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Original Research

Establishing the Median Infectious Dose and Characterizing the Clinical Manifestations of Mouse, Rat, Cow, and Human *Corynebacterium bovis* Isolates in Select Immunocompromised Mouse Strains

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Corynebacterium bovis **(Cb), the cause of hyperkeratotic dermatitis in various immunocompromised mouse strains, significantly impacts research outcomes if infected mice are used. Although Cb has been isolated from a variety of species, including mice, rats, cows, and humans, little is known about the differences in the infectivity and clinical disease that are associated with specific Cb isolates. The infectious dose that colonized 50% of the exposed population (ID_{50}) and any associated clinical disease was determined in athymic nude mice (Hsd:Athymic Nude-Foxn1^{nu}) inoculated with Cb isolates collected from mice (*n* = 5), rat (*n* = 1), cow (*n* = 1), and humans (*n* = 2) The same parameters were also determined for 2 of the mouse isolates in 2 furred immunocompromised mouse strains (NSG [NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/Sz] and NSG-S [NOD.Cg-Prkdc^{scid}Il2rgt^{m1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ]). To determine the ID_{50}, mice (*n* = 6/dose; 3 of each sex) were inoculated topically in 10-fold increments ranging from 1 to 10^8 bacteria. Mice were scored daily for 14 days for the severity of clinical signs. On days 7 and 14 after inoculation, buccal and dorsal skin swabs were evaluated by aerobic culture to determine infection status. The mouse isolates yielded lower ID_{50} values (58 to 1000 bacteria) than did the bovine (6460 to 7498 bacteria) and rat (10,000 bacteria) isolates. Human isolates did not colonize mice or cause disease. Mouse isolates produced clinical disease of varying severity in nude mice. Despite significant immunodeficiency, furred NSG and NSG-S mice required a 1000- to 3000-fold higher inoculum for colonization than did athymic nude mice. Once colonized, clinically detectable hyperkeratosis did not develop in the haired strains until 18 to 22 d after inoculation, whereas athymic nude mice that developed clinically detectable disease showed hyperkeratosis between 6 and 14 d after inoculation. In conclusion, there are significant differences in Cb's ID_{50}, disease course, and severity of clinical signs between Cb isolates and among immunodeficient mouse strains.**

Abbreviations: Cb, *Corynebacterium bovis*; CAH, *Corynebacterium*-associated hyperkeratosis; dpi, days postinoculation; ID_{50}, median infectious dose

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Introduction

Corynebacterium-associated hyperkeratosis (CAH) has been reported anecdotally as early as 1976 in athymic nude mice; subsequent global outbreaks were described in the 1980s and 1990s.[11,30] In 1998, *Corynebacterium bovis* (Cb), the causative agent of CAH, also known as scaly skin disease, was speciated by using 16S rRNA sequence analysis.[14] Cb, a gram-positive, rod-shaped bacterium, causes opportunistic infections in immunocompromised mice including, but not limited to, the *Foxn1$^{nu-/-}$* athymic nude (NU), NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl} (NSG), and Pkrdc^{scid} (SCID) strains.[11,31] Cb causes similar lesions in immunocompetent epidermal mutant *dep/dep* and hairless SKH1-Hr^{hr} mice, presumably due to sebaceous gland hyperplasia and altered follicle and hair shaft development.[11,26] In nude and furred immunodeficient strains, clinical signs include diffuse hyperkeratotic dermatitis and alopecia with adherent white keratin flakes on the skin, respectively.[5,40] The clinical syndrome in nude mice typically develops within 7 to 10 d after exposure and may include weight loss, dehydration, and pruritus.[11,13,30] In contrast, there are limited studies assessing Cb infection in furred immunocompromised mice, and there are no published studies that describe the disease course in NSG mice, which are commonly used and frequently infected with Cb.[31]

In mice with CAH, the epidermis is hyperplastic, displaying acanthosis and orthokeratotic hyperkeratosis, which may be

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accompanied by mild dermal mononuclear cell infiltrates.[35] Lesion severity is thought to be multifactorial and may be exacerbated by experimental manipulation including administration of chemotherapeutics, surgery, and irradiation.[5]

Cb is spread through direct contact with contaminated keratin flakes, fecal–oral and airborne transmission, fomites (cages, gloves, bedding, work surface, and instruments), and contaminated biologics, including cryopreserved tumors.[5,24,40] Cb has also been isolated from biosafety cabinets and the respiratory tract of animal husbandry staff.[6] Introduction of Cb into a vivarium generally leads to widespread colonization of susceptible stocks and strains with high prevalence of clinical disease in immunocompromised mice.[30] Once infected, NU mice remain colonized despite antibiotic treatment and clinical resolution.[5,25] Furthermore, the use of antibiotics in tumor models can alter the tumor microenvironment and potentially make tumors more resistant to future treatments, including chemotherapeutics.[22] Cb infection also affects the engraftment of chronic myelomonocytic leukemia in NSG-S mice and is thought to alter the immune profile of infected animals.[39]

Once CAH is suspected, diagnosis is confirmed by bacterial isolation or PCR analysis.[5,23] Cb can reliably be isolated from skin or buccal swabs with high sensitivity, reaching 100% and 94% in NU mice, respectively.[5] A thorough understanding of Cb's infectivity, pathogenicity, and epidemiology is critical to developing an effective strategy to prevent its introduction into susceptible colonies. Cb has been isolated from humans and various animal species, and genome-wide analyses have shed light on the differences in the genetic composition of these isolates and uncovered putative virulence factors and pathogenicity islands.[9] Based on their genetic sequences, Cb isolates cluster into 2 distinct groups: 1) rodent isolates and 2) bovine and human isolates.[9] However, relatively little is known about whether these genetic differences influence Cb infectivity, pathogenicity, infectivity, or interspecies transmission.

An important consideration in understanding the infectivity of a microorganism is to determine the median number of microbial units necessary to effectively colonize 50% of the exposed population; this number is the median infectious dose (ID_{50}).[2,7,28] Characterizing infectivity can help determine whether specific populations in a vivarium are more susceptible, and biosecurity protocols can be developed based on these findings. Historically, 3 methods—the Reed–Muench, Dragstedt–Behrens, and Spearman–Karber methods—have been used to estimate the ID_{50} from dose–response data.[19,28,29,38] More recently, probit, logistic regression, exponential, and approximate Beta Poisson models have been used for quantitative microbial risk assessment, allowing the probability of infection to be estimated based on any dose.[19]

This study was undertaken to determine the ID_{50} and dose–response relationships, and to characterize the clinical disease associated with Cb isolates cultured from mice, rats, bovines, and humans. In addition, the ID_{50} and associated clinical disease of 2 Cb isolates from mice were determined and compared in NU, NSG, and NSG-S mice.

Materials and Methods

Study design. The ID_{50} of 9 Cb isolates from various species and institutions (Table 1) were determined in NU mice. In addition, the ID_{50} were determined in NSG mice for isolates 7894 and 13-1426 and in NSG-S mice for isolate 7894. To establish the ID_{50} in NU mice, a maximum of 8 doses (10 to 10^8 in 10-fold increments) of each isolate in midlog growth phase were administered topically to 6 mice (3 of each sex). For isolate 7894 administered to NSG-S and NSG mice, the inocula administered ranged from 1 to 10^7 bacteria. For each isolate, 2 control mice (1 of each sex) received topical administration of uninfected media. After infection, each mouse was singly housed, monitored daily for 14 d, and scored based on clinical signs (Figure 1). On days 7 and 14 after inoculation, mice were weighed, and buccal and dorsal skin swabs were collected to assess Cb colonization through aerobic culture. All animal manipulation, including inoculation and testing, occurred during the light cycle (at approximately 0600 to 1200). Mice were euthanized by CO_2 overdose at 14 days postinoculation (dpi), body and spleen weights were determined, a postmortem examination was performed, macroscopic changes were documented, and full-thickness skin-punch (diameter, 6 mm) biopsy samples were collected from the nuchal crest and dorsal lumbar region for histopathology. Additional biopsy samples were taken from lesions when present.

To determine the clinical presentation of Cb in NSG mice, 4 female NSG mice were inoculated with either Cb isolate 7984 or

Table 1. Characteristics of the *Corynebacterium bovis* isolates used, including the species from which the isolate was cultured, isolation site, institutional source, and the number of putative virulence factors reported[9]

Isolate	Host	Isolation site	Institution	No. of putative virulence factors
7894	Athymic nude mouse	Skin disease (hyperkeratosis and acanthosis)	MSK	29
CUAMC1	Athymic nude mouse	Skin disease (hyperkeratosis and acanthosis)	UC	44
13-1426	NSG mouse	Skin disease (hyperkeratosis and acanthosis)	Biopharma	79
17-0240	Athymic nude mouse	Skin disease (hyperkeratosis and acanthosis)	MSK	28
Iso 24956	Athymic nude mouse	Skin (no clinical disease)	JAX	Unknown
16-2004	Athymic nude rat	Skin disease (dermatitis and ulcerative lesions)	MSK	75
4826	Bovine, cow	Mastitis, milk	CDC SBRL	33
F6900	Human, female	Wound	CDC SBRL	19
WCM 35	Human, female	Eye	NYPH-WCM	Unknown

CDC SBRL, Centers for Disease Control and Prevention, Special Bacteriology Reference Laboratory; JAX, The Jackson Laboratory; MSK, Memorial Sloan Kettering Cancer Center; NYPH-WCM, NY Presbyterian Hospital–Weill Cornell Medicine UC, University of Colorado–Anschutz Medical Campus.

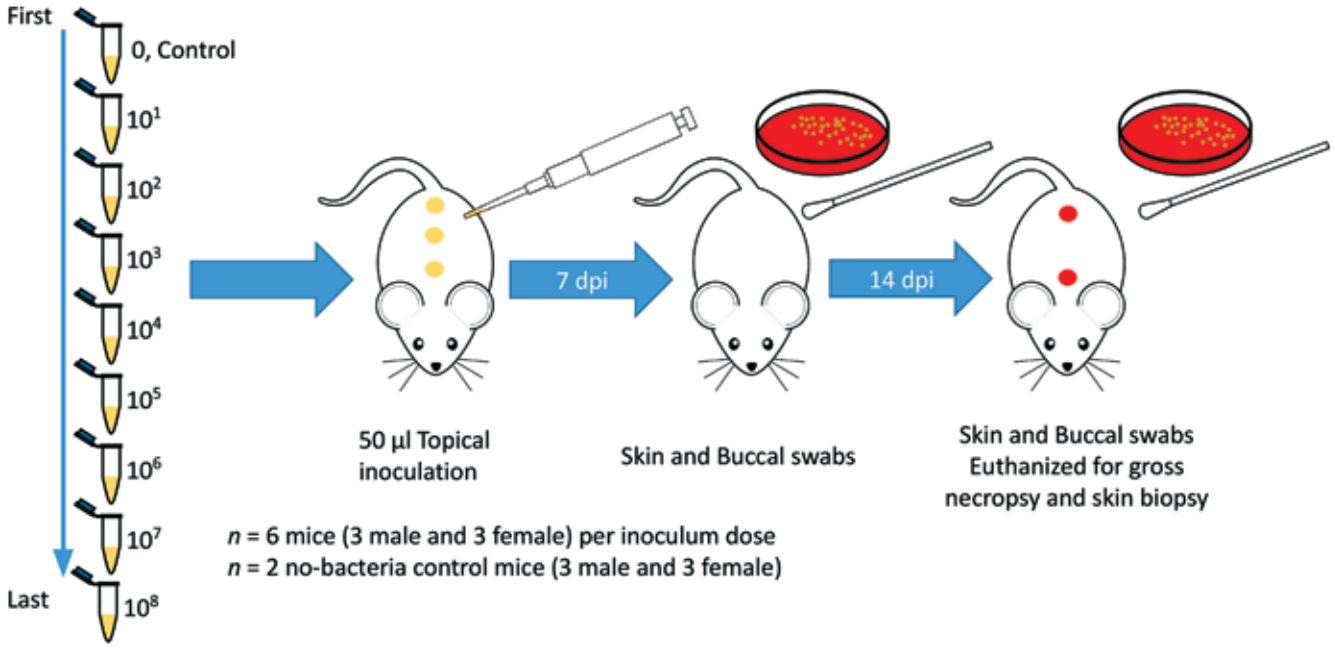

Figure 1. Experimental design: Eight groups of 6 nude mice were inoculated with increasing doses of Cb. The control group consisted of 2 mice that received media without bacteria. Mice were monitored daily for clinical signs. Skin and buccal cultures were conducted weekly until 14 dpi, the study endpoint.

13-1426 (10^8 bacteria per mouse). After inoculation, 2 mice that had received the same inoculum were cohoused with 2 naïve NSG mice; all 4 mice were monitored daily for signs of disease for 60 d. The mice were weighed, and dorsal skin swabs were collected from each mouse every 14 dpi to determine the presence and estimated quantity of Cb. Mice were euthanized at 60 dpi.

Mice. Three strains of mice were used: NU (*n* = 414; Hsd:Athymic Nude-Foxn1^{nu}, Envigo, Indianapolis, IN); NSG (*n* = 100; NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/Sz, The Jackson Laboratory, Bar Harbor, ME); and NSG-S (NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg (*n* = 50, CMV-IL3, CSF2, KITLG)1Eav/MloySzJ; The Jackson Laboratory). F1 generations were bred in house. Mice were free of the following agents based on inhouse surveillance: mouse hepatitis virus, Sendai virus, mouse parvovirus, minute virus of mice, murine norovirus, murine astrovirus 2, pneumonia virus of mice, Theiler meningoencephalitis virus, epizootic diarrhea of infant mice (mouse rotavirus), ectromelia virus, reovirus type 3, lymphocytic choriomeningitis virus, K virus, mouse adenovirus types 1 and 2, polyoma virus, murine cytomegalovirus, mouse thymic virus, Hantaan virus, *Mycoplasma pulmonis*, *Citrobacter rodentium*, *Salmonella* spp., *Filobacterium rodentium*, *Clostridium piliforme*, *Chlamydia muridarum*, *Corynebacterium bovis*, *Staphylococcus aureus*, fur mites (*Myobia musculi*, *Myocoptes musculinus*, and *Radfordia affinis*), pinworms (*Syphacia* spp. and *Aspicularis tetraptera*), and *Encephalitozoon cuniculi* when the studies were initiated, as determined by testing naïve outbred Swiss Webster (Tac:SW) mice exposed repetitively to soiled bedding from cages housing mice in the colony.

Husbandry and housing. Prior to inoculation, mice were housed in autoclaved, polysulfone IVC with stainless-steel wire-bar lids and filter tops (number 19, Thoren Caging Systems, Hazelton, PA) on autoclaved aspen chip bedding (PWI Industries, Quebec, Ontario, Canada) at a density of no greater than 5 mice per cage. After inoculation, mice were singly housed in autoclaved, solid-bottom and top, gasketed and sealed, polysulfone, positive-pressure, IVC (Isocage, Allentown Caging Equipment Company, Allentown, NJ) on autoclaved aspen-chip bedding (PWI Industries). Pre- and post-inoculated mice received autoclaved feed (5KA1, Purina Mills International, St Louis, MO) and autoclaved acidified (HCl) reverse-osmosis–purified water (pH 2.5 to 2.8) ad libitum. Each cage was provided with a sterile bag constructed of Glatfelter paper containing 6 g of crinkled paper strips (EnviroPak, WF Fisher and Son, Branchburg, NJ) for enrichment. Each cage was ventilated with HEPA-filtered air (filtration at cage level) at approximately 30 air changes hourly, and the rack effluent was exhausted directly into the building's exhaust system. Cages containing mice used for ID_{50} determination were not changed after inoculation until the mouse was euthanized at 14 dpi, to reduce the likelihood of cross-contamination. Cages containing inoculated NSG mice used to determine the clinical presentation of Cb were changed every 2 weeks. Cages were changed and all mouse manipulations were performed in a class II, type A2 biologic safety cabinet (LabGard S602-500, Nuaire, Plymouth, MN). The animal holding room was ventilated with 100% fresh air at a minimum of 10 air changes hourly and maintained at 72 ± 2 °F (21.5 ± 1 °C), relative humidity between 30% and 70%, and a 12:12-h light:dark photoperiod (lights on at 0600, off at 1800). All animal use described in this investigation was approved by Memorial Sloan Kettering's IACUC and conducted in accordance with AALAS's position statements on the Humane Care and Use of Laboratory Animals and Alleviating Pain and Distress in Laboratory Animals. The animal care and use program is AAALAC-accredited and operates in accordance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals* (8th edition).[20]

Bacterial culture methods. Cb isolates from skin and buccal swabs or frozen stocks were grown on trypticase soy agar supplemented with 5% sheep blood (BBL TSA II 5% SB, Becton Dickinson, Sparks, MD) at 37 °C with 5% CO_2 for 48 h, as previously described.[5,10] To determine the growth kinetics of each Cb isolate, 3 growth curves were established for every isolate except 7894 because its growth curve had been established

previously.[10] Frozen stocks (Microbank TM, Prolab Diagnostics, Rounds Rock, TX) of each Cb isolate were grown as described in Cheleuitte-Nieves et al.[10] Three colonies of each isolate were transferred to a 15-mL conical tube (Corning Falcon, Fisher Scientific, Hampton, NH) containing 1.5 mL of brain–heart infusion broth (Becton Dickinson) supplemented with 0.1% Tween 80 (VWR Chemicals, Solon, OH), and inoculated cultures were incubated at 37 °C with 5% CO_2, lying flat on a shaker (4625 Titer Shaker, Thermo Scientific, Waltham, MA) at 200 RPM. After 12 h, an aliquot was collected and diluted 1:100 into a 50-mL conical tube (Fisher Scientific) containing 5 mL of fresh brain–heart infusion broth supplemented with 0.1% Tween 80 and incubated as described above. At least 5 aliquots were removed from the broth culture at intervals of 6 or 12 h for up to 96 h. Each aliquot was diluted 1:10 in sterile saline (5 mL Saline Normal, Becton Dickinson). The McFarland density was determined for each diluted aliquot by using a densitometer (Den-1, Grantin Instruments, Shepreth, Cambridgeshire, United Kingdom), which measured the absorbance of light at a wavelength of 565 ± 15 nm and provided a numeric value corresponding to the McFarland interpretation. When required, an additional dilution was made to obtain a reading within the dynamic range of the densitometer. A Gram stain was performed on dilutions to confirm that the culture was free of contaminants. McFarland values were plotted, and the midlog was determined by using Prism (GraphPad Software, San Diego, CA) and a nonlinear sigmoidal 4PL best-fit analysis based on the 50% end point.

To determine the approximate concentration (cfu/mL) of Cb cultures in the exponential phase of growth, Cb isolates were grown in triplicate as described above. Aliquots were collected at time points corresponding to mid-exponential growth, and bacteria were counted using 10-fold serial dilutions and plating.

Mouse inoculation. The Cb inoculum was collected from the culture during the midlog of the exponential growth phase. All isolates were then titrated and serially diluted in 10-fold increments in brain–heart infusion broth to obtain 10 to 10^8 bacteria (±15%) per 50 µL. For isolates 7894, CUAMC1, and F6900, the highest doses of inoculum were 10^4, 10^6, and 10^6 bacteria, respectively. These were the initial 3 isolates evaluated. The dose range used for the earliest ID_{50} determinations was based on a pilot study conducted with isolate 7894; in that study, all inoculated mice were colonized with inocula below 10^3 bacteria. The dose range was expanded for CUAMC1 and F6900, the next isolates evaluated, to 10^6 bacteria and expanded further to 10^8 bacteria for all subsequent isolates evaluated after an inoculum of 10^6 organisms was found to be insufficient, necessitating the administration of a higher inocula (10^8 bacteria per mouse). For isolate 7894 administered to NSG-S and NSG mice, the inocula administered ranged from 1 to 10^7 bacteria. Cb inocula were placed in sterile 15-mL polypropylene centrifuge tubes (Fisher Scientific) and transferred to the vivarium on ice; mice were inoculated within an hour of preparation of the inocula. After inoculation, the concentration of each inoculum was confirmed using plate counts.

The day prior to Cb inoculation, the dorsal thoracolumbar skin of each mouse was swabbed, and mice were confirmed as negative for Cb by culture. Personnel wearing disinfectant-dampened (Clidox [1:4:1], Pharmacal Laboratories, Naugatuck, CT) plastic sleeves and disposable gloves performed all mouse procedures, including inoculation, swabbing, euthanasia, and cage changes. These activities were conducted in a class II, type A2 biologic safety cabinet (LabGard S602-500, Nuaire, Plymouth, MN) using sterile materials and aseptic methods. Inoculation and handling of mice after inoculation were performed in order of increasing inoculum dose. Cages that contained an experimentally naïve mouse were removed from the rack, and all sides of the cage were liberally sprayed with disinfectant and placed in the biologic safety cabinet. Each cage was open for less than 2 min. For bacterial inoculation, the mouse was restrained using the handler's nondominant hand by gently grasping the tail base. The hindquarters were elevated slightly as the mouse grasped the wire bar lid with its forelimbs. The lid was positioned to allow the restrained mouse to remain in the horizontal plane. With the dominant hand, the handler used a sterile filter micropipette (P200N, Marshal Scientific, Hampton, NH) to apply the bacterial inoculum directly to the skin along the dorsal midline, starting at the nuchal crest and proceeding to the tail (a distance of approximately 2 cm). In furred mice, the fur was parted along the midline before applying the inoculum. A 50-µL aliquot of bacterial suspension was evenly distributed along the dorsal midline at 4 or 5 sites, dispensing 10 to 15 µL per site by using a sterile filter 200-µL pipette tip (Universal Pipette Tips, Avantor, Bridgeport, NJ). This method ensured that the bacterial suspension did not flow off the mouse. The cage was then closed and again sprayed thoroughly with disinfectant before it was returned to the rack. Between cages, all interior surfaces of the biologic safety cabinet were sprayed with disinfectant, and the handler donned a new pair of disinfectant-wetted gloves and sleeves. A new cage was placed in the biologic safety cabinet as described above, and the same steps were repeated until all inoculations (0 to 10^8 bacteria per mouse) were completed. Media control mice were inoculated with an equivalent amount of bacteria-free brain–heart infusion broth.

Clinical scoring system. NU, NSG, and NSG-S mice used for ID_{50} determination and NSG mice used to assess clinical presentation were monitored daily for 14 or 60 d, respectively. Mice were evaluated without opening the cage and were scored using the system shown in Tables 2 and 3.

Bacterial sampling after mouse inoculation. Infection was assessed by weekly aerobic culture of the skin and buccal region for 2 wk after inoculation and the skin only once every 14 dpi in the 60-d NSG clinical presentation study. These sites were selected based on a previous study.[5] All mice were sampled in order from lowest to highest inoculum. To prevent cross-contamination within each dose group, animals with lower clinical scores were swabbed first. When animals had similar scores, cages were selected at random. All cages were handled aseptically as described in the animal inoculation section. Each cage was open

Table 2. Clinical scoring system for nude mice

Grade	Clinical signs
0	No clinical signs observed.
1	Few flakes or scales adhered to the skin or mildly hunched posture with no erythema.
2	Few flakes or scales adhered to skin or mildly hunched posture and mild erythema associated with flaking or scaly lesions.
3	Moderate erythema or moderate number of white flakes adherent to the skin or few white flakes at 3 or more additional locations affected, including the ventrum, muzzle, cheeks, crown, and limbs.
4	Moderate erythema or moderate number of white flakes adherent to the skin and moderately hunched posture.
5	Numerous flakes or scales adhered to skin (thick layer) or severe erythema or severely hunched posture.

Mice were scored 0 to 5 depending on clinical signs representing mild, moderate, and severe disease

Table 3. Clinical scoring system for furred mice (e.g., NSG, NSG-S)

Grade	Clinical signs
0	No clinical signs observed.
1	Vessel dilation at ear base or periorbital swelling or rough or unkempt hair coat.
2	Auricular hyperemia or periorbital swelling or rough or unkempt hair coat.
3	Periorbital swelling or auricular hyperemia or facial alopecia.
4	Severe periocular erythema or alopecia or copious auricular white flaking and truncal alopecia.
5	Severe periocular erythema or alopecia or copious auricular white flaking with thickened skin.

Mice were scored 0 to 5 depending on clinical signs representing mild, moderate, and severe disease

for no longer than 5 min. The animal was grasped gently by the base of the tail, allowing all 4 limbs to grasp the wire bar lid. A sterile culture swab (Remel Bactiswab, Fisher Scientific) was held perpendicular to the animal and swabbed along the dorsal midline from the base of the tail cranially to the nuchal crest and caudally back to the tail base, with rotating of the swab as it was advanced. For the caudal buccal swab, a sterile cotton-tipped applicator (24-106-2S, McKesson, Irving, TX) was used as previously described.[5] Briefly, the handler used a 3-finger technique to restrain the mouse with the nondominant hand. The mouse was then weighed and returned to its cage; the scale was wiped with chlorine dioxide disinfectant and allowed to dry for 5 min prior to the next use. The cage was closed, sprayed, and wiped down with disinfectant and returned to the rack. The biologic safety cabinet was cleaned as described above, the handler donned a new pair of disinfected gloves, and the next cage was processed after waiting 5 min.

Confirmation of infection. Skin swabs from days −1, 7, 14, and, for the NSG disease presentation study, every 14 d thereafter and buccal swabs from days 7 and 14 were each streaked in a 4-quadrant pattern onto agar plates (TSA II 5% SB, Becton Dickinson).[3] The plates were incubated for 72 h at 37 °C with 5% CO_2. When present, distinct colonies were speciated by using MALDI-TOF spectroscopy (MALDI Biotyper Sirius CA System, Bruker, Billerica, MA). Plates without growth were held for an additional 7 d before being considered negative. Plates were scored from 0 to 4 according to the number of quadrants with bacterial growth.

Postmortem gross examination and skin histopathology. On day 14 of the ID_{50} study, mice were euthanized by CO_2 overdose. The carcass and spleen were weighed, and each mouse underwent a complete postmortem gross examination, including macroscopic inspection of the skin and all internal organs. Full-thickness skin samples (diameter, 6 mm) were collected by using a punch biopsy (Integra, Mansfield, MA) at the nuchal crest and approximately 2 cm caudal from the crest in the dorsal lumbar region. Additional samples were taken from lesions when present. Specimens were fixed in 10 % neutral buffered formalin, routinely processed in alcohol and xylene, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Histologic examination was performed on all skin biopsies to confirm the presence or absence of Cb-related lesions and to determine the magnitude of the histologic changes. Slides were evaluated and lesions recorded by a board-certified veterinary pathologist (ICM) who was blind to groups and doses. Samples were semiquantitatively scored as normal (0), minimal (1), mild (2), moderate (3), or severe (4), depending on the degree of acanthosis, orthokeratotic hyperkeratosis (orthokeratosis), bacteria, and inflammation. Specifically, acanthosis refers to an elevated number of keratinocytes and indicates an increase in epidermal thickness resulting from hyperplasia and often hypertrophy of cells of the stratum spinosum. Minimal acanthosis was defined as an increased thickness of up to a 3-cell stratum spinosum layer; mild as 4 to 7 cell layers; moderate as 8 to 10 cell layers; and, severe as greater than 10 cell layers. Orthokeratosis refers to increased thickness of the stratum corneum without retention of keratinocyte nuclei and was mostly identified in a compact to laminated pattern. Minimal orthokeratosis was defined as an increased thickness of up to 50 µm; mild as 100 to 200 µm; moderate as to 200 to 300 µm; and, severe as greater than 300 µm. Bacterial colonies were assessed based on the presence of distinguishable clusters of short bacilli in the stratum corneum or the lumen of hair follicles. Minimal presence of bacteria was defined as small amounts of scattered bacterial rods with no formation of characteristic clusters; mild as 1 to 2 clusters; moderate as 3 to 5 clusters; and, severe as greater than 6 clusters. Inflammation was mostly comprised of a mixture of mononuclear cells and neutrophils, associated both with and without hair follicle rupture. Minimal inflammation was defined as single superficial pustules or small numbers of inflammatory cells scattered in the dermis; mild as slightly larger or increased numbers of superficial pustules or slightly greater numbers of inflammatory cells scattered in the dermis; moderate as larger or increased numbers of pustules or increased numbers of inflammatory cells in the dermis, frequently forming clusters around capillaries or adnexal units; and, severe as large and multiple pustules with high numbers of inflammatory cells forming coalescing clusters or sheets in the dermis, often infiltrating the epidermis or adnexal epithelia. For all isolates, the average of each of the 4 microscopic characteristics was calculated for each inoculum based on 2 biopsies per mouse. The total histopathology score was calculated by adding the averages for all 4 histologic characteristics. On day 60 of the NSG clinical disease study, mice were euthanized with CO_2, and an external examination was conducted.

ID_{50} determination according to the Reed–Muench, Dragstedt–Behrens, and Spearman–Karber methods, and probit regression. The frequency of positive mice based on culture results per dose was determined and used to calculate the ID_{50}. For each isolate and strain of mouse used, the ID_{50} was calculated by using the skrmbd package obtained from the USDA in R 4.2.1 statistical computing for Windows (https://www.r-project.org/about.html).[28,38] The Reed–Muench method was used to calculate the ID_{50} using the 2-step equation below. The method uses the cumulative sums of infected animals per dose. First, the proportional distance (PD) between the percentage of positive animals above and below the 50% end point is calculated. The ID_{50} is then calculated by taking the inverse log of the proportional distance and the log of the dose in which fewer than 50% of the mice became infected.[28,38]

$$\mathrm{PD} = \frac{(50\% - \text{Percentage infected dilution next below 50\%})}{(\text{Percentage infected dilution next above 50\%} - \text{Percentage infected dilution next below 50\%})}$$

$$ID_{50} = \text{inverse log}\,([\text{Log10}\,(\text{dose at which less than 50\% of the mice become infected}) + \mathrm{PD}])$$

The Dragstedt–Behrens method relies on the frequency of positive animals at each dose, and the ID_{50} is estimated by

connecting the hypothetical fractions between the doses and determining the intermediate value.[38] The Spearman–Karber method estimates the 50% end point by the probability mass function of the dose distribution between 0% and 100% response. This method requires at least a single experimental dose to have a 100% positive response to pathogen exposure.[38] For isolates that did not reach 100% positive responses, the skrmbd package assumes the next lower or higher dose will produce a zero or complete response, respectively. Probit regression analysis was performed by using statistical software (MedCalc Software Ltd, Ostend, Belgium).[19] The probit regression procedures fit a sigmoidal dose–response curve and calculate values with 95% confidence interval of the dose variable corresponding to a series of possibilities.

Statistical analysis. The buccal and dorsal culture scores were compared on days 7 and 14 after inoculation by using paired *t* tests. For each isolate, differences in the percentage change in body weight and the spleen:body weight ratio and all histologic characteristics (including hyperkeratosis, acanthosis, bacterial groups, inflammation, and the total histopathology score) were compared by dose relative to the respective control by using one-way ANOVA. Posthoc testing was performed using Dunnett's multiple comparison test. A *P* value less than or equal to 0.05 was considered statistically significant. All calculations were performed by using Prism 9 for Windows (version 9.3.0, GraphPad Software).

Results

Growth kinetics of *Corynebacterium bovis* isolates. The Cb isolates displayed different growth rates and characteristics (Figure 2). Isolates 7894, 17-0240, Iso 24956, 16-2004, and WCM 35 aggregated, forming clumps when grown in broth, whereas the other isolates showed uniform turbidity. Clumped bacteria were dispersed by gentle manipulation with a pipette before taking densitometer readings.

The Cb isolates used in this study were slow growing compared with other bacterial species, such as *Staphylococcus* spp.[18] All isolates remained in the lag phase for the first 0 to 12 h, which was followed by an exponential phase that lasted between 12 and 60 h. The midlog phase ranged from 13 and 35 h (Table 4). The mouse-derived isolates reached midlog at 13 to 29 h, whereas the bovine and rat isolates reached midlog at 16 and 20 h, respectively. The human isolates had the slowest growth rates, reaching midlog at 30 or 35 h. The stationary phase varied between 24 and 60 h.

ID_{50} and dose–response model. The frequency of mice testing positive by buccal, dorsal, or both culture sites was used to calculate ID_{50} and dose–response curves by using probit regression analysis. Probit regression models were used to generate dose–response curves with 95% CI (Figure 3). Because isolates 7894, CUAMC1, and 16-2004 in NU mice had doses in which 50% of the mice were colonized, 95% CI could not be generated. Dose–response curves could not be generated for the human isolates F6900 and WCM 35, or for NSG mice inoculated with 13-1426, because they remained uninfected after inoculation with as many as 10^8 bacteria or because they did not have sufficient positive mice to perform the calculations.

In NU mice, the ID_{50} for Cb varied by isolate and source species (Table 5). Mouse-derived isolates had lower ID_{50} (58 to 1000 bacteria) compared to isolates obtained from other species. Isolate 17-0240 was the only isolate whose probit curve indicated a higher probability (20%) of infection at lower doses in NU mice. Furthermore, 17-0240's ID_{50} was 100 bacteria according to the Reed–Muench and Dragstedt–Behrens methods, but was almost half of that based on probit analysis. The rat (16-2004) and bovine (4826) isolates had ID_{50} between 6000 and 10,000 bacteria, 60 to 100 times greater than that of mouse isolates 7894 and 17-0240, which had the lowest ID_{50}. The human isolates (F6900 and WCM 35) did not colonize any mice at either 7 or 14 dpi at doses as high as 10^8 bacteria. Therefore, an ID_{50} could not be determined for those isolates.

Inoculation of NSG and NSG-S mice with mouse isolate 7894 generated an ID_{50} of 91,056 to 133,325 and 284,000 to 363,998 bacteria, respectively, depending on the method used to calculate the ID_{50} (Table 5). These doses were approximately 13,000- and 23,000-fold higher than the ID_{50} dose for NU mice. The ID_{50} for NSG mouse isolate 13-1426 could not be determined in the NSG strain because only 2 of the 6 mice inoculated with 10^8 bacteria were positive on culture. Therefore, the ID_{50} in NSG mice was probably greater than 10^8, whereas the ID_{50} for this isolate was 433 to 464 bacteria in NU mice. Probit analysis revealed a rightward shift of the probability curve in furred mice as compared with NU mice, with the slope increasing starting at an inoculum of 10,000 bacteria.

Disease course in immunocompromised mice. Disease progression and severity differed by Cb isolate in NU mice (Figure 4). When the isolate caused disease, larger inocula generally yielded an earlier disease onset, higher disease scores, and earlier clinical resolution. Depending on the inoculum dose, mice receiving a Cb isolate that caused moderate to severe disease (e.g., 7894, 13-1426, 16-2004, 17-0240) generally reached peak disease and showed or began to show clinical resolution by study termination at 14 dpi (Figure 4). The isolates were ranked from most to least severe when compared by a dose that all isolates shared (10,000 bacteria): 7894, 17-0240, 13-1426, 16-2004, CUAMC1, and 4826 (Figure 5 A). Mouse isolate CUAMC1 and bovine isolate 4826 produced minimal disease, with only a single mouse per isolate developing a disease score of 2 over the 14 dpi observation period, and then only in mice that had received the highest inocula of 10^6 and 10^8 bacteria, respectively (Figure 4). When the disease scores were plotted by the ID_{50} inocula, or the dose just above the calculated ID_{50} when the ID_{50} did not correspond to the inoculum administered, as well as the lowest dose resulting in clinical signs over the 14-day observation period, disease progression was similar among isolates (Figure 5B and C). Despite clinical resolution, some mice receiving higher inocula remained highly culture positive (culture grade 3 or 4). NU mice that were positive for Iso 24956 by buccal and dorsal cultures did not develop disease. The human-derived isolates F6900 and WCM 35 failed to colonize mice, therefore none of the mice inoculated with these isolates displayed clinical signs. Disease severity was not associated with lower midlog growth rates; however, mice inoculated with isolates with lower ID_{50} (7894, 17-0240, 13-1426) tended to develop more severe clinical disease, with the exception of Iso 24956.

Buccal and dorsal culture sites did not differ significantly with regard to the identification of Cb-colonized mice (data not shown). Culture-positive NSG and NSG-S mice inoculated with either mouse isolate (7894 or 13-1426) did not develop clinical signs over the 14-d observation period. Culture-positive furred immunodeficient mice consistently had fewer bacteria recovered (culture grade 1 to 3) than did culture-positive NU mice (culture grade 3 or 4) at 14 dpi. Based on observation, more bacteria were isolated from the NSG-S strain at 14 dpi (culture grade 1 to 3, with 1 mouse scoring a 4 on buccal culture) as compared with 7 dpi (culture grade 1 or 2). However, this pattern was not consistently observed in NSG mice, which consistently had low

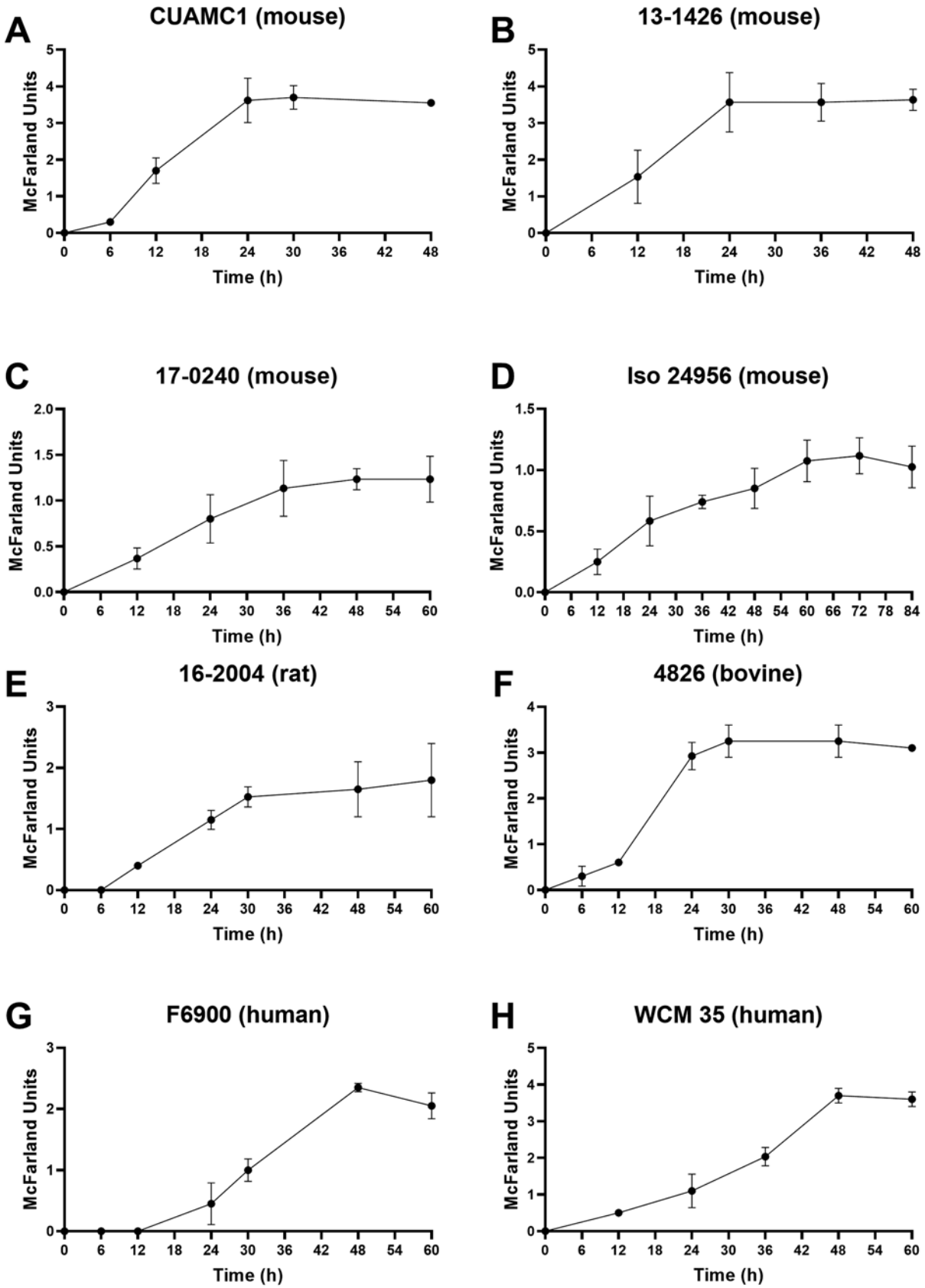

Figure 2. Growth kinetics of *Corynebacterium bovis* isolated from various species. Densometer readings correspond to the average McFarland standard values of 3 independent experiments, and errors bars represent the SEM (n = 3). The growth kinetics of isolate 7894 were reported previously[1] and therefore do not appear in the figure.

Table 4. Time to reach midlog growth for 9 *Corynebacterium bovis* isolates from various species[10]

Isolate	Source	Midlog (h)
7894	Nude mouse	18
CUAMC1	Nude mouse	13
13-1426	NSG mouse	13
17-0240	Nude mouse	20
Iso 24956	Nude mouse	29
16-2004	Nude rat	20
4826	Bovine	16
F6900	Human	30
WCM 35	Human	35

scores (grade 1 or 2, with only 2 mice scoring 2) and with only a few colonies present at both 7 and 14 dpi.

In the 60-d study, NSG mice inoculated with 10^8 bacteria of isolate 7894 showed clinical disease at 18 or 22 dpi, depending on the cage (Figure 6). All inoculated and contact sentinel mice in both cages developed clinical disease by day 26 and reached the clinical end point (disease score of 5) by day 55. NSG mice inoculated with 10^8 bacteria of isolate 13-1426 remained culture negative and did not develop clinical disease over the 60-d observation period, even though this isolate had originally been cultured from clinically affected NSG mice.

Postmortem gross examination and histopathology. When present, gross lesions at euthanasia were limited to the skin. Macroscopic skin lesions in NU mice at 14 dpi matched the clinical findings and included varying degrees of white flaking and erythema of the skin over the entire carcass, but especially affecting the ventrum, muzzle, cheeks, and limbs (Figure 7). No gross lesions were seen in NSG and NSG-S mice inoculated with mouse-derived isolates 7894 and 13-1426 over the 14-d observation period. However, histologic lesions were seen at day 14 in some mice infected with 7894 (Figure 8). In the 60-d study, the NSG mice inoculated with isolate 7894 displayed various degrees of periorbital swelling, erythema, or alopecia, especially affecting the face, ears, and trunk, at necropsy. Gross lesions were not seen in NSG mice inoculated with isolate 13-1426.

There were no significant differences in either the percentage change of body weight or the spleen:body weight ratio for any isolate administered at any dose to NU, NSG, and NSG-S mice when compared with the corresponding controls that were inoculated with bacteria-free media with the exception of NU mice inoculated with ≥ 10^6 bacteria of isolate 13-1426. NU mice inoculated with 10^6, 10^7, and 10^8 bacteria of isolate 13-1426 bacteria showed significant ($P = 0.02$) decreases in body weight of 2.1%, 3.8%, and 0.6%, respectively, compared with the media inoculated control group.

On histology, bacteria were observed in 39 of 528 mice inoculated with any Cb isolate at any dose. If bacteria were noted on histology, they were found on mice inoculated with Cb isolates Iso 7894, 13-1426, and 17-0240 at the peak of that animal's disease curve (Supplemental Table S1 and Figure 4). Bacterial colonies were observed in NSG (10^4 and 10^7) and NSG-S (>10^6) mice infected with isolate 7894 (Supplemental Table S2). No bacteria were seen microscopically in NU mice infected with mouse isolate CUAMC1 or human isolate F6900 (Supplemental Tables S1 and S3). Small bacterial colonies were observed in a single mouse that had received 10^2 bacteria of the human isolate WCM35. These colonies had similar morphology and distribution as other Cb isolates, but WCM35 colonization was not confirmed because these mice were never culture positive.

Dermatitis occurred in NU mice inoculated with select doses of mouse isolates 7894, 13-1426, 17-0240, and CUAMC1 and in media inoculated controls (Supplemental Table S1). When present, dermatitis was most often characterized by mixed mononuclear and neutrophilic infiltrates that were primarily associated with hair follicle rupture and not necessarily with

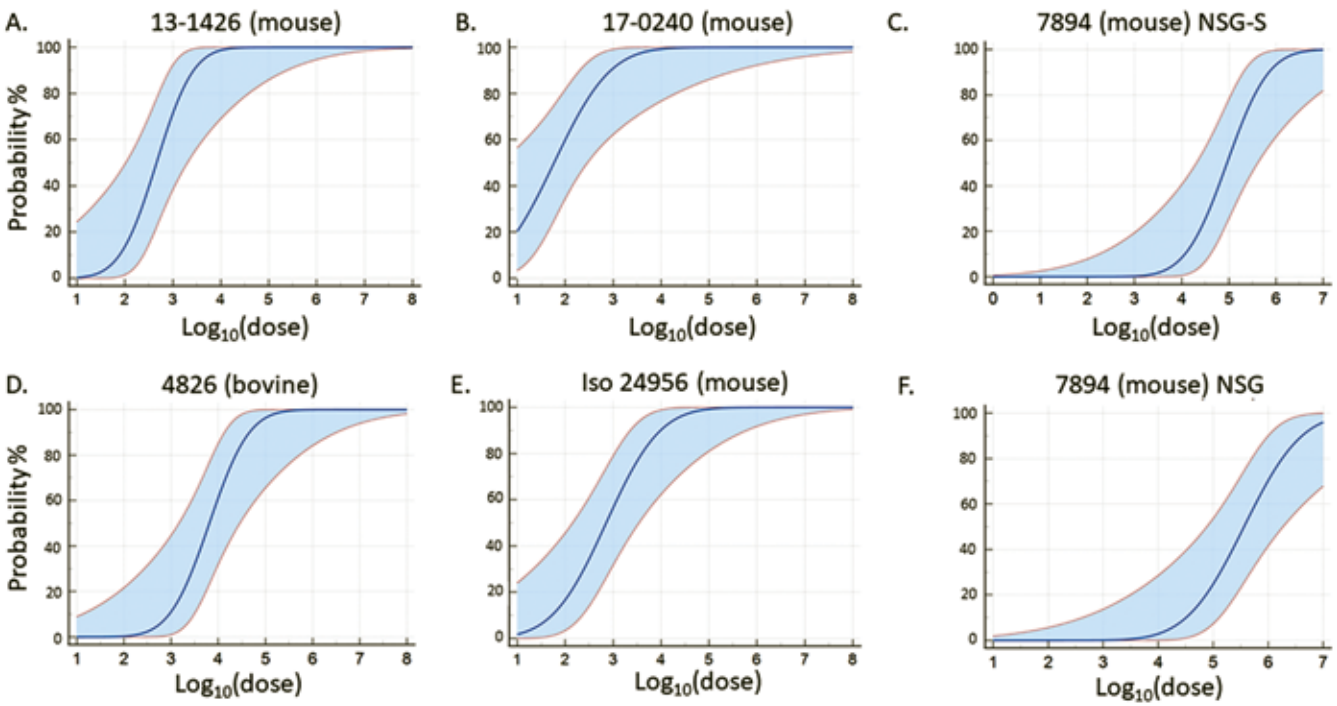

Figure 3. Dose–response curves of *Corynebacterium bovis* isolates generated by probit regression analysis. Curves showing the probability of infection and maximum likelihood estimation, ranging from 0 to 100, for different Cb isolates and strain of mouse inoculated indicated in parentheses. Dashed lines and blue shading depict the 95% confidence interval for the dose corresponding to a particular probability.

Table 5. ID_{50} of *Corynebacterium bovis* isolates from different species in various immunodeficient mouse strains

Isolate	7894	CUAMC1	13-1426	17-0240	Iso 24956	16-2004	4826	7894	7894
Source	Mouse	Mouse	Mouse	Mouse	Mouse	Rat	Bovine	Mouse	Mouse
Recipient / Method	NU	NU	NU	NU	NU	NU	NU	NSGS	NSG
Reed–Muench	100	1000	464	100	599	10,000	7498	125,892	284,803
Spearman–Karber	100	1000	464	68	681	10,000	6813	100,000	316,227
Dragstedt–Behrens	100	1000	433	100	571	10,000	6995	133,352	307,471
Probit	100	1000	456	58	712	10,000	6460	91,056	363,998

The ID_{50} for isolates F6900 (human) and WCM35 (human) in nude mice and 13-1426 (mouse) in NSG mice could not be calculated and therefore are not shown.

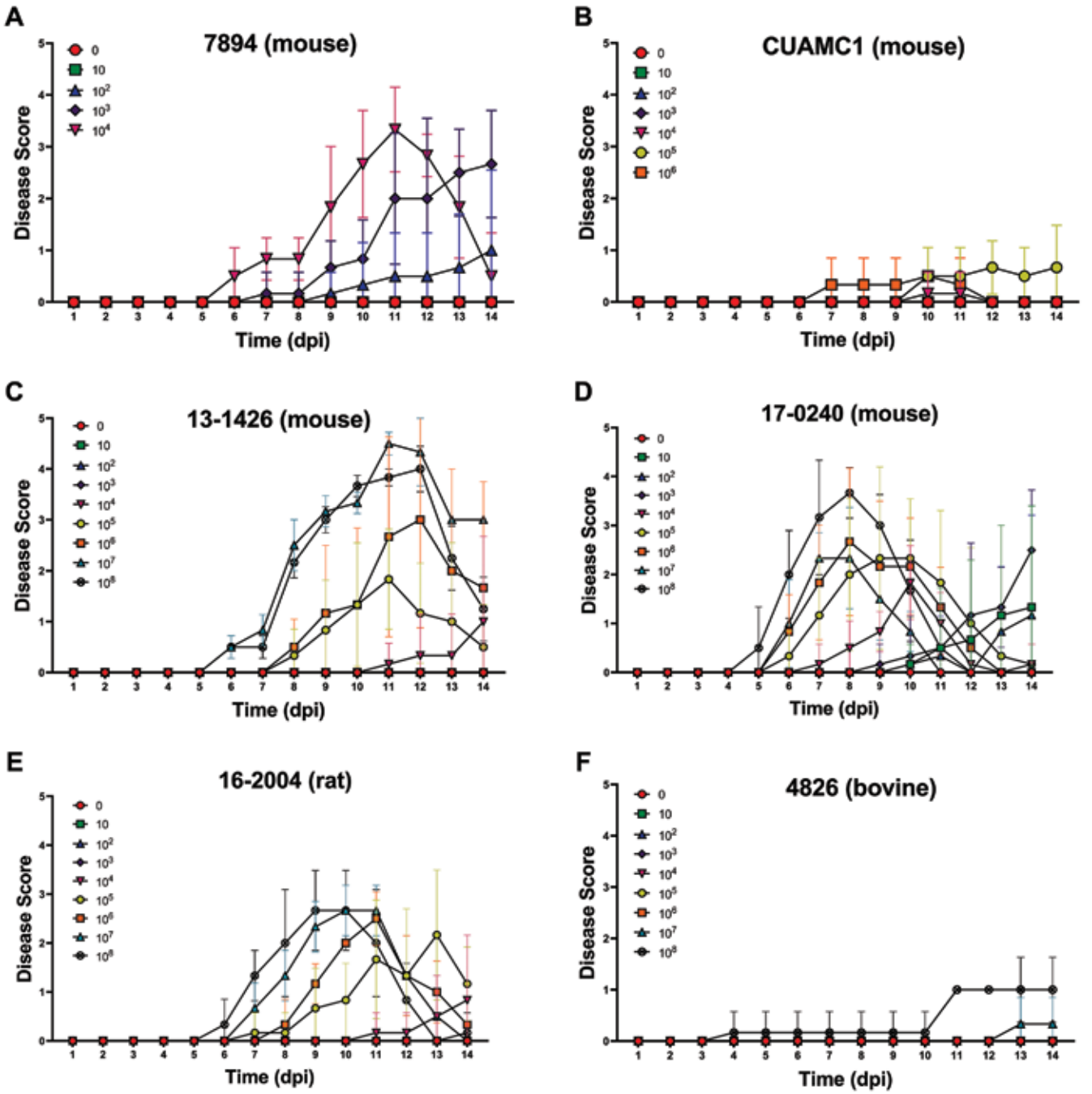

Figure 4. Disease scores by dose for nude mice inoculated with various *Corynebacterium bovis* isolates. Mice were inoculated with 0 to 10^8 bacteria, except for isolates 7894, F6900, and CUAMC1 which were administered maximum inocula of 10^4, 10^6, and 10^6, respectively. The mice were monitored and scored daily for 14 d. The scores shown are the mean ± 1 SD of 6 mice receiving the same inoculum.

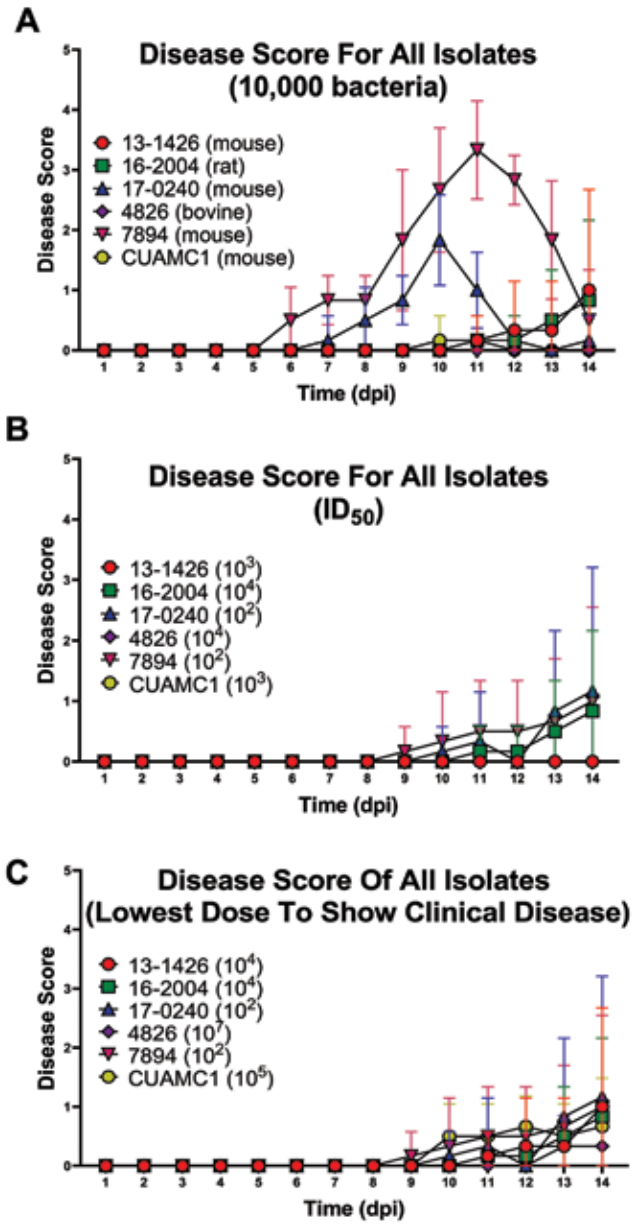

Figure 5. Daily disease scores for 14 dpi for all *Corynebacterium bovis* isolates in athymic nude mice (A) infected with 10,000 bacteria, (B) based on ID_{50}, and (C) based on the lowest dose resulting in clinical disease. Mice were inoculated with 0 to 10^8 bacteria except for isolates 7894, F6900, and CUAMC1, which were administered as maximum inocula of 10^4, 10^6, and 10^6, respectively. The scores shown are the mean ± SD of 6 mice receiving the same inoculum.

Cb infection; similar findings were observed in the uninfected controls. The remaining isolates rarely caused inflammatory lesions. Inflammation was not observed in any of the NSG or NSG-S mice examined. (Supplemental Table S2).

NU mice inoculated with mouse Cb isolates had the highest total histology scores, primarily due to higher scores for acanthosis and hyperkeratosis (Supplemental Table S1, Figure 9). For all isolates that caused disease in NU mice, the highest total histology scores were associated with higher clinical disease scores at the time of euthanasia and not necessarily with higher doses of inoculum (Supplemental Tables S1 and S3, and Figure 4). The highest total histology scores occurred in mice inoculated with isolates 7894 (7.6), 13-1426 (8.7), and 17-0240 (6.0; Supplemental Table S1). For Cb isolates 7894 and 13-1426, the highest scores, 7.6 and 8.7, were observed in mice receiving 10^3 and 10^7 bacteria, respectively (Supplemental Table S1). The lesions observed in all NSG and NSG-S mice inoculated with Cb were significantly lower than the NU mice infected with the same isolate ($P < 0.0001$). The NSG and NSG-S mice infected with isolate 7894 (mouse-derived) had histologic scores similar to the media inoculated controls (Supplemental Table S2). NSG mice infected with 13-1426 did not have any histologic lesions and therefore had a mean score of 0. Regardless of dose, Iso 24956 had a lower total histopathologic score compared to other mouse isolates that were able to colonize NU mice (Supplemental Table S1). However, despite not displaying overt clinical disease, NU mice inoculated with Iso 24956 had significantly higher mean total scores at doses greater than 10^4 as compared with the media inoculated control group ($P < 0.05$). Similarly, mouse isolates 7894, 13-1426, and CUAMC1 had significantly higher mean total scores than did media inoculated cage mates at doses greater than or equal to 10^2, 10^6, and 10^3, respectively ($P < 0.05$).

Isolates cultured from humans and cows (WCM35 [human], F6900 [human], and 4826 [bovine]) produced minimal histologic changes associated with Cb colonization despite inoculums as large as 10^8 bacteria (Supplemental Table S3). When comparing only Cb isolates obtained from other species, NU mice inoculated with the rat isolate 16-2004 at doses of 10^2 or greater had the most significantly different hyperkeratosis and total histopathologic scores as compared with media inoculated controls (Supplemental Table S3).

Discussion

Cb has been and continues to be an extraordinary challenge to institutions using immunocompromised mice as models in various scientific disciplines. Once introduced into a naïve colony, Cb is extremely difficult to eradicate.[24] Even though Cb was first described more than 40 y ago, relatively little is known about the bacterium. The current study provides new and important insights into Cb's biology. We recently reported genetic differences among Cb isolates from various species, but did not know whether these differences resulted in differences in an isolate's virulence (its ability to colonize a host, infect and transmit between species, and cause clinical disease).[9] In the current study, we demonstrated that these characteristics vary considerably among isolates that were cultured from both the same and different species.

In public health, quantitative microbial risk assessment is used to determine the overall risk a potential pathogen poses to the public, particularly for water- or food-borne illnesses.[19,38] The assessment involves hazard identification, exposure route characterization, dose response analysis, risk evaluation, and management.[19] A few of these characteristics have already been determined for Cb and have been used to develop strategies to prevent its initial introduction into mouse colonies. However, an important consideration in understanding the infectivity of a microorganism is to determine the number of microbial units necessary to effectively colonize and infect its host—that is, the infectious dose or the minimum number of organisms required to produce infection in a host and allow reisolation after the host has had time to eliminate the microbe.[2,7,8] The infective dose capable of producing infection in 50% of a population (ID_{50}) is often determined to help define the risk to a specific population.[7,29] The ID_{50} of Cb was unknown previously; therefore, the principal aims of this study were to calculate the ID_{50}, develop a dose–response curve for Cb in immunodeficient mice, and determine the interspecies transmissibility of Cb isolates isolated from various host species. In addition, we aimed to

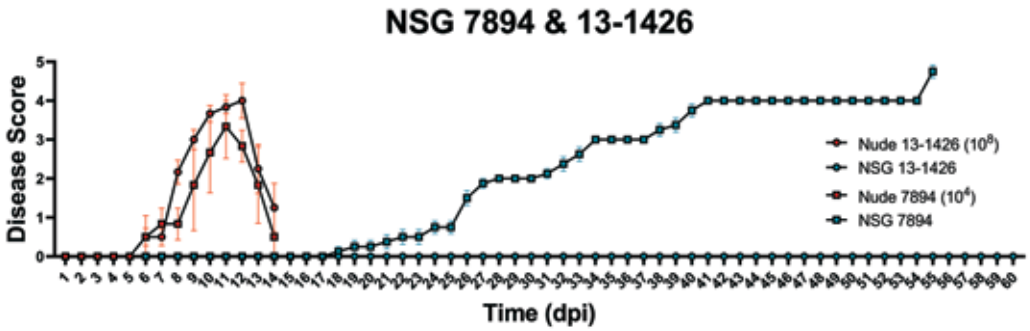

Figure 6. Daily disease scores for 60 dpi for NSG mice infected with *Corynebacterium bovis* 7894 and 13-1426 with 10^8 bacteria. The scores shown are the mean ± SD of 6 mice receiving the same inoculum.

determine whether susceptibility to Cb differed between immunodeficient mouse strains. We used 3 methods—Reed–Muench, Dragstedt–Behrens, and Spearman–Karber—to estimate the ID_{50} from dose–response data.[38] We also used a probit logistic regression model to define the probability of infection associated with any dose, not just the ID_{50}.[19]

The murine Cb isolates that we evaluated differed markedly in the number of bacteria needed to colonize a murine host. Among the 5 mouse isolates we evaluated, as much as a 10-fold difference in the median dose was necessary to colonize NU mice. Isolate 7894 not only required one of the lowest doses for colonization, but was also the most virulent in that it caused the most severe clinical disease with the earliest onset as compared with other isolates at the same dose. In contrast, CUAMC1 required 10 times more bacteria to colonize NU mice and caused minimal clinical disease. Neither of the 2 human isolates we evaluated resulted in colonization, even though the mice received as many as 10^8 bacteria. Although additional human isolates should be evaluated, our findings suggest that human Cb isolates pose little to no biosecurity risk to mice.

Our data showed that mouse isolate 7894 was one of the most virulent and most infectious isolates based on its clinical scores and ID_{50} in NU mice; interestingly, the ID_{50} was several magnitudes higher in the furred highly immunocompromised NSG and NSG-S strains. Despite the NSG and NSG-S strains being considerably more immunodeficient than NU mice, 1000 to 3000 times more Cb was needed to achieve colonization in those 2 strains, and neither developed clinical signs during the 14-d observation period. As a result of these findings and our suspicions based on clinical experience that the onset of clinical disease is considerably later in NSG mice, NSG mice were inoculated with either isolate 7894 or 13-2426 and observed for 60 days. NSG mice administered isolate 7894 began to show clinical signs as late as 21 dpi, which is considerably later than when given to NU mice (6-10 dpi). However in contrast to NU mice, clinical disease in NSG mice progressed until they reached the humane endpoint at 55 dpi. A recent study described requiring 10-fold higher doses of CUAMC1 to infect NSG (2×10^7 cfu) as compared with NU mice (2×10^6 cfu), and none of the mice developed clinical disease during a 70-d observation period.[25] Despite these contrasts in median infectious dose among these commonly used immunocompromised strains, infection and the resulting clinical disease remain high in enzootically infected vivaria using these and other highly immunocompromised strains.[6,26,30] One consideration is that natural infection may occur because of multiple exposures to Cb and could be facilitated by altered skin biology and flora resulting from hair removal by depilatory agents or shaving during tumor implantation or surgery.

The ID_{50} of isolate 13-1426, which was originally cultured from an NSG mouse with Cb-associated clinical disease, was greater than 10^8 in NSG mice. In contrast, fewer than 500 bacteria were needed to colonize 50% of the NU mice. None of the 8 NSG mice inoculated with this isolate were colonized within 60 dpi. However, 2 of 6 NSG mice did become colonized when 10^8 bacteria were administered during our determination of the ID_{50}. It would be valuable to understand how long NSG mice require after colonization with this isolate to develop clinical signs. Alternatively, this finding may have resulted from loss of virulence factors during *in vitro* culture.[33]

An understanding of each isolates' growth kinetics was necessary to determine the ID_{50}, as bacteria needed to be inoculated when their growth was at midlog, a time when they are actively dividing. All Cb isolates grew slowly, which was consistent with our previous findings for this species and is the reason that cultures should be held for 5 to 7 d before concluding that Cb is not present in samples.[10] The majority of isolates tended to cluster together with regard to midlog growth times, which were similar in most mouse and human isolates (13 to 20 and 30 to 35 h, respectively). Although the growth rate was slow overall, isolates grew at different rates, with only the mouse isolates CUAMC1, 13-1426, and 17-0240 and the rat isolate 16-2004 having similar midlog growth times of 13 and 20 h, respectively. However, a shorter time interval to midlog growth was not associated with a lower ID_{50}, a shorter onset of clinical disease, or greater disease severity. Mouse isolates CUAMC1 and 13-1426 had similar times to attain midlog growth, but 13-1426 displayed severe disease as compared with CUAMC1, which produced minimal disease. Similarly, a longer interval to reach midlog growth did not correlate with a lack of colonization, given that mouse isolate Iso 24956, 1 of the 3 slowest growing Cb isolates, colonized NU mice, but did not develop clinical disease during the postinoculation observation period. However, both human isolates (F6900 and WCM 35), which had the slowest growth rates, failed to colonize NU mice. Whether the growth characteristics of these isolates was related to their lack of colonization remains unknown. Furthermore, Cb growth kinetics likely differ *in vitro* and *in vivo* and probably vary by isolate and mouse strain.

Isolates 7894, 16-2004, Iso 24956, 17-0240, and WCM 35 showed autoaggregation in vitro, a feature demonstrated by other *Corynebacterium* spp. and other bacteria.[34] The process is mediated by self-recognizing surface structures that link proteins and exopolysaccharides.[34] The biologic function of autoaggregation is not fully understood but it is thought to protect bacteria from environmental stress or host responses by promoting biofilm formation, increasing tolerance to antimicrobial agents, impeding phagocytosis by the host immune

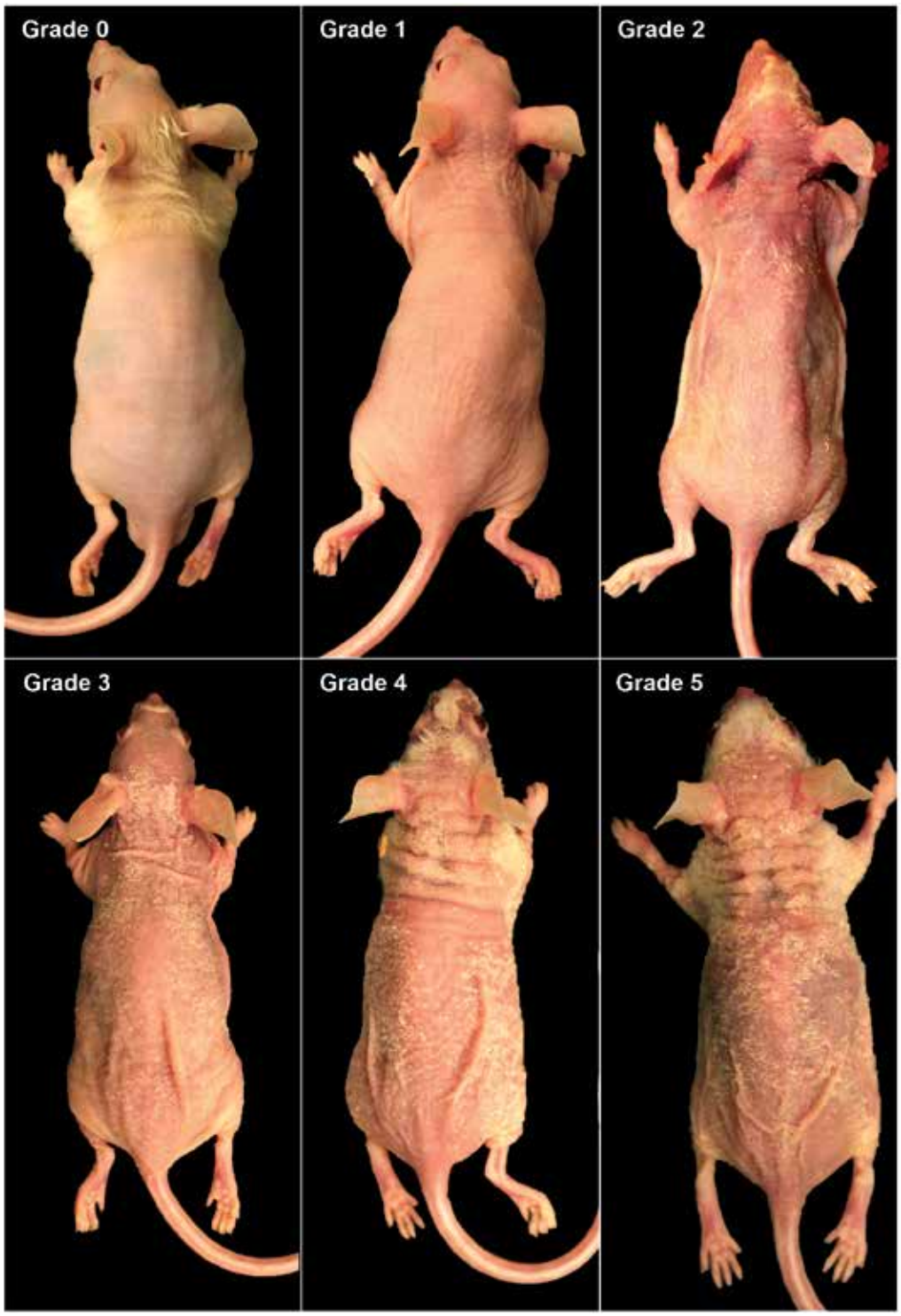

Figure 7. Photographs showing clinical disease by score at gross necropsy of athymic nude mice. Mice were scored 0 to 5 based on clinical signs representing mild, moderate, and severe disease.

system, or elevating the invasion frequency of the host's cells.[1,12,17,37] Although Cb isolates such as 7894 produced severe disease and developed aggregates, others that aggregated in vitro, such as Iso 24956, showed colonization without disease or, as with WCM 35, did not colonize at all. Thus, autoaggregation alone cannot be used to predict Cb's ability to infect or cause disease.

NU mice are overtly susceptible to Cb, which accounts for the high morbidity that has been previously reported.[11] However, the clinical signs presented by the infected NU mice used in this study varied by isolate and dose. For example, mice colonized with isolates 7894 and 17-0240 displayed signs of CAH when given 10^3 bacteria; however, CUAMC1, Iso 24956, and 13-1426 did not. At higher inocula, mice colonized with isolates 7894, 13-1426, and 17-0240 showed marked disease, with some mice reaching a clinical score of 5. In contrast, mice colonized with the other isolates had mild, minimal, or no disease. In this study, mice colonized with CUAMC1 had minimal disease, with only 8

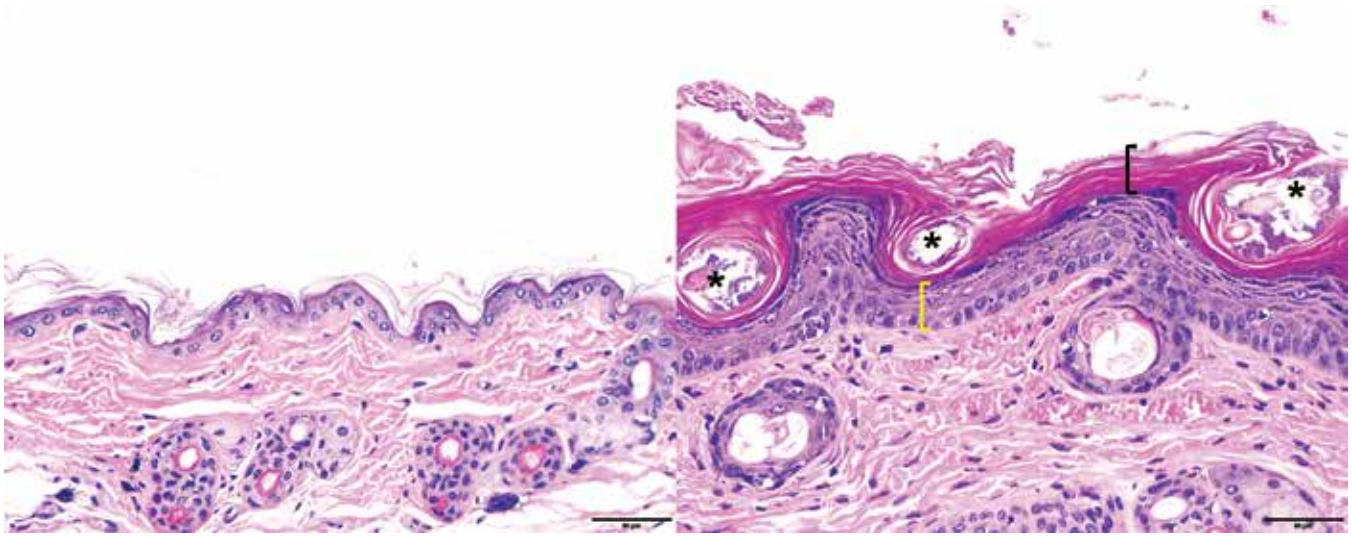

Figure 8. Skin histology at day 14 after inoculation of NSG mice infected with Cb 7894 at 10^7 bacteria. The left panel depicts normal skin histology. The right panel depicts histologic changes associated with Cb infection. *, bacteria; black bracket, hyperkeratosis; yellow bracket, acanthosis.

of 30 mice showing clinical score of 1, and an additional mouse that scored 2 at 14 dpi. In contrast, in a recently published article in which NU mice were inoculated with this isolate, the authors reported no obvious clinical signs; however, hyperkeratosis was identified microscopically over the 70-d observation period.[25] We hypothesize that differences in inoculation methods, housing conditions, the phase of growth at which the bacteria were administered (24 h), the skin flora, and the media used (PBS) likely accounted for this difference.[25]

When NU mice developed clinical disease in the current study, CAH was observed between 6 to 14 dpi, depending on the isolate and dose, with some mice showing clinical resolution within the 14-dpi period. These results were similar to those in previous reports.[11,13,30] Dole et al. showed that NU mice that were inoculated with a virulent Cb strain that was isolated from a NU mouse with severe CAH showed no clinical differences from mice inoculated with a less-virulent NU isolate, nor with the bovine ATCC isolate, as all isolates caused mild CAH.[13] The authors speculated that this finding may have resulted from attenuation of the virulent isolate when it was grown in vitro. The cause of differing CAH presentations in NU mice is hypothesized to be multifactorial, with experimental manipulation, skin flora, humidity, type of caging, and hair growth cycles all contributing to the clinical phenotype.[5,11,25] Our study clearly demonstrated that the resulting clinical phenotype depends on the isolate used. We could not identify specific putative virulence factors contributing to the observed differences, because many of the previously identified factors of interest were shared among the isolates causing markedly different clinical phenotypes.[9] Additional studies are needed to identify factors that contribute to Cb's pathogenicity.[35]

The interspecies transmissibility of bovine adapted Cb has been reported previously.[13] To our knowledge, the current study is the first to evaluate the potential of Cb isolated from humans and rats to infect immunocompromised mice. Compared with Cb isolated from mice, the ID_{50} of Cb isolated from rats and cows are as much as 10 times higher. Even though their ID_{50} are higher, the potential remains for these species to act as reservoirs, and therefore appropriate biosecurity precautions should be taken. Caution should be exercised when housing nude rats in the same room as immunocompromised mice. Moderate to severe clinical disease was noted in NU mice colonized with the rat Cb isolate 16-2004, whereas the rat from which this isolate was cultured originally had ulcerative lesions on its limbs, dorsum, and paws; whether these were due to an active Cb infection or the result of other bacteria isolated, which included *Staphylococcus aureus* and *Staphylococcus xylosus*, is unknown. Also unknown is whether nude rats will develop Cb-associated disease or will remain colonized with Cb. Interestingly, the rats from which isolate 16-2004 was obtained were housed in an animal room also housing NU mice. The bovine isolate we evaluated produced mild disease only at the highest doses (10^7 to 10^8 bacteria). The isolates cultured from humans did not colonize NU mice at doses as high as 10^8 bacteria. These isolates were cultured from the eye and a skin wound and had the longest growth times. Humans are not likely to act as reservoirs of isolates capable of causing Cb-associated disease in immunocompromised mice. However, Cb (presumably of mouse origin) was isolated from the nasal passages of an animal care staff member who worked extensively with enzootically infected immunocompromised mice.[6] Therefore, humans could potentially serve as fomites in the transmission of Cb.

Iso 24956, which colonized NU mice but did not cause disease, was initially cultured from a commercial breeding colony of highly immunocompromised mice that never developed clinical signs. The breeder subsequently inoculated various hairless and furred immunocompromised and immunocompetent mice with the isolate and did not observe clinical disease despite some of the mouse strains becoming colonized. Whole-genome sequencing of the isolate revealed that it clustered with previously sequenced cow and human isolates but was closest to sequenced human isolates. Although the isolate's ID_{50} was closest to the rodent isolates, its growth kinetics were similar to that of the human isolates. The commercial breeder considered the possibility that the isolate had been introduced into the affected barrier by a staff member, some of whom had contact with cows, but the definitive source of this isolate remains unknown. The question remains regarding whether human isolates at a sufficient dose or with prolonged or repeated exposure could adapt to colonize mouse skin and cause disease. Future studies with additional human isolates, higher inocula, and repeat administration should be undertaken to confirm that human Cb isolates cannot colonize mice.

Hairlessness, the degree of immunosuppression, and altered skin homeostasis are all thought to contribute to mouse

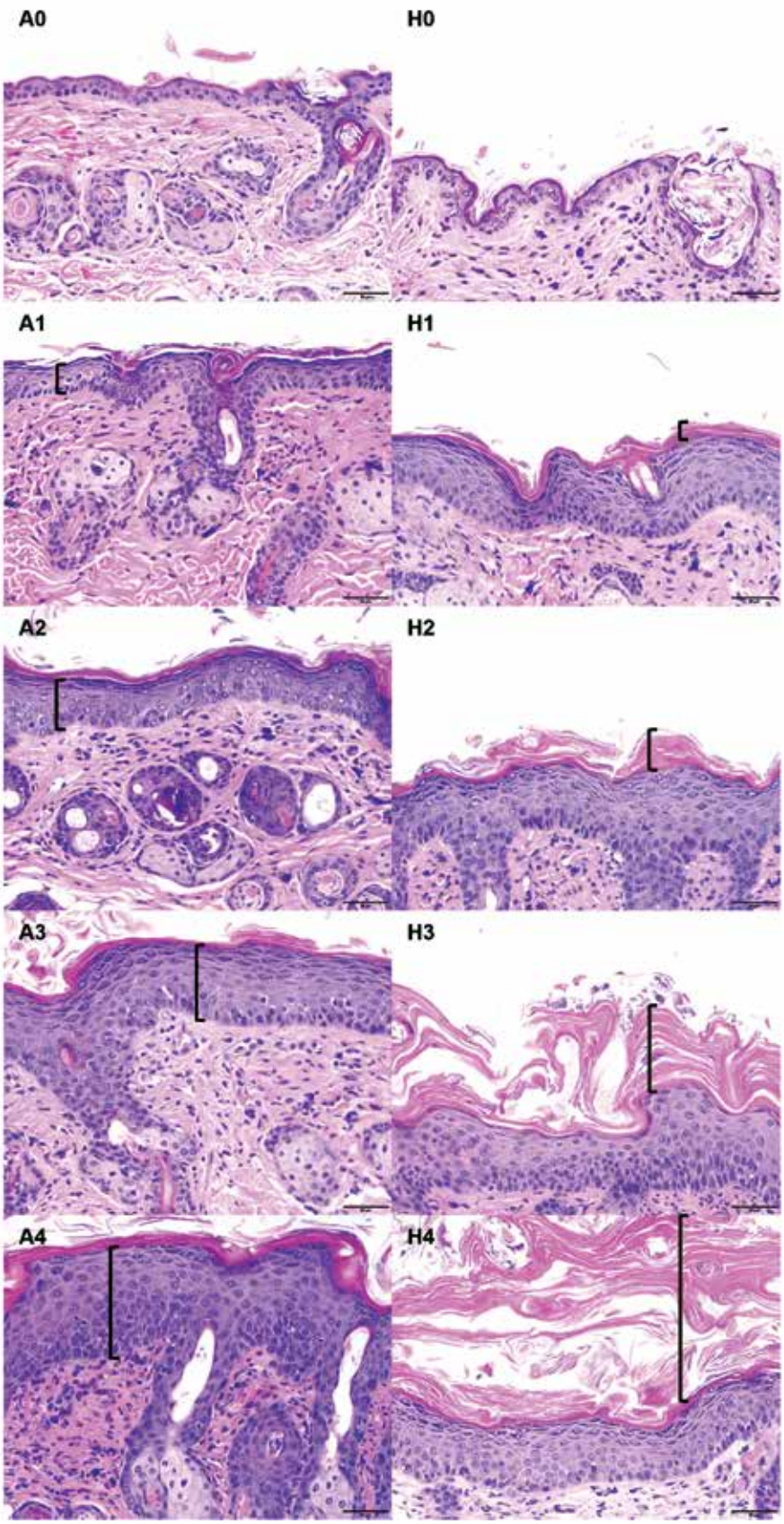

Figure 9. Photomicrographs demonstrating histologic scoring system for acanthosis and hyperkeratosis over 14 dpi in nude mice infected with Cb. Samples were semiquantitatively scored as normal (0), minimal (1), mild (2), moderate (3), or severe (4), based on the intensity of acanthosis and orthokeratotic hyperkeratosis (orthokeratosis). A 0 to 4, acanthosis grade; H 0 to 4, hyperkeratosis grade; black bracket represents the location and amount of acanthosis (A) or hyperkeratosis (H).

susceptibility to Cb, given that hairless immunocompetent SKH1-Hr^{hr} and epidermal-mutant *dep/dep* mice become colonized with the bacterium.[11,26,36] Skin homeostasis is complex and relies on a variety of factors, including the skin's microbiome, the hair growth cycle, disintegrating sebocytes releasing lipid-rich sebum, continuous replication of epithelial cells, and innate antimicrobial peptide expression.[4,16] Because Cb cannot produce its own lipids, it requires the lipid-rich sebum in the stratum corneum for growth and survival.[32] Persistent colonization of the immunocompetent *dep/dep* mouse strain with Cb is thought to be mediated by excessive sebum production from hyperplastic sebaceous glands and an abundant amount of lipids in the interfollicular epidermis.[26] To our knowledge, differences in the amount of and function of sebaceous glandular tissue or sebum production between NU and furred immunocompromised mice have not been investigated. However, a previous study has shown that Cb grows more rapidly and in greater numbers on the skin of NU compared with NSG mice.[25] In the current study, the mouse's fur was parted and Cb was inoculated directly onto the skin; therefore, the higher ID_{50} determined in the furred immunocompromised strains are potentially associated with innate skin defenses, such as an increased surface area for antimicrobial peptide accumulation, less sebum production, or differences in the skin microbiome.[25] The NSG-S strain appeared to be more susceptible to infection than the NSG strain, due to the NSG-S's lower ID_{50} and slightly higher histologic scores when inoculated with isolate 7894 at doses equal to or above 10^6.

The most notable histologic changes in NU mice were associated with rodent isolates, including mouse isolates 7894, 13-1426, and 17-0240 and rat isolate 16-2004. Hyperkeratosis and acanthosis were the major contributors to the total histology scores for all isolates in this study (Supplemental Tables S1 through 3). Bacteria were seen infrequently, despite severe clinical disease in some mice. Histology is a relatively insensitive method for detection of pathogens, and failure to observe bacteria in the examined sections does not rule out their direct involvement in the histologic lesions. Several control mice had hyperkeratosis, acanthosis, and inflammation, which were attributed to the genetic mutation associated with the NU phenotype of dysfunctional hair development and differentiation.[15,21,27] Consequently, the inflammation observed in this study was principally associated with folliculitis and rupture of hair follicles, which are spontaneous background lesions in this strain secondary to their dysfunctional follicular structures, and not overtly associated with Cb infection. Iso 24956 produced significantly higher total histology scores for doses greater than 10^4 as compared with controls, despite absence of clinical signs and suggesting that Cb isolates that do not cause overt disease may still alter the skin. Cross-isolate histologic comparisons were not possible because clinical disease varied at the study endpoint, with some mice having higher clinical scores in the lower dose groups and others at higher doses in which clinical signs had begun to regress or had completely resolved.

In conclusion, our study revealed considerable differences in the ID_{50} of individual Cb isolates, their ability to cause disease, and the severity of disease that developed. Severely immunocompromised mice required higher inocula to become colonized, and clinical disease developed later than in NU mice infected with the same isolate. NU mice could not be colonized with Cb isolated from humans with Cb-associated disease. At least some bovine and rat isolates were infectious to mice, although higher doses were needed to achieve colonization. We were unable to identify any genetic or in vitro growth characteristics that could be attributed to any of our findings. Future studies are necessary to elucidate the etiologies of these differences, focusing on those that can be harnessed to eradicate Cb from enzootically infected colonies or mitigate its effects.

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