-gloved hands with the outer pair of gloves changed before each cage to prevent cross-contamination.

Microbiologic techniques: bacterial collection and identification and blood culture technique. Three bacterial collection techniques were used during this study in conscious rodents: skin swab, skin scrape, and buccal swab. The skin swab technique consisted of firmly rubbing a dry, sterile cotton-tipped applicator (Puritan Medical Products, Guilford, ME) over the dorsum and flanks of the mice. The skin scrape technique used a scalpel blade (no. 20 Bard–Parker Rib-Back Carbon Steel Surgical Blade, Becton Dickinson AcuteCare, Frankin Lakes, NJ) to scrape the superficial dermis over the dorsum of the mouse to elicit slight flaking. A sterile urethrogenital calcium alginate tipped applicator (Puritan Medical Products) was rubbed along the caudal buccal mucosa for the buccal swab collection technique. Both clinically diseased and apparently healthy mice were sampled by using these methods. All mice within a cage were sampled with the same applicator or scalpel blade to generate a single pooled culture per cage. For the skin scrape technique, the scalpel blade was transferred and pressed onto agar plates by using sterile forceps.

All samples were streaked onto Columbia colistin–nalidixic acid agar with 5% sheep blood (BBL, BD Diagnostic Systems, Sparks, MD) and incubated at 37 °C with 5% CO_2. Colonies that displayed characteristic morphology (1 to 2 mm, smooth, punctiform, white, nonhemolytic) and gram-stain appearance (short, gram-positive rods arranged in V forms)[14] were subcultured onto Columbia agar with 5% sheep blood (BBL, BD Diagnostic Systems) for speciation. Cultures were incubated for 7 d before declared negative for *Corynebacterium* spp.

Isolates were identified by colorimetric biochemical testing (API Coryne, bioMerieux, Marcy l'Etoile, France) after 48 h of incubation at 37 °C[12] to ensure accurate sugar metabolism. At the onset of the study, representative samples from each colorimetric biochemical profile that were identified as *C. bovis* were submitted for confirmatory testing with colorimetric carbon fingerprinting (Biolog, Hayward, CA) and 16S rRNA sequencing. Either a generic bacterial PCR assay or one specific for *Corynebacterium bovis*, which amplifies a segment of the 16S rRNA gene, was performed and the resulting sequences compared with genomic sequences in GenBank by using BLAST software (University of Missouri Research Animal Diagnostic Laboratory, Columbia, MO).

For various studies, blood for culture was collected by cardiocentesis at the time of euthanasia. After carbon dioxide euthanasia, chest skin was sprayed with 70% isopropyl alcohol and wiped in one direction by using a 70% isopropyl alcohol prep pad (Dukal Corporation, Hauppauge, NY) for a contact time of 30 s. Approximately 0.5 mL blood was collected by a percutaneous cardiac stick. Due to concerns of contamination from inadequate skin sterilization, the method was changed early in the study such that the chest skin was seared with a hot scalpel blade (heated in BactiCinerator, Oxford Labware, St Louis, MO) immediately prior to cardiocentesis in lieu of alcohol disinfection. Blood cultures (BBL Septi-Chek TSB, 20 mL, BD Diagnostic Systems) were incubated for 7 to 10 d at 37 °C according to the manufacturer's recommendations and supporting literature[7] before declared negative. The incubated broth was streaked onto a broad selection of agar media, including Columbia colistin–nalidixic acid agar with 5% sheep blood, on day 2 and day 7 to 10 and incubated at 37 °C with 5% CO_2 for 7 d. Any characteristic *Corynebacterium* spp. colonies were identified as described previously. Also at these time points, culture bottles were evaluated for turbidity and a gram-stained smear evaluated for bacteria.

Collection technique evaluation and infection prevalence. Three bacterial collection techniques were evaluated in rodents, furred strains as well as those carrying the nude mutation, housed in 2 immunodeficient holding rooms to determine the most sensitive *C. bovis* detection technique and to assess infection prevalence. The 2 holding rooms contained approximately 450 and 350 rodent cages in individually ventilated caging systems. The holding rooms housed immunodeficient mice [primarily athymic nude, beige-nude-xid (NIHS-*Lyst*^{bg}*Foxn1*^{nu}*Btk*^{xid}), NOD-scid (NOD.CB17-*Prkdc*^{scid}), and NOD-scid IL2Rγ^{null} (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ)] and, in one room, a small population of immunodeficient and immunocompetent rats. These rodents were included on other approved research protocols within the institution and housed at a density of 1 to 5 animals per cage; 10% of the population of each room (total: n = 80 cages; 312 rodents) was sampled for *C. bovis*. Collection techniques included skin swab and buccal swab for rodent strains carrying the nude

mutation (n = 43 cages; 169 rodents) and skin swab, skin scrape, and buccal swab techniques for furred rodents (n = 37 cages; 143 rodents) by using the methods described.

Antibiotic prophylaxis. We evaluated a 2-wk course of antibiotic prophylaxis for prevention of *C. bovis* infection and disease in experimentally naïve 6-wk-old male athymic nude mice. Amoxicillin was selected based on published antibiotic susceptibility results[5,30] as well as personal experience with susceptibility testing and resolution of clinical disease in cases within our mouse colony when treated with amoxicillin. Athymic nude mice (23 cages; n = 47 mice total) were placed on 2 wk of amoxicillin diet [1200 ppm amoxicillin (200 mg/kg effective dose), TestDiet, PMI Nutrition International] prophylaxis immediately on arrival in our vivarium, while 24 athymic nude mice in 7 cages served as controls. Mice were housed at a density ranging from 1 to 5 mice per cage due to mild male aggression and resultant separation (average of 2 mice per cage in the treatment group and 3 mice per cage in the control group). The amoxicillin concentration was selected based on a twice-daily dose recommendation of 100 mg/kg body weight for mice,[15] assuming that a 30-g mouse eats approximately 5 g of food daily.[2]

Mice were confirmed culture negative for *C. bovis* upon arrival; their skin was swabbed once weekly for the initial 2 wk and 3 times weekly thereafter to monitor for the presence of *C. bovis*. In addition, mice were evaluated 3 times weekly for manifestation of hyperkeratotic dermatitis. Initially, mice that developed CAH (n = 33; 16 control and 17 amoxicillin-treated) were euthanized for blood culture and skin biopsies. The remaining mice (n = 38; 8 control and 30 amoxicillin-treated) were monitored for character and length of clinical disease. Clinical disease was graded at each observation on a scale of 1 to 3 representing mild, moderate, and severe disease, respectively. Grade 1 disease consisted of a minimal amount of white flakes adherent to the dorsal skin with the appearance of dusting. Grade 2 disease was consistent with a moderate amount of white flakes adherent to the dorsal skin, with additional locations affected including the ventrum, muzzle, cheeks, crown, and limbs. Erythema variably manifested at grade 2 in locations consistent with flaking lesions and became more pronounced with grade 3 disease. Grade 3 represented the most severe disease phenotype, with a thicker layer of white flakes adherent over the body.

Clinical therapy. Three treatments—amoxicillin diet (1200 ppm amoxicillin; 200 mg/kg effective dose), penicillin–streptomycin topical spray (Penicillin–Streptomycin Solution 100×, JR Scientific, Woodland, CA) diluted to 2500 units/mL penicillin and 2500 µg/mL streptomycin in sterile water, and sterile water topical spray (B Braun Medical, Irvine, CA)—and no treatment were evaluated for their ability to reduce the duration of clinical disease in experimentally naïve 4-wk-old male athymic nude mice (n = 57 mice; 21 cages). Mice primarily were housed at a density of 3 per cage; however, 4 cages included in the study housed 1 or 2 mice total due to mild male aggression and resultant separation. Within 2 d of arrival in the vivarium, mice were exposed to *C. bovis* by 1 of 3 methods: (1) contact exposure for 1 min to a nude mouse with CAH (n = 6 cages; 18 mice), (2) contact exposure for 1 min to subclinically infected nude mice (n = 7 cages; 19 mice), and (3) the addition to their cage of 1 oz of 4-d-old soiled bedding collected from subclinically infected nude mice (n = 8 cages; 20 mice). Prior to inoculation of the study animals, subclinical infection in mice was determined by skin swab and culture as described earlier.

At the onset of CAH, mice were skin swabbed to confirm *C. bovis* infection and randomly assigned by cage to 1 of the 4 treatment groups: amoxicillin diet (n = 5 cages; 12 mice), penicillin–streptomycin topical spray (n = 5 cages; 15 mice), sterile water topical spray (n = 5 cages; 13 mice), and control (n = 6 cages; 17 mice). Mice were evaluated, their disease graded on the aforementioned disease scale, and treated twice daily (0800 and 1700) until resolution of clinical signs. Topical sprays were delivered by using small, plastic spray-pump bottles (item no. 176705, CVS Distributor, Woonsocket, RI) to saturate the dorsum and ventrum of the mice with approximately 0.5 mL total volume.

Bacterial eradication. A prolonged course of antibiotic treatment was evaluated for its potential to eradicate subclinical *C. bovis* infection in male and female athymic nude mice. Subclinically infected nude mice that had previously exhibited CAH were treated with either 28 (n = 15), 62 (n = 15), or 79 (n = 7) d of amoxicillin diet (1200 ppm amoxicillin; 200 mg/kg effective dose). Treatment length was categorized into 2 groups for evaluation: 4 wk (n = 15 male mice in 10 cages; 112 d old) or more than 8 wk (n = 22 female mice in 7 cages; 156 d old). All mice were confirmed *C. bovis* culture-positive by skin swab prior to initiation of this study and were used once a minimal group size of 15 subclinically infected mice was established. Mice were housed at a density ranging from 1 to 5 mice per cage to maintain previous cage groupings. A treatment duration of greater than 8 wk was evaluated in the second group in light of encouraging skin culture results from the 4-wk treatment group.

One (>8-wk study) to 2 (4-wk study) wk prior to completion of the amoxicillin treatment in each group, half of the cages (n = 5 cages, 6 mice in the 4-wk group; n = 4 cages, 11 mice in the >8-wk group, respectively) were transferred out of the immunodeficient mouse holding room with endemic *C. bovis* infection into an isolated holding room in order to prevent the possibility of reinfection after discontinuation of the antibiotic, according to standard husbandry operating practices. Those housed in the isolated holding room (located outside of the main barrier facility) were maintained in complete autoclaved caging with weekly husbandry changes performed by the first author according to strict microisolation technique and using 1:5:1 chlorine dioxide solution, the standard concentration used in this facility. All mice were skin swabbed twice weekly during and after treatment to monitor for *C. bovis*. After approximately 2 (>8-wk study) or 3 (4-wk study) mo, monitoring for *C. bovis* was reduced in those mice still culture negative. Mice were cultured twice monthly for 2 (>8-wk study) or 3 (4-wk study) mo and then monthly for 3 additional months. At the conclusion of the study, mice were euthanized for histologic skin analysis.

Antibiotic susceptibility testing. The Clinical and Laboratory Standards Institute (CLSI) has not published guidelines for susceptibility testing of coryneform bacteria given their variable or delayed growth patterns and different media or atmospheric requirements.[13,18] The methods used in this study were based on recommended techniques and interpretation criteria established for *Streptococcus* spp.[13] or for *Staphylococcus* spp. when streptococcal interpretive criteria were not provided for the antibiotic. Antibiotic susceptibility testing was performed by using the Kirby–Bauer disk diffusion method (BBL SensiDisc Susceptibility Test Discs, BD Diagnostic Systems) with Mueller–Hinton agar containing 5% sheep blood (BBL, BD Diagnostic Systems). Testing was conducted on isolates collected from cages (n = 11) containing *C. bovis*-infected mice before and after 4 or >8 wk of amoxicillin diet administration. In addition, isolates were divided and tested as small and large colony-types, a phenotypic difference that appeared with subculturing. Therefore, 4 isolates were tested per cage sample: the small colony-type, both before

and after amoxicillin treatment, and the large colony-type, both before and after amoxicillin treatment (n = 44 isolates total). Isolates were inoculated in Mueller–Hinton broth (BBL, BD Diagnostic Systems) at a concentration of 6 McFarland standard. This suspension is heavier than the standard recommendation for antibiotic susceptibility testing of many bacterial species (0.5 McFarland standard)[6] but was necessary to visualize growth of *C. bovis*. Antibiotics were chosen based on previous antibiotic susceptibility testing for *C. bovis*[5,30] and a spectrum of activity that included gram-positive bacteria. Tests were incubated for 48 h at 37 °C with 5% CO_2 prior to evaluation. A CO_2-enriched atmosphere is recommended to enhance growth of lipophilic corynebacteria.[13]

Provocation testing. In an attempt to replicate severe *C. bovis* disease previously seen in experimental mice in our vivarium and to better understand disease manifestation, 112-d-old subclinically infected male athymic nude mice (n = 14) were given 50 mg/kg cyclophosphamide [Cyclophosphamide for Injection, USP (Baxter Healthcare Corporation, Deerfield, IL) reconstituted in 0.9% sodium chloride injectable USP (Hospira, Lake Forest, IL)] intraperitoneally every 48 to 72 h (based on a 3 times per week dosing schedule) for 90 d. Mice previously had exhibited CAH within 6 wk prior to initiation of provocation testing and were skin-culture-positive for *C. bovis*. The dosing regimen was selected based on review of the literature.[1,16,29] Mice were weighed weekly, and skin lesions were scored 3 times weekly. In addition, untreated mice (n = 13) from the same cohort were monitored during this time for disease recrudescence without cyclophosphamide-induced immunosuppression. After 90 d, complete necropsies with histopathologic evaluation were conducted for the experimental mice and a subset of the control mice (n = 5). Blood cultures were evaluated as described previously.

Histopathology. Complete histologic analysis of all tissues was conducted for mice included in the provocation study. Mice from the remaining studies were evaluated for skin pathology only. Skin biopsies were collected from both the interscapular region as well as the flank, each section measuring 1 × 2.5 cm. Formalin-fixed, paraffin-embedded 5-µm tissue sections were processed routinely and stained with hematoxylin and eosin; skin sections were in addition evaluated with a tissue Gram stain (modified Twort or Brown and Brenn). Histologic analysis of the entire skin section included evaluation of epidermal thickness, bacterial load, and degree of inflammation present. Epidermal thickness was considered normal at 1 to 3 viable, nucleated cells visible from the basal layer to the stratum granulosum, mildly thickened at 4 to 6 cells, and moderately hyperplastic at 7 to 9 cells. Bacterial burden and inflammation were each graded on a scale of 1 to 3, equivalent to minimal, mild, and moderate lesions, respectively. A bacterial burden score of 1 indicated minimal bacteria that were difficult to locate within the stratum corneum, grade 2 indicated few bacteria within the section that were easy to identify, and grade 3 represented a moderate bacterial load that were easily identifiable and aggregating. Grade 1 inflammation indicated a minimal increase in dermal inflammatory cells compared with adjacent nonlesional skin. Grade 2 inflammation was mild, with some aggregating of inflammatory cells within the dermis, and grade 3 indicated moderate dermal aggregates of inflammatory cells, as well as the presence of serocellular crusts or necrotic or apoptotic cells within the epidermis. Regarding bacterial assessment, intracorneal bacteria were scored only if they resembled gram-positive rods. Because other *Corynebacterium* spp. are considered part of the normal flora of the skin and mucus membranes of animals[3] and frequently are isolated from laboratory rodents,[14] the presence of *C. bovis* was confirmed with culture and PCR.

Statistical methods. Nested mixed-model ANOVA, with mice as a random effect, was used to assess outcomes of the antibiotic prophylaxis study and outcomes of the clinical therapy study. The treatment group was the fixed effect and mice in the cage were the nested random effect in both studies. Marginal least-squares means (± SE) and overall P values for the fixed effects were estimated.

The bacterial eradication study outcomes were censored data. A Cox regression that accounted for the intracage correlation by using robust sandwich covariance estimates[21] was conducted to examine the effect of the length of trial on the outcomes. We evaluated estimated hazard ratios (95% confidence interval) between the treatment groups along with the corresponding P value.

To evaluate the results of antibiotic susceptibility testing, differences between paired large colony and small colony isolates and between pretreatment and posttreatment isolates were calculated and summarized by median and range. Small and large colony isolates were paired based on origination from a single mouse cage sample. The Wilcoxon signed-rank test was used to evaluate the significance of the difference.

All statistical tests were 2-sided; associations and differences were considered significant if the P value was less than 0.05. All analyses were performed with SAS 9.2 software (SAS Institute, Cary, NC).

Results

Microbiologic techniques: bacterial identification. *C. bovis* isolates revealed phenotypic differences in colony morphology. Subculturing induced a large and small colony-type, distinguishable by size after 48 h of growth at 37 °C with 5% CO_2 (Figure 1). The large colony-type produced heavier growth on Columbia agar with 5% sheep blood when compared with the small colony-type, each measuring approximately 2 mm or 1 mm, respectively. In addition, the small-colony phenotype was unstable as subculturing consistently yielded a mixed population of the small and large colony-types. The small and large colony-types produced colorimetric biochemical profiles that differed in their enzymatic reactions and carbohydrate utilization. The large colony-type was consistently positive for β-galactosidase enzyme and metabolized glucose, whereas the small colony-type was β-galactosidase negative, variably metabolized glucose, and was positive for the enzyme pyrolidonyl arylamidase. Both colony-types were urease-positive, catalase-positive, and alkaline-phosphatase-positive. In total, 9 biochemical profiles were generated from isolates confirmed to be *C. bovis* with 16S rRNA sequencing. Profiles generated most consistently by the small and large colony-types were 4101004 and 0501104, respectively. Colorimetric carbon fingerprinting identified both the small and large colony-types as *C. bovis*. 16S rRNA sequences from the large and small colony-types were identical and displayed 100% identity to 44 *C. bovis* sequences, the majority of those deposited in GenBank. For example, the sequences displayed 100% identity to *C. bovis* strain (GenBank accession, NR_037042) over basepairs 956 through 1254 (University of Missouri Research Animal Diagnostic Laboratory, Columbia, MO).

Collection technique evaluation and infection prevalence. Bacterial collection techniques were evaluated in immunodeficient rodents—furred strains and those carrying the nude mutation—that were part of other research protocols to determine the most sensitive collection technique for detecting *C. bovis* and to

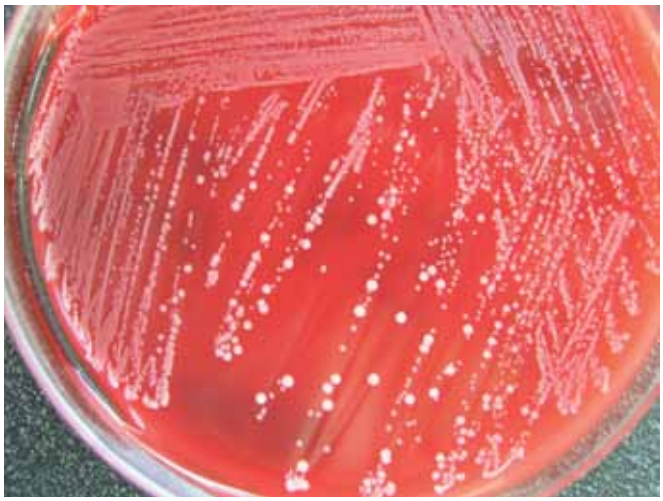

Figure 1. Growth of *C. bovis* on Columbia agar with 5% sheep blood. The small and large colony-types are distinguishable, measuring approximately 1 mm and 2 mm, respectively, in diameter.

assess infection prevalence within the institution. In nude mouse strains, 32 of 42 cages sampled in 2 immunodeficient rodent holding rooms were found to be culture-positive for *C. bovis*. The skin swab technique was positive 100% of the time (32 of 32) whereas the buccal swab was positive 94% of the time (30 of 32). Notably, one cage of mice that was negative on buccal culture was also on amoxicillin diet (1200 ppm amoxicillin; 200 mg/kg effective dose). In addition, 6 of 35 furred immunodeficient mouse cages were culture-positive for *C. bovis*. The buccal swab technique detected 83% of cages (5 of 6) to be culture-positive for *C. bovis*, followed by 67% (4 of 6) with the skin swab and 17% (1 of 6) with the skin scrape techniques. Three rat cages were sampled, 2 of which housed immunocompetent furred rats (Sprague–Dawley and Copenhagen) and 1 housed an athymic nude rat (Crl: NIH-*Foxn1*^{rnu}). Two (Sprague–Dawley and athymic nude) of the 3 rats were culture-positive, and only by the buccal swab technique, which was positive 100% (2 of 2) of the time. Neither the skin swab nor skin scrape techniques isolated *C. bovis* in rats.

Overall infection prevalence in the 2 immunodeficient mouse holding rooms was 30% and 65%. In nude mice, infection prevalence was 57% and 86%, notably not 100% in one of the holding rooms likely due to 3 mouse cages sampled that were on amoxicillin diet and one cage sampled that had just entered the vivarium the day prior. Strains of mice and rats infected with *C. bovis* included athymic nude, beige-nude-xid, NOD-scid IL2Rγ^{null}, SCID-beige (CB17.Cg-*Prkdc*^{scid}*Lyst* ^{bg}), and NOD-scid mice, as well as Sprague–Dawley and athymic nude rats. Beige-nude-xid mice routinely displayed hyperkeratotic dermatitis grossly identical to the disease of athymic nude mice, whereas NOD-scid and NOD-scid IL2Rγ^{null} mice often displayed a less pronounced hyperkeratotic dermatitis with patchy alopecia, a roughened hair coat, hunched posture, and periocular erythema.

Antibiotic prophylaxis. Two weeks of amoxicillin prophylaxis for incoming athymic nude mice prevented neither *C. bovis* infection nor clinical disease; 100% of the mice in both the control and amoxicillin groups became infected with *C. bovis* and exhibited hyperkeratotic dermatitis. No significant difference regarding time to infection after arrival, time to CAH manifestation, or length of CAH was observed between the 2 groups (Table 1). All mice also developed CAH regardless of male aggression.

Mice (n = 33; 16 control and 17 amoxicillin-treated) were euthanized at disease onset for evaluation of blood cultures and skin biopsies. Treated mice were euthanized between 13 to 34 d after discontinuation of the amoxicillin diet. In 25 mice, blood was collected by percutaneous cardiac stick after alcohol disinfection of the chest skin. Blood cultures from 14 of 25 of these mice (8 control; 6 amoxicillin-treated mice) were positive for *C. bovis*. Signs of septicemia were not apparent; mice appeared healthy except for hyperkeratotic dermatitis. The positive blood cultures therefore were dismissed as skin contamination. Blood cultures from the remaining mice (n = 8) in this group, all in similar health condition as the initial subset, were negative after cardiocentesis that was preceded by searing of the chest skin in lieu of alcohol disinfection. In addition to macroscopic lesions, histologic evaluation of the skin revealed lesions consistent with CAH, including orthokeratotic hyperkeratosis with or without intracorneal gram-positive bacterial rods, acanthosis, and a mild mononuclear cell infiltrate (Figures 2 and 3). No appreciable difference was noted between the amoxicillin-diet or control groups (Table 2).

Clinical disease was characterized and graded in the remaining mice (n = 38, 8 control and 30 amoxicillin-treated). In experimentally naïve mice across groups, clinical disease remained mild to moderate (grade 1 or 2; Figure 4), and loss of body condition was not detected (data not shown). Mice monitored twice weekly by skin culture were found to remain persistently infected for 2.5 mo. With continued testing by culture approximately every 2 mo, mice remained *C.-bovis*–positive for a total of 7 mo before euthanasia.

Clinical therapy. Three treatments were evaluated regarding their abilities to reduce disease severity and duration in experimentally naïve athymic nude mice. No significant difference among treatments was detected with regard to disease duration, the most severe disease grade exhibited over course of clinical CAH, the time to disease manifestation after known exposure, or the duration of treatment required to clear disease (Table 3). Clinical disease cleared in all mice in approximately the same length of time regardless of treatment or lack thereof. In addition, each method of exposure to *C. bovis* resulted in infection with subsequent disease manifestation. All mice developed CAH regardless of minimal male aggression in this study.

Bacterial eradication. Subclinically infected male and female athymic nude mice were treated with 4 wk or more than 8 wk of amoxicillin in efforts to eradicate *C. bovis* infection. While on amoxicillin diet, 78% of mice became *C. bovis* culture-negative within 3 wk. In the >8-wk study, 7 mice in 2 cages required 51 d of antibiotic treatment before cultures were negative for *C. bovis* and in the 4-wk study, one cage of mice remained culture-positive throughout amoxicillin treatment. The 2 studies did not differ significantly in terms of time to clearance of infection (4-wk treatment compared with >8-wk treatment: hazard ratio = 1.6; 95% CI, 0.6 to 3.9; P = 0.34).

After discontinuation of amoxicillin, all but 2 cages in the 4-wk study (n = 2 mice) and one cage in the >8-wk study (n = 3 mice) gradually became culture-positive for *C. bovis*. In the 4-wk study, cultures became *C. bovis*-positive between 0 to 46 d (mean = 17 d) after treatment, and in the >8-wk study, cultures were positive between 0 to 55 d (mean = 21 d) after discontinuation of the antibiotic. Statistically, there was no significant difference in the time to reexpression of *C. bovis* infection (4-wk treatment compared with >8-wk treatment: hazard ratio = 1.2; 95% CI, 0.5 to 3.0; P = 0.75). Furthermore, within the 4-wk and >8-wk studies, there was no difference in time to detection of infection after treatment between mice that had been transferred

Table 1. Evaluation of amoxicillin diet (1200 ppm amoxicillin; 200 mg/kg effective dose) to prevent *C. bovis* infection and clinical disease in experimentally naïve athymic nude mice

	Amoxicillin diet	Control diet	*P*
No. of mice in group	47	24	not applicable
Time to infection (d)	34.5 ± 1.8 (20–55)	30.6 ± 2.7 (15–38)	0.104
Additional time to CAH (d)	5.2 ± 0.5 (0–12)	6.2 ± 0.8 (3–9)	0.202
No. of mice for evaluation of CAH duration	30	8	not applicable
Duration of CAH (d)	5.4 ± 0.4 (2–7)	6.4 ± 0.8 (4–7)	0.287

Data are given as mean ± SE (range).

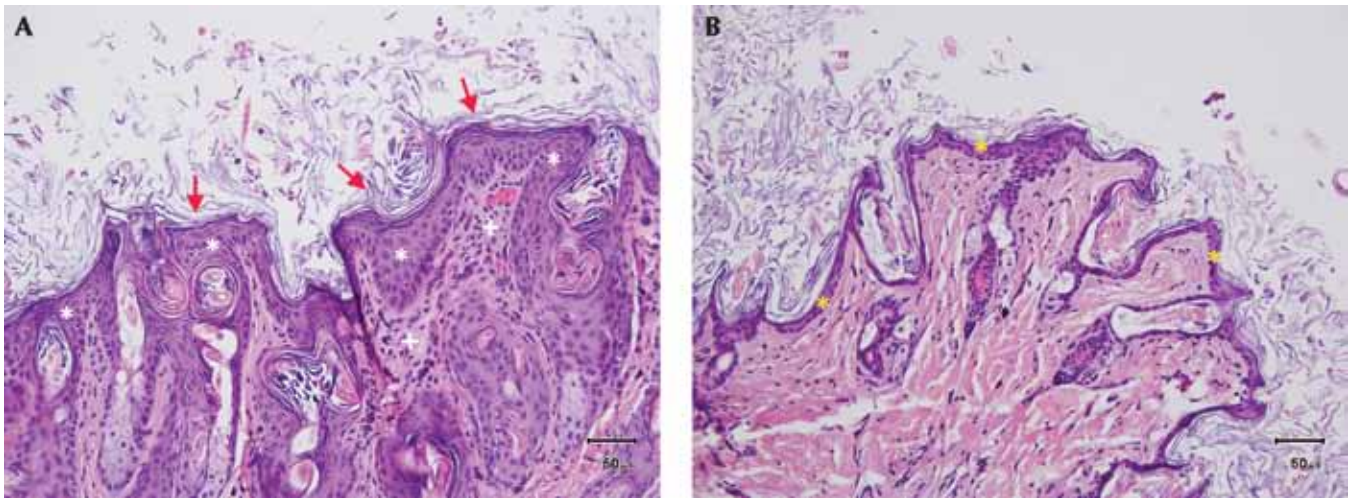

Figure 2. Skin; mouse. (A) Section of lesional skin demonstrating acanthosis (white asterisks; consistent with average epidermal cell thickness of 5), orthokeratotic hyperkeratosis (red arrows), and a mild infiltrate of lymphocytes and plasma cells with fewer mast cells and neutrophils within the superficial dermis (surrounding white crosses; consistent with inflammation score of 2). Hematoxylin and eosin stain; bar, 50 µm. (B) Section from periphery of lesional skin, demonstrating relatively normal epidermal thickness (yellow asterisks) and paucity of inflammatory cells within the dermis. Hematoxylin and eosin stain; bar, 50 µm.

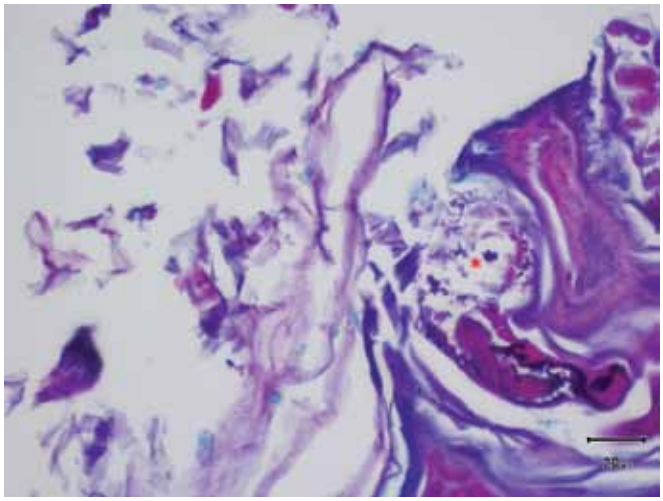

Figure 3. Skin; mouse. Intracorneal clusters of gram-positive, rod-shaped bacteria with fewer gram-positive cocci (red asterisk; consistent with bacterial burden score of 3). Modified Twort stain; bar, 20 µm.

from the immunodeficient mouse holding room to an isolated holding room compared with those that remained in the immunodeficient mouse holding room. Two mice that received 4-wk treatment and 3 mice that received >8 wk of treatment remained serially *C. bovis* culture-negative for at least 284 and 206 d after treatment, respectively.

Both culture-negative and culture-positive mice were submitted after amoxicillin treatment for histologic skin evaluation to evaluate the effects of long-term antibiotics on the classic lesion associated with *C. bovis*. In both treatment groups, all *C. bovis* culture-positive mice were subclinical; histologically, epidermal thickness, bacterial load, and degree of inflammation were quite mild, consistent with macroscopic findings. In addition, mild skin lesions, consisting of slightly thickened epidermis, mild inflammation, and rare bacteria, were seen in those mice in which prolonged antibiotic treatment had resulted in successful eradication of *C. bovis* (Table 2).

Antibiotic susceptibility testing. Antibiotic susceptibility testing was conducted on small and large colony-type *C. bovis* isolates, collected before and after 4 or >8 wk of amoxicillin diet administration ($n = 11$ cages; 44 isolates). A significant ($P < 0.001$) difference in susceptibility was noted between small and large colony-type isolates for all antibiotics; the small colony-type had reduced susceptibility regardless of amoxicillin administration (Table 4). Data are presented as median zone diameters and ranges for the 2 colony phenotypes to show trends in antibiotic susceptibility rather than the designation of susceptible, intermediate, or resistant, given that interpretative criteria were based on *Streptococcus* spp. and *Staphylococcus* spp. In addition, susceptibilities of individual large and small colony-type isolates were compared before and after prolonged amoxicillin administration to monitor for resistance development. There was no significant difference in *C. bovis* isolate susceptibility to ampicillin (median difference, –0.5 mm; range, –12 to 5 mm; $P = 0.06$), the test correlate for amoxicillin (raw data not shown).

Table 2. Mean histologic skin analysis of athymic nude mice across experimental studies

	Group	Clinical CAH?	Epidermal thickness (no. of cells)	Bacteria	Inflammation
Antibiotic prophylaxis study	2 wk Amoxicillin (*n* = 17)	Yes	7.1	2.1	2.4
	Control (*n* = 16)	Yes	6.6	1.6	2.6
Bacterial eradication studies	4 wk Amoxicillin (*n* = 13)	No	3.9	0.77	1.4
	>8 wk Amoxicillin (*n* = 19)	No	3.4	0.8	2.1
	Successful eradication[a] (*n* = 5)	No	3.7	0.2	1.9
Provocation studies	Cyclophosphamide (*n* = 10)	Yes (*n* = 8)	6.2	2.0	2.0
		No (*n* = 2)	3.0	1.0	1.0
	Cyclophosphamide and amoxicillin (*n* = 4)	No	2.5	0.75	0.5
	Control (*n* = 5)	No	3.6	1	2

Macroscopic lesions of *Corynebacterium*-associated hyperkeratosis are indicated if present at necropsy. Epidermal thickness was considered normal at 1 to 3 viable nucleated cells visible from the basal layer to the stratum granulosum, mildly thickened at 4 to 6 cells, and moderately hyperplastic at 7 to 9 cells. Bacterial burden and inflammation were graded on a scale of 1 to 3, equivalent to minimal, mild, and moderate.
[a]Includes 2 mice from the 4-wk study and 3 mice from the >8-wk study of amoxicillin diet administration.

Provocation testing. Subclinically infected male athymic nude mice (*n* = 14) that had exhibited CAH previously were given cyclophosphamide (50 mg/kg IP every 48 to 72 h) for 90 d in efforts to replicate severe disease previously seen within our vivarium and to better understand disease manifestation. All but one mouse exhibited hyperkeratotic dermatitis during the course of cyclophosphamide administration. Hyperkeratotic dermatitis was appreciable on average 41 d after the start of cyclophosphamide administration, with a range of onset of 18 to 70 d after drug administration. Skin lesions waxed and waned with intervening periods of resolution. Recrudescence of disease occurred as many as 4 times in one mouse, with an average of 2 disease recurrences per mouse. Duration of hyperkeratotic dermatitis ranged from 3 to 49 d. A prolonged course of hyperkeratotic dermatitis lasting longer than 7 to 10 d was observed in 12 of 14 mice, with an average duration of disease of 21.5 d.

While receiving cyclophosphamide, mice exhibited undulating weight loss. Although at times weight loss appeared to be associated with manifestation of CAH, there also was pronounced weight loss without skin lesions. Amoxicillin diet treatment (1200 ppm amoxicillin; 200 mg/kg effective dose) was implemented in 4 mice that exhibited marked weight loss with concurrent hyperkeratotic dermatitis; skin lesions resolved and body condition quickly improved. At the time of euthanasia, mice receiving cyclophosphamide alone had an average weight loss of 16.6%, those receiving concurrent amoxicillin had an average weight loss of 6.9%, and control mice had an average weight gain of 1.1%.

Complete necropsies were conducted on control (*n* = 5) and experimental mice (*n* = 14) with skin lesions of primary interest. Of the 10 mice receiving cyclophosphamide alone, 8 exhibited hyperkeratotic dermatitis at necropsy. Neither control mice nor mice receiving both cyclophosphamide and amoxicillin exhibited hyperkeratotic dermatitis at the time of euthanasia. Histologic score was consistent with the presence or absence of CAH (Table 2). In addition, all experimental mice had moderate to marked lymphoid depletion of the spleen and lymph nodes, consistent with chronic cyclophosphamide administration; no other opportunistic infections were detected. Blood cultures were conducted for all mice at the time of euthanasia; all cultures were negative for bacterial growth.

Histopathology. Histologic skin analysis was conducted in all studies to monitor disease progression and antibiotic effect on pathology. Across studies, results from histologic skin analyses were consistent with the presence or absence of macroscopic hyperkeratosis. Mice had regionally extensive changes of varying severity of orthokeratotic hyperkeratosis with or without intracorneal gram-positive bacterial rods, acanthosis, and a mild infiltrate of lymphocytes, plasma cells, and neutrophils with fewer mast cells within the superficial dermis (Figures 2 and 3). Bacteria were found in clusters in the stratum corneum, and in more severely clinically affected mice, bacteria extended into the superficial layers of hair follicles at times. Mice exhibiting CAH at the time of necropsy had pronounced epidermal hyperplasia with increased bacterial load and inflammation. Compared with mice with overt disease, subclinically infected mice had lower scores across evaluation categories, including those mice in which *C. bovis* had been eradicated by lengthy antibiotic treatment (Table 2).

Discussion

Once established, *C. bovis* is a difficult pathogen to manage in facilities housing nude and other immunodeficient mouse strains and as a result continues to present a major challenge. Limited information is available regarding effective strategies for management and clearance of the bacterium other than colony depopulation and disinfection.[27] Our research explored various detection, preventive, treatment, and eradication measures aimed to better manage the introduction and spread of *C. bovis*.

Small and large colony phenotypes have not been previously reported for *C. bovis*. These 2 colony types were detected in nearly every isolate after subculturing for speciation. The clinical significance of this finding is not readily apparent, in that nearly all isolates showed both phenotypes whether collected from clinical or subclinical nude mice. A difference in virulence or disease expression, therefore, does not seem likely but deserves further investigation. Small-colony variants have been described in a number of bacteria and most extensively studied in staphylococci.[24] They are a slow-growing subpopulation of bacteria with atypical colony morphology, unusual biochemical characteristics, increased antibiotic resistance, and an unstable colony phenotype,[24] all characteristics consistent with our small

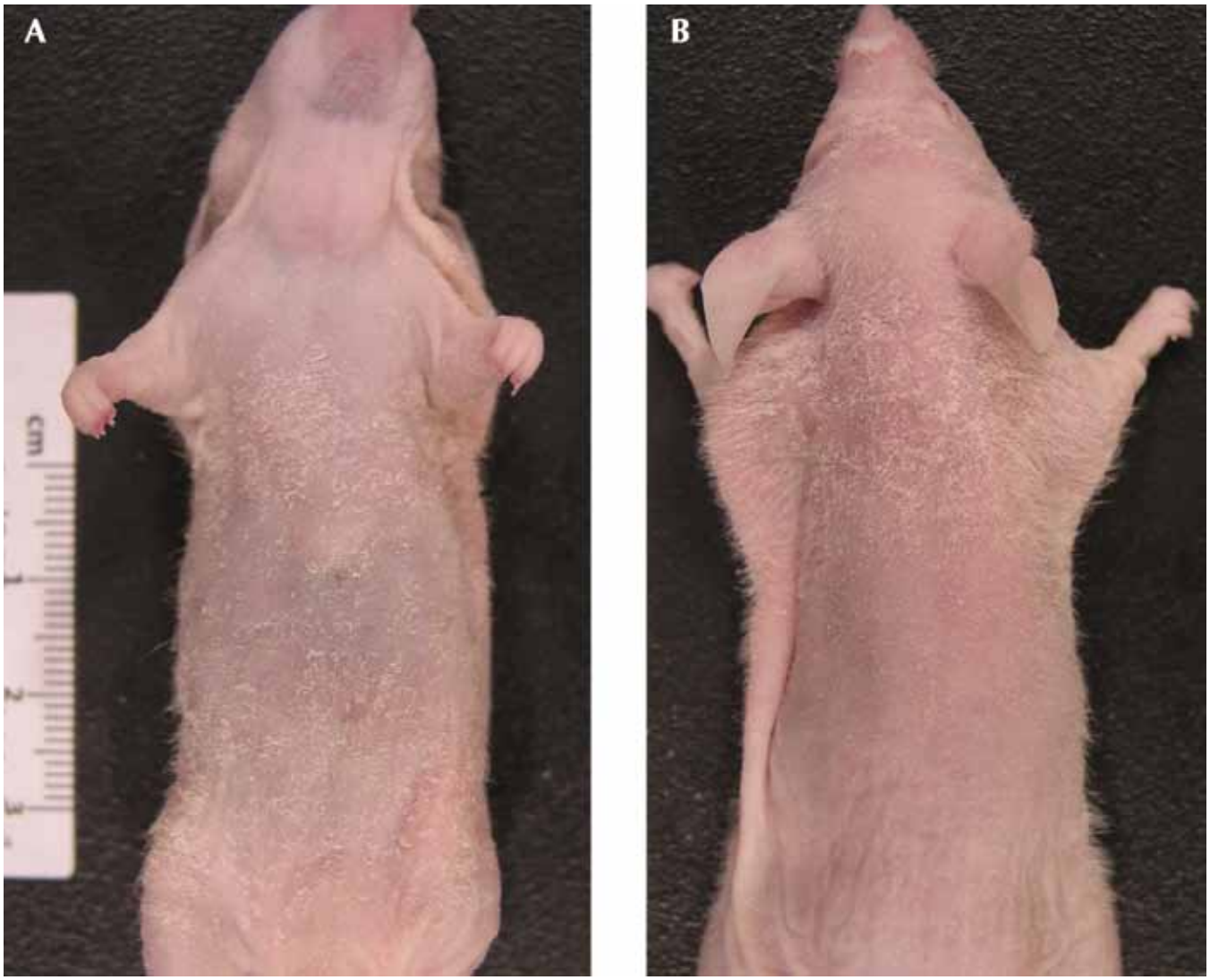

Figure 4. Classic manifestation of *Corynebacterium*-associated hyperkeratosis. Experimentally naïve mice typically display mild to moderate skin lesions (Grade 2 disease shown here) with white flakes adherent over the dorsum, ventrum, and muzzle.

colony-type *C. bovis* strain. 16S rRNA sequences of other bacterial small colony variants have also been identical to that of the normal-colony morphology.[23] In addition, small colony variants are associated with persistent, recurrent infections,[24] and this phenomenon may contribute to the persistence of *C. bovis* in nude mice in addition to its lipophilic nature and localization to the stratum corneum.

Earlier research demonstrated the skin swab to be significantly more sensitive than the buccal swab in athymic nude mice.[5] Evaluation of collection techniques for *C. bovis* in the current study revealed that in mouse strains carrying the nude mutation, the skin swab was comparable to the buccal swab. In furred mice, the buccal swab detected the highest number of infected cages, but the low number of *C. bovis* infections made it difficult to ascertain the significance of this finding. Although the hair coat was deliberately parted for maximal access to the skin during collection, reduced contact with the skin surface may account for the lower detection rate of the skin collection techniques. In addition, the skin scrape was a very superficial technique conducted on conscious rodents, thus preventing firm collection against the skin that may be improved by using either anesthetized or euthanized rodents.

Throughout the duration of all studies reported here, neither of the skin collection techniques resulted in bacterial overgrowth that hindered *C. bovis* detection, as has been previously reported.[27] Alcohol disinfection of the skin prior to sampling was not conducted, although this technique has been suggested to reduce commensal bacterial flora.[3] In addition, although cultures were incubated for 7 d, colonies consistent with and subsequently identified as *C. bovis* were identifiable after 48 h incubation with the methods described above. We believe our skin swab technique detected *C. bovis* infection early in nude mice. Initial collections yielded just a few characteristic colonies whereas serial skin cultures in nude mice over time resulted in increased colony numbers and manifestation of clinical signs. Our research demonstrates consistent methods for identifying *C. bovis* that are of value for laboratories unfamiliar with this pathogen.

The methods we evaluated for prevention, treatment, and eradication of *C. bovis* infection and disease in experimentally naive athymic nude mice ultimately were ineffective. Antibiotic prophylaxis appeared to delay infection, but no significant difference from the control group was noted, and ultimately all mice became infected after discontinuation of amoxicillin. The slight delay in time to infection may be due to prevention of

Table 3. Evaluation of clinical therapy to reduce disease severity (scale, 1 to 3; mean ± SE) and duration (mean ± SE) in experimentally naive athymic nude mice.

	No. of mice	Time to disease manifestation (d)	Duration of disease (d)	Most severe disease grade	Duration of treatment (d)
Water spray	13	13.2 ± 0.4	4.1 ± 0.3	1.4 ± 0.2	3.8 ± 0.3
Penicillin–streptomycin spray	15	11.8 ± 0.4	4.5 ± 0.3	1.5 ± 0.1	4.0 ± 0.3
Amoxicillin diet	12	12.6 ± 0.4	4.1 ± 0.3	1.1 ± 0.2	4.0 ± 0.3
Control	17	12.2 ± 0.3	4.1 ± 0.3	1.3 ± 0.1	not applicable
P	not done	0.070	0.729	0.193	0.837

Disease severity was graded on a scale of 1 to 3, representative of mild, moderate, and severe skin lesions. The most severe disease grade for each mouse over their course of clinical CAH was averaged in each treatment group and is reflected here.

Table 4. Antibiotic susceptibilities of small and large colony-type isolates of *C. bovis*.

			Median (range; *n* = 44 isolates)		
Antibiotic	Concentration	Susceptible	Large colony (diameter, mm)	Small colony (diameter, mm)	Difference (mm)
Amoxicillin– clavulanic acid	20/10 µg	≥20 mm[b]	33 (28–42)	25 (20–30)	8 (2–17)
Ampicillin	10 µg	≥24 mm[a]	30 (25–40)	25 (20–27)	7 (–1–15)
Gentamicin	10 µg	≥15 mm[b]	30 (23–38)	25 (15–28)	6 (0–13)
Penicillin	10 U	≥24 mm[a]	33 (25–40)	25 (20–29)	8 (1–13)
Tetracycline	30 µg	≥23 mm[a]	34 (30–42)	25 (20–32)	10 (4–18)
Trimethoprim– sulfamethoxazole	1.25/23.75 µg	≥19 mm[a]	28 (0–38)	0 (0–0)	28 (0–38)
Erythromycin	15 µg	≥21 mm[a]	36 (30–38)	25 (22–30)	10 (5–15)
Enrofloxacin	5 µg	≥20 mm[b]	30 (22–36)	15 (12–20)	14 (7–21)
Ceftriaxone	30 µg	≥24 mm[a]	34 (30–42)	24 (20–30)	10 (4–20)

Criteria for susceptibility were based on the zone diameters for either [a]streptococci as recommended or [b]staphylococci when streptococcal standards were not provided for the antibiotic. Because interpretive criteria were nonspecific to *C. bovis*, classification of susceptibility (susceptible, intermediate, or resistant) was not assigned to each isolate.
For all antibiotics, difference between large and small colonies was significant (Wilcoxon signed-rank test; $P < 0.001$).

infection during treatment or to antibiotic suppression masking infection. A previously published abstract implied that antibiotic treatment in the drinking water prevents disease but not persistent infection in a colony.[25] In our study, all mice manifested clinical disease characterized by hyperkeratotic dermatitis, consistent with morbidity reports of 80% to 100%.[11,27] We did not evaluate the efficacy of longer-term antibiotic prophylaxis on arrival, but this regimen likely would only mask infection and presents an undesirable variable for ongoing research in the vivarium. In a previous inoculation study, all *C.-bovis*–infected mice did not develop clinical disease, although histologic lesions were present in many mice that lacked gross lesions.[5] Factors such as cage environment and humidity have been suggested to play a role in manifestation of disease.[5] Our provocation testing results further suggest that several factors, including concurrent research and opportunistic infections, may have roles in the development and severity of clinical disease. It is likely that close monitoring and observation of mild disease accounted for the high percentage of clinical disease reported in our current studies.

Antibiotic treatment in drinking water is suggested to ameliorate clinical lesions, thereby preventing dispersal of contaminated keratin flakes and transmission of the bacterium.[3] Our study found that topical and oral antibiotic treatments were ineffective at reducing disease severity or duration in experimentally naïve mice. In our experience, some researchers use topical penicillin–streptomycin spray to avoid a variable due to systemic antibiotic administration in their research. We hypothesized that topical treatment would prove effective due to the increased antibiotic concentrations localized to the epidermis. Regardless of the treatment used, however, our findings were consistent with published work that hyperkeratotic dermatitis resolves spontaneously.[5,27] Perhaps topical treatment is more effective when treating experimentally manipulated mice that exhibit more severe disease, thus accounting for its continued use by some researchers. In that case, further work with a topical ointment, rather than a liquid, may be warranted to ensure greater contact time with the skin. Due to its labor-intensive nature and the potential to confound research, topical treatment on a large scale is undesirable. In addition, due to grooming behavior, mice may ingest the topical antibiotic.

Although treatment was unnecessary for experimentally naïve mice, clinical experience in our vivaria shows amoxicillin diet to be effective at ameliorating clinical disease in experimentally manipulated mice with moderate hyperkeratotic dermatitis and poor body condition. In addition, 4 mice that developed pronounced weight loss and hyperkeratotic dermatitis while receiving cyclophosphamide quickly regained body condition with resolution of skin lesions when treated with amoxicillin. We did not subsequently discontinue antibiotic treatment for assessment of disease progression. Disease recrudescence would likely depend on the current health and experimental status of the mouse.

To date, bacterial eradication measures have focused on colony depopulation and facility disinfection with variable success.[27] Our efforts to eradicate *C. bovis* from subclinically infected mice with prolonged amoxicillin diet administration were ineffective at the colony level, although a few mice were successfully cleared of *C. bovis* infection, a result that has not been reported previously. We considered mice to have been

successfully cleared of *C. bovis* given the extended length of time that skin cultures remained negative. We are confident in our culture technique that results were not false-negatives. Histopathologic examination of the skin for bacteria supported culture results; rare bacteria were detected, and skin cultures yielded other *Corynebacterium* spp. Eradication failure in most mice may be due to inadequate antibiotic concentration in the stratum corneum, where the bacterium localizes, as well as the possibility of reexposure during treatment due to environmental persistence. In addition, mice in which the infection cleared may have had a low bacterial burden.

Previous antibiotic susceptibility testing of *C. bovis* isolates of mouse origin demonstrated susceptibility to most common antibiotics, excluding nafcillin, sulfamethoxazole–trimethoprim, and nalidixic acid.[5] *C. bovis* strains isolated from bovine mammary glands were susceptible to all antibiotics tested excluding tilmicosin.[30] Based on interpretive criteria for *Streptococcus* spp. and *Staphylococcus* spp., we demonstrated the large colony-type isolate susceptible to all antibiotics except for trimethoprim–sulfamethoxazole to which it is variably resistant; an important finding as use of this antibiotic may interfere with detection of *C. bovis* contrary to earlier reports.[3,5] The small colony-type isolate demonstrated a significant reduction in susceptibility to all antibiotics, consistent with the behavior of small colony variants of other bacterial species.[24] Increased resistance to ampicillin in either the small or large colony-type isolates after prolonged amoxicillin administration was not observed. We considered whether routine amoxicillin prophylactic and treatment use in our facility had already established a resistant state within the small colony-type isolate. We do not believe this situation to be true, given that the small colony-type isolate did not predominate on culture as would be expected with selection pressures and demonstrated reduced susceptibility to all antibiotics. In addition, individual cases of CAH in nude mice remain responsive to amoxicillin treatment within our facility, as demonstrated during provocation tests. We therefore believe that failure of amoxicillin to eradicate *C. bovis* from subclinically infected mice is not the result of resistance development.

A considerable limitation to our antibiotic susceptibility testing data is the lack of published interpretive criteria and an evaluation standard. Susceptibility testing of coryneform bacteria is difficult, due to slow, inconsistent growth and variable atmospheric requirements.[13] Poor growth of *C. bovis* on the Mueller–Hinton agar with 5% sheep blood required an increased McFarland standard of the test inoculums, which can result in falsely reduced zone diameters.[9,20] We feel the possibility of smaller zone diameters is inconsequential due to standardized inoculum concentration across all tests. We did not seek to establish *C. bovis* interpretive criteria, which would have been aided by determination of minimum inhibitory concentration and breakpoints; instead, our objective was to screen for resistance and distinguish susceptibility differences between the large and small colony-types. Conversely, an excessively dilute inoculum can result in falsely large zone diameters and false susceptibility.[9,20] Other supplemental techniques have been reported to aid in susceptibility testing of *C. bovis*, including Mueller–Hinton broth supplemented with 1% Tween 80.[30]

Provocation testing of subclinically infected athymic nude mice that had previously exhibited CAH resulted in a prolonged, more pronounced clinical disease in most mice but was not associated with mortality as had occurred in mice used experimentally in our vivarium. Cyclophosphamide-induced immunosuppression alone did not induce severe disease and mortality or cause a systemic spread of the bacterium, as previously hypothesized in light of our and others' past experience with blood cultures.[22] The immunosuppressive dose and or duration could perhaps be further modified to mimic experimental stress and to elicit a more severe disease phenotype. Previous cases of severe CAH disease within our vivarium may have resulted from research-related complications of xenograft work and tumor burden as well as other unknown opportunistic infections. In addition, enhanced toxicity of chemotherapeutic agents in nude mice with CAH has been reported,[11] an important consideration in cancer research. Negative blood cultures from the mice in our study dispelled our thoughts that endpoint disease may be associated with systemic spread of *C. bovis*.

Histologic skin analysis across groups was consistent with the presence or absence of macroscopic skin lesions. Eradication of *C. bovis* in athymic nude mice has never been described; we therefore were interested to learn whether clearance of the bacterium would cause resolution of acanthosis that has been reported to remain in persistent infections.[5] No significant differences were detected in histologic findings from mice in which *C. bovis* had been eradicated compared with mice that remained persistently infected with or without antibiotic treatment, with both groups demonstrating mild epidermal hyperplasia. This finding suggests either that the presence of the bacterium elicits lasting skin pathology or that other mechanisms can account for the mild epidermal hyperplasia and inflammation seen in mice in which *C. bovis* infection had been cleared. Acanthosis is a nonspecific reaction to chronic inflammation, and normally the skin of athymic nude mice has a more prominent and irregular stratum corneum than that in furred mice.[5] In our studies, mice culture-negative for *C. bovis* were culture-positive for other *Corynebacterium* spp., *Staphylococcus* spp., and *Enterococcus* spp. on their skin. *Staphylococcus xylosus* and the fungus *Paecilomyces* have been described as causes of dermatitis in athymic nude or hairless mice.[4,19,27] Therefore, the persistence of acanthosis could be due to concurrent infection with one or more of these agents.

C. bovis remains a pervasive pathogen with challenging epidemiology, as demonstrated by the number of strains of furred and nude, immunodeficient and immunocompetent rodents from which the bacterium was isolated in this study. A short course of antibiotic prophylaxis was shown to be ineffective at infection or disease prevention. Various treatments to lessen clinical CAH or to eradicate subclinical infection were determined to be ineffective at the colony level. More severe disease phenotypes, previously believed to be associated with *C. bovis*, may in fact be due to other opportunistic infections and complications from experimental use, given that cyclophosphamide-induced immunosuppression was unable to induce severe CAH disease. More work is needed to evaluate sources of persistence of *C. bovis* in vivaria and additional strategies for prevention and treatment of the disease.

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