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Pathogenicity and Genetic Variation of 3 Strains of *Corynebacterium bovis* in Immunodeficient Mice

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Corynebacterium bovis **has been associated with hyperkeratotic dermatitis and acanthosis in mice. We studied 3 different strains of *C. bovis*: one previously described to cause hyperkeratotic dermatitis (HAC), one that infected athymic nude mice without leading to the classic clinical signs, and one of bovine origin (ATCC 7715). The 3 strains showed a few biochemical and genetic differences. Immunodeficient nude mice were housed in 3 independent isolators and inoculated with pure cultures of the 3 strains. We studied the transmission of these *C. bovis* studies to isolator-bedding and contact sentinels housed for 5 to 12 wk in filter-top or wire-top cages in the respective isolators. Using a *16S rRNA*-based qPCR assay, we did not find consistent differences in growth and transmission among the 3 *C. bovis* strains, and neither the incidence nor severity of hyperkeratosis or acanthosis differed between strains. Housing in filter-top compared with wire-top cages did not alter the morbidity associated with any of the strains. Our findings confirmed the variability in the gross and histologic changes associated with *C. bovis* infection of mice. Although bacteriology was a sensitive method for the detection of *Corynebacterium* spp., standard algorithms occasionally misidentified *C. bovis* and several related species. Our study demonstrates that PCR of skin swabs or feces is a sensitive and specific method for the detection of *C. bovis* infection in mice. An *rpoB*-based screen of samples from North American vivaria revealed that HAC is the predominant *C. bovis* strain in laboratory mice.**

Abbreviations: HAC, hyperkeratosis-associated *C. bovis* strain; NHAC, *C. bovis* strain that did not cause clinical disease in the source colony; ATCC 7715, *C. bovis* ATCC 7715; rpoB, RNA polymerase β subunit; qPCR, quantitative real-time PCR.

Scaly skin disease of athymic nude mice, first reported in 1976, was later found to be caused by *Corynebacterium bovis* infection.[5] The skin condition, also called hyperkeratotic dermatitis,[16,17] most often occurs in mice that are both immunodeficient and hairless but also has been reported to occur in hairless immunocompetent mice, such as SKH1.[5] In addition, haired immunodeficient mice, such as SCID mice, may show alopecia and clinical signs.[17] Infected immunodeficient mice often lose weight, probably due to anorexia, and dehydration. The most prominent clinical sign of the disease is a severe scaly appearance to the skin, which has been informally likened to a cornmeal coating. Clinical signs are often very mild or nonexistent with many asymptomatic carriers.[3,5] Clinical signs usually disappear over time, but infection persists, and all infected mice, including those that never developed signs, can spread the bacteria to naïve mice. Variability in clinical signs and in the *C. bovis* strains recovered from infected laboratory mice has been reported.[3]

Histopathologic changes associated with *C. bovis* infection include orthokeratotic hyperkeratosis that correlates with the scaling observed grossly, diffuse acanthosis (thickening of stratum spinosum and stratum basale), and mild mononuclear cell infiltration in the dermis. Most mice survive infection, and the clinical signs resolve, but acanthosis is persistent.[3,5] The term 'hyperkeratosis-associated coryneform (HAC) infection' has been widely adopted in the field since it was first coined in 1995, but hyperkeratosis is neither specific for *C. bovis* infection nor is it as persistent as the acanthosis.[3,5]

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The infection renders mice unusable for many research applications: for example, there are reports of decreased xenograft growth in infected mice.[10] Various treatment strategies including amoxicillin-treated diet, penicillin–streptomycin topical spray, and antibiotic prophylaxis are ineffective at eradicating the infection.[3] Aseptic hysterectomy or embryo transfer can rid a line of mice of the bacteria, but the bacteria survive well in the environment, and reinfection can occur if the same facility is repopulated. Various decontamination methods[3,16] frequently have proven unsuccessful, due to the widespread environmental dissemination and resistance to desiccation of the organism. These challenges demand reliable diagnosis and a better understanding of disease progression and transmission. Bacteriology and histology have been the traditional methods for detection and diagnosis of *C. bovis* infection.[3,5,17] In addition, the *16S rRNA*[9,11] and *rpoB*[12] genes have been targeted for PCR-based identification of *C. bovis.*

In the current study, we sought to investigate whether the reported differences in clinical signs associated with *C. bovis* infection might be attributed to different *C. bovis* strains in circulation or to different housing conditions, as suggested by earlier studies.[3,5] We investigated whether the time course for transmission and disease severity varies among isolates of *C. bovis*. The 3 isolates we evaluated in the current study were the hyperkeratosis-associated *C. bovis* isolate described previously[5] (the HAC strain), a *C. bovis* isolate from a large group of asymptomatic nude mice (the nonHAC [NHAC] strain), and an isolate of bovine origin (ATCC 7715). We identified sequence variations in the 16*S rRNA* and *rpoB* genes of the 3 isolates. After experimental infection, all 3 strains caused the diffuse acanthosis that traditionally is associated with *C. bovis* infection,[5] low-grade hyperkeratosis, and inflammation.

Evaluation of various diagnostic methods demonstrated that real-time PCR of skin swabs or feces is more reliable than is bacteriology or histology for the detection and diagnosis of *C. bovis* infection.

Materials and Methods

Animals. Female SPF Crl:NU-^{Foxn1nu} (nude) mice (age, 4 to 5 wk; Charles River, Wilmington, MA) were used for these studies. The mice were raised in semirigid isolators. The mice were free of murine viruses, pathogenic bacteria including *C. bovis*, and endoparasites and ectoparasites. Nonpathogenic bacteria detected on some of the mice included *Staphylococcus epidermidis*, *S. warneri*, *Enterococcus gallinarum*, and *C. urealyticum*. The mice were maintained in 3 semirigid isolators that were sterilized prior to the start of study. The isolators were provided with γ-irradiated aspen shavings as bedding (Northeastern Products, Warrensburg, NY) and HEPA-filtered air (12 air changes daily) with 14:10-h light:dark cycle. The mice had ad libitum access to γ-irradiated feed (Lab Diet 5L79, Purina, St Louis, MO) and γ-irradiated water. All the materials introduced into or removed from the isolators were disinfected with chlorine dioxide solution (Clidox S, Pharmacal Research Laboratories, Naugatuck, CT). The bedding was changed weekly, and 10% of the dirty bedding from each cage was transferred to each sentinel cage. The study mice were observed daily for clinical signs and weighed weekly to monitor weight loss. All animal use procedures were approved by Charles River's IACUC.

***C. bovis* inoculum.** Glycerol stocks of the HAC, NHAC, and ATCC 7715 strains that had been maintained at −70 °C were streaked onto blood agar fortified with 5% sheep RBC and incubated for 48 h at 37 °C with 5% CO_2. A single colony of each was inoculated in CM broth (brain–heart infusion broth supplemented with 0.5% yeast extract and 20% horse serum; Becton Dickinson, Franklin Lakes, NJ) and grown overnight; turbidity was adjusted to 1 McFarland by using saline (0.85%) prior to inoculation on mice.

Study designs. The 3 studies described below were done in succession in independent isolators assigned to each strain: HAC, NHAC, and ATTC 7715(Figure 1).

Study 1. Female nude mice (n = 12; age, 4 to 5 wk) in wire-top cages were introduced into each of the 3 isolators. Within each isolator, 6 mice (3 each in cages 3 and 4) were inoculated with approximately 5×10^7 bacteria applied gently to the dorsal midline skin by using a sterile cotton swab. The remaining 6 mice (3 each in cages 1 and 2) were used as sentinels and received dirty bedding (10% by volume) from inoculated cages once each week. Sentinel mice also were exposed to *C. bovis* via the isolator environment and handlers' gloves (Figure 1) and henceforth are referred to as 'isolator–bedding sentinels.'

Study 2. Female nude mice (n = 20, 5 mice per cage; age, 4 to 5 wk) in open-top or filter-top cages were introduced in each of the 3 isolators. In each isolator, 5 mice were infected by cohousing them with study 1 mice for 3 d and henceforth are referred to as 'contact sentinels.' After exposure, the contact sentinels were moved to a clean wire-top cage and subsequently served as the dirty bedding source for 2 filter-top cages (5 mice per cage) housed for 5 or 12 wk and for one wire-top cage that housed 5 mice for 12 wk. Bedding sentinels received 10% dirty bedding weekly. The mice were observed daily for clinical signs and were monitored weekly for *C. bovis* load by performing qPCR on skin swabs.

Study 3. Female nude mice (n = 5; age, 4 to 5 wk) housed in a filter-top cage were introduced in each of the 3 isolators. These mice were infected by cohousing them with study 2 mice for 3 d.

Sample collection. Swabs for PCR testing and bacteriology were collected by firmly rubbing a sterile cotton swab on the dorsal and ventral skin of the mice. Samples were collected on day 0 (immediately before inoculation), day 3 or 4, day 7, and weekly thereafter for the duration of the study. Feces and skin swabs for the comparison study were collected by the same technician. For collection of fecal samples, mice were held vertically, and the fecal pellets were allowed to drop directly into the sample tube, to avoid cross contamination. At the end of the experiment, the mice were euthanized by using CO_2, and full-thickness skin samples were collected for histopathology.

Bacteriology and biochemical analysis. For detection of *Corynebacterium* spp. on skin, culture swabs were streaked onto CM agar and blood agar fortified with 5% sheep red blood cells (Becton Dickinson). The plates were incubated for 48 h at 35 °C to 37 °C with 5% CO_2. All colonies with morphology consistent with *Corynebacterium* spp. were subcultured for another 48 h on CM agar. Isolated colonies were identified by using VITEK 2 *Corynebacterium* identification cards (Biomérieux, Marcy l'Etoile, France).

For detection of other bacteria prior to introduction into the isolator, skin swabs were streaked onto Columbia agar fortified with 5% sheep red blood cells with nalidixic acid to select for gram-positive bacteria (Becton Dickinson) and incubated for 24 to 48 h at 35 °C to 37 °C. Bacterial colonies were identified by their characteristic morphology. Biochemical characterization followed subculturing for another 24 to 48 h on the desired agar plate; a single-colony suspension was applied to a VITEK 2 Gram-Positive card (Biomérieux) and analyzed on a VITEK 2 compact instrument (Biomérieux).

DNA extraction and sequencing of *16S rRNA* and *rpoB* genes. DNA was extracted by using a MagMAX Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA) and a Qiagen robotic extraction station (Thermo Labsystems, Franklin, MA) according to manufacturers' instructions. A 1393-bp region of *16S rRNA* gene was amplified by using the primers CORYN16S9F (5′ GAT CCT GGC TCA GGA CGA AC 3′) and CORYN16S1399R (5′ GTT ACC AAC TTT CAT GAC GTG AC 3′); primer nomenclature is based on the nucleotide positions in GenBank accession NR_037042.[14] PCR analysis was performed by using long-range *Taq* polymerase (Qiagen, Hilden, Germany). The thermal cycling conditions used were: initial denaturation at 93 °C for 3 min, followed by 35 cycles of denaturation at 93 °C for 15 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min.

A region of the *rpoB* gene was amplified by using HotStar *Taq* polymerase (Qiagen) with the primers CB1959F (5′ AAG TTC GAG CGC ACC AAC CA 3′) and CB3125R (5′ TGG CCC ACA CCT CCA TCT 3′), which generate an expected product of 1216 bp; primer nomenclature is based on the nucleotide positions of a *C. bovis* reference sequence (GenBank accession no., AY492236). Thermal cycling conditions were: denaturation at 95 °C for 15 min, followed by an initial 5 cycles of denaturation at 95 °C for 30 s, primer annealing at 65 °C for 30 s, and extension at 72 °C for 60 s. The annealing temperature was decreased to 60 °C for 5 cycles and to 54 °C for 35 cycles; all other parameters remained as described for the initial 5 cycles.

PCR products were separated on 1% agarose gels, eluted from the gel (Minelute Kit, Qiagen), and sequenced (ABI 3130XL DNA sequencer, Tufts University Core Facility, Boston, MA).

Sequence analysis. Vector NTI software (Life Technologies) was used for sequence analysis and alignment. Reference sequence data of the *16S rRNA* (NR_037042) and *rpoB* (AY492236) genes of *C. bovis* were included.

	Bedding suppliers				Isolator–bedding sentinels		
Study	Mode of exposure	No. of mice	Cage type	Study duration (wk)	No. of mice	Cage type	Study duration (wk)
1	inoculation	6	wire-top	5	6	wire-top	5
2	cohousing	5	wire-top	12	5	filter-top	5
					5	wire-top	12
					5	filter-top	12
3	cohousing	5	filter-top	6	–	–	–

Figure 1. Scheme for *C. bovis* transmission studies. Independent isolators were assigned for each strain (HAC, NHAC, and ATCC 7715). Mode of exposure, number of mice, cage type, and weeks on study are shown for studies 1, 2, and 3.

Submission to GenBank. *16S rRNA* and *rpoB* gene sequences for the *C. bovis* strains obtained in this study were submitted to GenBank (accession nos., JX456367 to JX456372).

Quantitative PCR (qPCR) for *C. bovis* detection and real-time PCR assays for differentiation of the HAC strain. *C. bovis* was detected in nucleic acid samples by using a proprietary *16S rRNA*-based fluorogenic real-time PCR assay (Charles River Laboratories). The *C. bovis* PCR assay targeting the *16S rRNA* gene is specific to *C. bovis* and does not detect any known sequences for other *Corynebacterium* spp.

Sequence polymorphism in the *rpoB* gene was used to develop a pair of real-time PCR assays for distinguishing the HAC strain from other strains lacking the targeted polymorphism; a common forward primer (900 nM; 5′ CTC GCT CAC CGC GAC G 3′) and reverse primers (300 nM each) specific for HAC (5′ GGA GGA CCG GAG CAG A 3′) and other (nonHAC) *C. bovis* strains (5′ GGA GGA CCG GAG CAG G 3′) were used along with a common minor grove binder probe (200 nM; 5′ CGA CGG CGC CTC 3′ bound to 6-carboxyfluroscein and a nonfluorescent quencher) for distinguishing the HAC strain. The thermodynamic properties of the primers (Sigma-Aldrich, St Louis, MO) and the probes (Applied Biosytems, Foster city, CA) were evaluated by using Net Primer software (Premier BioSoft International, Palo Alto, CA) and Primer express software (Life Technologies), respectively.

DNA isolated from Chinese hamster kidney cells was used as a negative-control template, and 100 copies of cloned fragments of the assay targets were used as positive controls. Ten-fold serial dilutions of the cloned target amplicon (range, 10^1 to 10^7 copies) were used to generate a standard curve for calculating the copy numbers of the *C. bovis 16S rRNA* gene target in each qPCR analysis well, as previously described.[1] Absolute copy numbers per swab were calculated by accounting for the dilution factors used during DNA extraction and PCR analysis. In addition, we performed a DNA-spike control assay to confirm the absence of PCR inhibitors in the fecal sample.[8] The analytical sensitivity of all assays was determined to be 1 to 10 copies per well (data not shown). The assays were run on an ABI 7300 thermocycler (Life Technologies), with 5 cycles of denaturation at 95 °C for 15 s and primer annealing–extension at 64 °C for 1 min, followed by 55 cycles of denaturation at 95 °C for 15 s and primer annealing–extension at 58 °C for 1 min. Real-time PCR results were analyzed by using vendor-supplied software (Life Technologies).

Histopathology. Full-thickness samples of skin were excised from the back of the neck for histopathology and fixed in 10% neutral buffered formalin. Tissues were routinely processed into paraffin blocks, and then 5-µm thick sections were cut and stained with hematoxylin and eosin. Sections were evaluated by a board-certified veterinary pathologist who was blinded to the study groups. The skin was scored for severity of hyperkeratosis, acanthosis, inflammation, and visible bacteria on an ascending subjective scale of 0 to 5 representing no, minimal (barely detectable), mild, moderate, and severe change (Figure 2). In addition, hair follicle growth phase was noted for each section.

Statistics. Paired *t* test or one-way ANOVA followed by post-hoc analysis was performed to calculate statistical significance by using SigmaPlot software (Systat Software, San Jose, CA). A *P* value of less than 0.05 was used to define statistical significance.

Results

Sequence analysis of *16S rRNA* and *rpoB* genes and development of real-time PCR assays to distinguish the *C. bovis* HAC strain. We sequenced the *16S rRNA* (1289 bp) and *rpoB* (1008 bp) genes of the 3 *C. bovis* strains to look for possible differentiation markers (Figure 3). Both the *16S rRNA* (NR_037042)[14] and *rpoB* (AY492236)[12] gene sequences are available for the ATCC 7715 strain. Comparison of the *16S rRNA* sequences of our 3 strains to that of the reference strain (NR_037042) identified 2 adenine deletions at positions 60 and 66 in all 3 strains and a guanine-to-thymine substitution at position 125 in the NHAC strain. In our 3 strains, *rpoB* was more variable than was *16S rRNA* gene, as has been reported earlier for *Corynebacterium* spp.[12] and other bacteria.[8] The *rpoB* gene sequence for the ATTC 7715 strain was identical to the reference sequence AY492236, which was derived from a *C. bovis* strain of bovine origin. There were 5 common single-base mismatches in the *rpoB* gene sequence that distinguished the HAC strain from the NHAC and ATCC 7715 strains. We targeted one of these (cytosine compared with thymine at position 2909 of the reference strain) to design 2 primers for use in 2 different assays to distinguish the HAC strain from the other 2 *C. bovis* strains.

The *rpoB* gene was sequenced to authenticate HAC, NHAC, and ATCC 7715 inocula prior to the start of study 1. Similarly, *rpoB* sequencing of representative isolates taken from each isolator after completion of study 3 confirmed no cross-contamination between the study isolators.

Transmission of 3 *C. bovis* strains (study 1) on nude mice. We infected each study group with one of the 3 *C. bovis* strains: HAC, NHAC, and ATCC 7715. At day 4 after inoculation, the generic qPCR for *C. bovis* detected an average of 10^3 DNA copies per swab collected from the inoculated mice ($n = 6$, 3 mice per cage) in all 3 isolators, indicating a uniform baseline infection. On day 4, prior to the first bedding transfer, the sentinel mice ($n = 6$, 3 mice per cage) were already weakly positive (approximately 10^2 DNA copies per swab), likely from the bacteria transferred by the handlers' gloves or from scattered particulate matter containing the bacteria in the isolator. Subsequently, weekly swabbing of the skin followed by qPCR was performed for 5 wk to monitor the bacterial load on inoculated and sentinel mice (Figure 4). In both inoculated (Figure 4 A) and isolator–bedding sentinel cages (Figure 4 B), the infection peaked between weeks 3 and 4 for all bacterial strains to about 10^5 target copies per swab, approximately 1000-fold higher than the levels detected at day 4. The HAC load on isolator–bedding sentinel mice peaked at week 3, a week earlier than did the NHAC and ATCC 7715

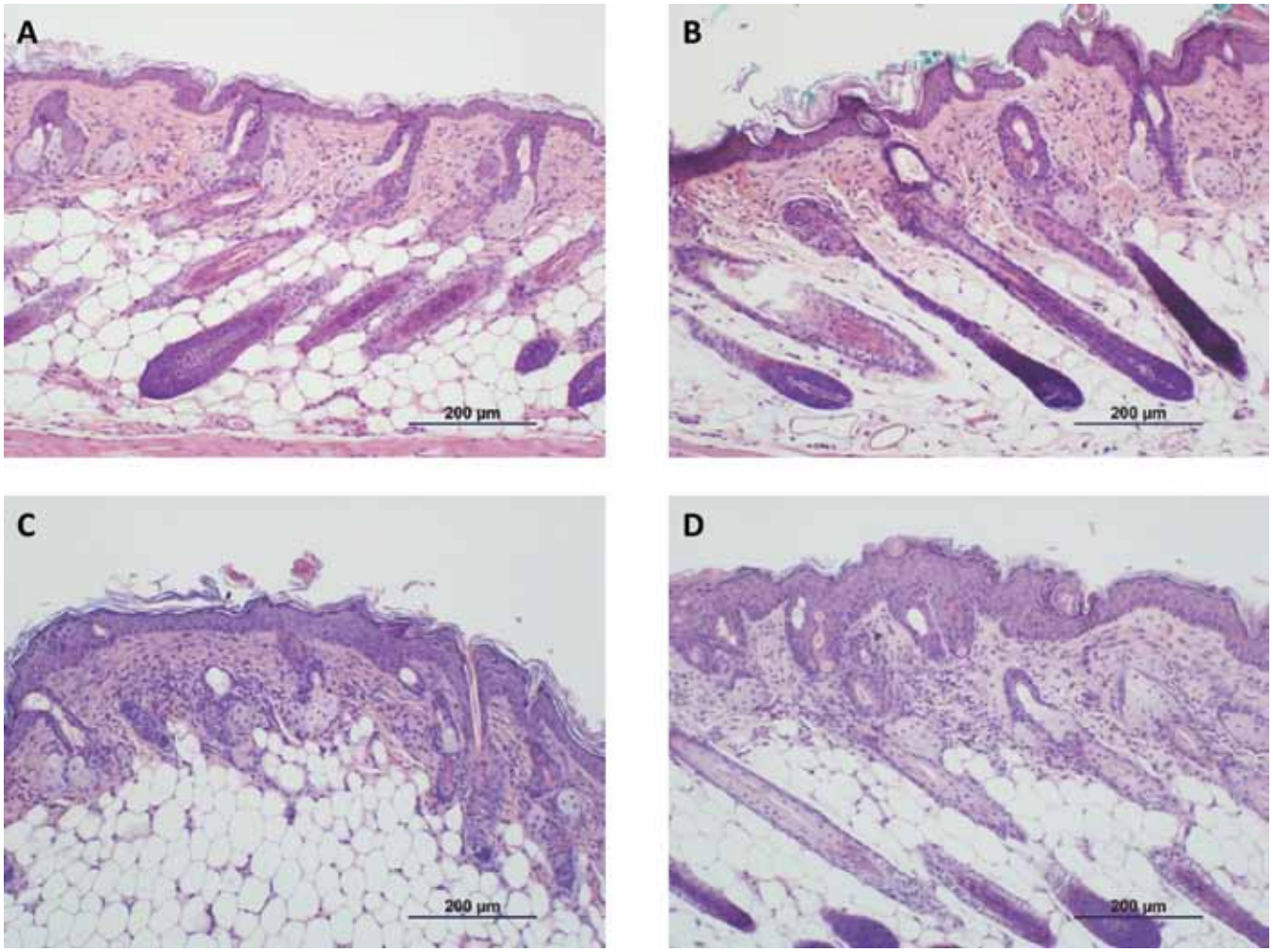

Figure 2. Skin sections from HAC-infected mice displaying different severity grades of acanthosis: (A) grade 0 (none). (B) Grade 1 (minimal). (C) Grade 2 (mild). (D) Grade 3 (moderate). Hematoxylin and eosin stain; bar, 200 µm.

strains. The HAC load on isolator–bedding sentinel mice was significantly ($P < 0.05$) higher compared with both NHAC and ATCC 7715 counts on isolator–bedding sentinels at week 2 and significantly ($P < 0.05$) higher than the ATCC 7715 counts on isolator–bedding sentinels at week 3. However, by week 5, bacterial loads on sentinel mice were similar in all 3 isolators, indicating that all 3 strains of *C. bovis* were proliferating and being transferred efficiently to sentinels via bedding or other environmental means within isolators.

Extended exposure to 3 *C. bovis* strains in different housing environments (study 2). Distinct differences among the 3 strains in the transmission and progression of clinical disease were not evident in study 1. Therefore, we used 2 different cage environments and an extended observation period of 12 wk in study 2. In addition to wire-top caging, we introduced filter-top caging to evaluate whether it exacerbated clinical signs by changing the intracage humidity or by altering the number of bacteria present on the skin, as previously suggested.[5] Prior to exposure, the load of *C. bovis* on the skin of the infected cohorts was determined to be 2×10^3 to 4×10^3 copies per swab. qPCR data indicated only subtle differences in the growth of the 3 strains; no significant differences in *C. bovis* load at week 5 were seen between strains in contact sentinels or isolator–bedding sentinels in wire-top cages. However, at week 12, HAC contact sentinels had significantly ($P < 0.05$) higher bacterial load than did ATCC 7715 contact sentinels, and HAC isolator–bedding sentinels had significantly ($P < 0.05$) higher bacterial load than did NHAC isolator–bedding sentinels. The *C. bovis* load appeared to approximately double every 3 to 4 wk in HAC and NHAC contact sentinels, whereas there was only a marginal increase for the ATCC contact sentinels (Figure 5 A). The typical scaly skin appearance associated with *C. bovis* was not seen in any group. After an initial delay of 2 wk, isolator–bedding sentinels mice in wire-top cages (Figure 5 B) and filter-top cages (data not shown) demonstrated a similar pattern of increase in *C. bovis* load as that of infected cohorts. For all 3 strains, the initial peak in *C. bovis* load occurred 1 to 2 wk earlier in contact sentinels than in isolator–bedding sentinels (data not shown).

Exposure of mice to a high load of *C. bovis* (study 3). To determine whether rapid exposure to a high dose of *C. bovis* produced clinical disease, we introduced 5 naive mice into each isolator during week 11 of study 2, when the *C. bovis* load on the existing mice was near peak for all 3 strains (10^5 to 10^6 copies per swab). In contrast to studies 1 and 2, in which the initial *C. bovis* load on day 3 or 4 after exposure was 10^3 to 10^4 copies per swab in all 3 isolators, we determined that the initial load of *C. bovis* on study 3 mice was approximately 100-fold greater, at 1×10^5 to 2×10^6 copies per swab on day 3. Peak infection for all 3 strains occurred during week 1, followed by a decline during week 2 and a second peak at week 4 (data not shown). Three mice exposed to the HAC strain developed mild to moderate scaly skin during week 1 after exposure and were submitted

	Nucleotide (position)								
AY492236	T (2018)	C (2129)	C (2327)	C (2630)	C (2661)	T (2687)	A (2777)	C (2828)	C (2909)
ATCC 7715	T	C	C	C	C	T	A	C	C
NHAC	T	C	T	C	T	C	G	C	C
HAC	C	T	C	G	C	C	G	G	T

Figure 3. Polymorphisms present in the *rpoB* gene sequence. A 1008-bp sequence (nucleotides 2014 to 3021) was compared with the reference sequence (AY492236); polymorphic nucleotides and their positions are indicated.

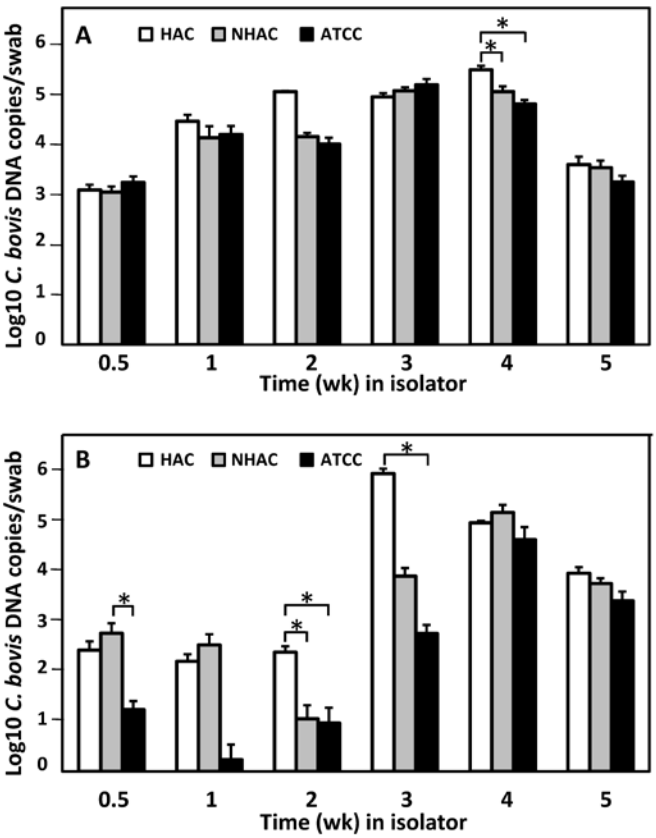

Figure 4. Time course of *C. bovis* load (mean ± SEM) as detected by qPCR of (A) inoculated and (B) isolator-bedding sentinel mice in wire-top cages (study 1). The mice were inoculated with the HAC, NHAC, or ATCC 7715 strain in independent isolators. The isolator-bedding sentinel mice in the respective isolators received 10% dirty bedding weekly from the inoculated cages. Each data point represents 6 mice (except that for HAC sentinels at week 5 represents 5 mice). *, Values were significantly ($P < 0.05$) different.

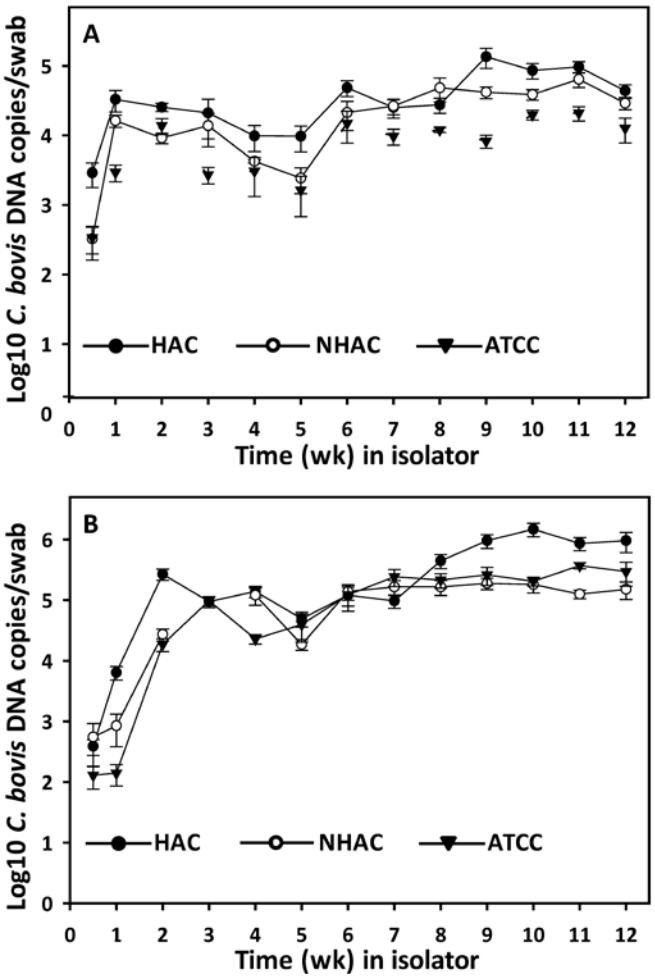

Figure 5. Time course of *C. bovis* load (mean ± SEM) as detected by qPCR of (A) contact sentinels and (B) isolator-bedding sentinels housed in wire-top cages for 12 wk (study 2). Each data point represents 5 mice (except that for NHAC contact sentinels at week 3 represents 4 mice, and corresponding data were not collected for isolator-bedding sentinels.

for necropsy. The remaining mice were monitored for clinical signs until week 6, and the skin was swabbed weekly for PCR analysis. The remaining 2 mice exposed to the HAC strain and all mice exposed to other 2 strains remained free of clinical signs of disease throughout the study.

Histopathology. All mice in all 3 studies, including the 3 mice in the HAC isolator that displayed clinical signs after 1 wk (study 3), had minimal to mild acanthosis, no to minimal hyperkeratosis (microscopically), and no to mild inflammation. No significant difference was noted between various groups (different strains and cage types) in the 3 studies. Table 1 summarizes the histopathology results from study 2 mice after 12 wk of exposure.

To test whether development of clinical signs or histopathologic lesions correlated with follicular activity, we assessed the phase of the hair growth cycle (anagen, telogen) in various groups from the 3 studies. Approximately 24% of the sections had hair in the telogen phase and 61% were in the anagen phase of growth, whereas the remaining skin sections included portions of skin showing anagen and telogen phases of hair growth. The scores for visible bacterial load in sections showing anagenic or telogenic hair growth were averaged by study group, and no difference between groups was noted.

Comparison of qPCR and biochemical methods for the detection of *C. bovis*. In addition to weekly qPCR, skin swabs were evaluated by bacteriologic and biochemical methods during week 1 of study 1, when the load of *C. bovis* was very low (10^1 to 10^3 DNA copies per swab). The results suggested similar diagnostic sensitivity of qPCR and bacteriology for detection of *C. bovis*. However, bacteriology and biochemical analysis detected additional *Corynebacterium* spp. (not *C. bovis*, as expected) on many mice at the end of studies 1, 2, and 3 (week 5, 12, and 6, respectively); all of these mice were diagnosed as positive for *C. bovis* according to qPCR analysis (Figure 6). The organisms from HAC, NHAC, and ATCC 7715 isolators

Table 1. Microscopic evaluation of skin sections (mean score) from isolator–bedding sentinels (study 2) housed in wire-top cages for 12 wk

	HAC		NHAC		ATCC 7715		
	cohabitation	sentinels	cohabitation	sentinels	cohabitation	sentinels	Controls
Hyperkeratosis	0.2	0.6	0.2	0.2	0.8	0.4	0
Acanthosis	1.4	1.6	1.2	1.0	1.2	1.4	0.2
Inflammation	0.2	0.2	0.8	0.8	0.8	1.2	0.2

Each group contained 5 mice; controls were 5-wk-old nude mice that were not exposed to any *C. bovis* strain.
No significant differences between groups were present.

Study	# of mice	No. of weeks of exposure	No. of mice positive for *C. bovis* according to qPCR	No. of mice positive for *C. bovis* according to VITEK	No. of mice identified by VITEK 2 as having a species other than *C. bovis* (species name)
1	36	1	25[a]	28[a]	None
		5	36	25	11 (*C. mastitidis* or other *Corynebacterium* spp.)
2	15	5	15	1	14 (*C. mastitidis* or *C. jeikeium*)
	45	12	45	45	None
3	12	6	12	1	11 (*C. mastitidis*)

VITEK, automated analysis system
[a]All of the samples determined to be negative at week 1, prior to transfer of dirty bedding, were from the sentinel cages. All of the inoculated mice (6 per strain) tested positive by both methods at week 1.

Figure 6. Comparison of qPCR and biochemical methods for the detection and identification of *C. bovis*.

were identified as *C. mastitidis* in 36%, 33%, and 62% of cases, respectively (Figure 7). Therefore, to authenticate identification, we extracted DNA from 4 isolates from studies 1 and 2 that were identified as *C. mastitidis* or *C. jeikeium*. The *16S rRNA*-based *C. bovis* real-time PCR assay identified these isolates as *C. bovis*. The HAC, NHAC, and ATCC 7715 strains are biochemically very similar as indicated in Figure 7, with a few differences in the activity of urease and several arylamidases (L-proline arylamidase, L-pyrrolydonyl arylamidase, alanine–phenylalanine–proline arylamidase). No interstrain differences were noted in regard to the fermentation of various sugars such as D-glucose, D-xylose, D-maltose, sucrose, D-ribose, and D-mannitol.

Detection of *C. bovis* in feces. We investigated whether *C. bovis* is detected as readily from feces as skin swabs. Samples were collected from HAC contact–exposed mice ($n = 5$) at weeks 8 and 10 of study 2; all mice were PCR-positive according to both sample types, indicating similar diagnostic sensitivity. However, 10-fold more template on average was detected in feces ($[1.46 \pm 0.84] \times 10^5$ *C. bovis* target DNA copies) than in skin swabs ($[0.14 \pm 0.09] \times 10^5$ *C. bovis* target DNA copies) at week 8 ($n = 5$; $P < 0.05$), but similar amounts of *C. bovis* DNA were detected in the 2 sample types at week 10 ($[0.29 \pm 0.26] \times 10^5$ versus $[0.39 \pm 0.26] \times 10^5$ *C. bovis* target DNA copies in feces and swabs, respectively; $n = 5$).

Characterization of field strains of *C. bovis*. We screened a total of 176 skin swabs submitted for *C. bovis* screening to our commercial PCR testing laboratory during 2010 through 2011. The samples were tested by using the *16S rRNA*-based real-time PCR assay; 71 samples from 13 clients were positive for *C. bovis*. The *rpoB* gene-based assay was performed on these 71 positive samples, of which all except 1 were identified as HAC, indicating that this strain is the predominant variant in laboratory mice. Because the *rpoB*-based primers we use cannot distinguish between the 2 nonHAC strains (NHAC and ATCC 7715), we sequenced the *rpoB* gene from the only nonHAC-positive sample and determined it to be an NHAC isolate; the client facility did not note any clinical signs in the mouse. Notably, no *C. bovis* isolate consistent with the ATCC 7715 strain of bovine origin was found among the 71 positive samples from the laboratory mice that we screened in the current study. In addition, we sequenced the *rpoB* gene from HAC-positive samples obtained from 5 independent locations; all 5 isolates were identical to the HAC strain in the current study. A total of 7 of 12 clients having HAC outbreaks in their facilities reported observing subclinical to severe clinical signs in nude mice (Figure 8).

Discussion

The classic *C. bovis*-associated disease in mice is a pronounced hyperkeratotic, 'scaly' skin, although more often the disease is milder, with fewer whitish flakes scattered over the skin. The current study compares the pathogenicity of 3 *C. bovis* strains in nude mice. We here extend our previous work on biochemical characterization of the HAC strain (and 2 other strains) to DNA sequencing of regions of the *rpoB* and *16S rRNA* genes. Nucleotide differences present in the HAC and NHAC strains allow their differentiation from each other and from a *C. bovis* strain of bovine origin (ATCC 7715). In addition, the 3 strains demonstrated several biochemical differences (Figure 7). In vivo, the extent of colonization by *C. bovis* of sentinel mice exposed to soiled bedding from infected isolators was proportional to the bacterial load on the source mice. The proliferation, transmission, and histopathologic changes induced by the NHAC strain (which we had hypothesized to be relatively benign) and the bovine-derived *C. bovis* (ATCC 7715) were similar to those of the HAC strain.

Proliferation of *C. bovis* on nude mice was evidenced by the increase in DNA copies throughout the 22 wk spanning the 3 studies. The increasing bacterial population was not generally accompanied by clinical signs. The high dose of *C. bovis* transferred onto naïve mice at the start of study 3 might have contributed to the clinical signs in 3 mice (Figure 8 A) exposed to the HAC strain. In contrast to our 1995 study,[5] surprisingly few mice were clinically affected in the current study, perhaps because the isolate had become attenuated by successive passages over the years or because necessary cofactors were not present in the recent studies. However, this pattern is typical in *C. bovis* outbreaks: often only a few of the colony mice show

Biochemical test	HAC	NHAC	ATCC 7715
Urease	+	−	+
L-Proline arylamidase	+	−	+
L-Pyrrolydonyl arylamidase	+	−	±
Alanine–phenylalanine–proline arylamidase	+	−	−
Tyrosine arylamidase	+	+	±
Phenylalanine arylamidase	+	+	+
β-Galactosidase	±	+	±
β-Glucuronidase	−	−	−
Esculin hydrolase	−	−	−
L-Lactate alkalinization	−	−	−
% of isolates identified as *C. mastitidis*	36% (4 of 9)	33% (3 of 9)	62% (8 of 13)

Biochemical profile of the isolates identified as *C. mastitidis* is not shown

Figure 7. Representative biochemical profiles, obtained by using VITEK 2 *Corynebacterium* identification cards, of the 3 *C. bovis* strains. The HAC, NHAC, and ATCC 7715 isolates occasionally were misidentified as *C. mastitidis*. Percentages and total numbers are shown.

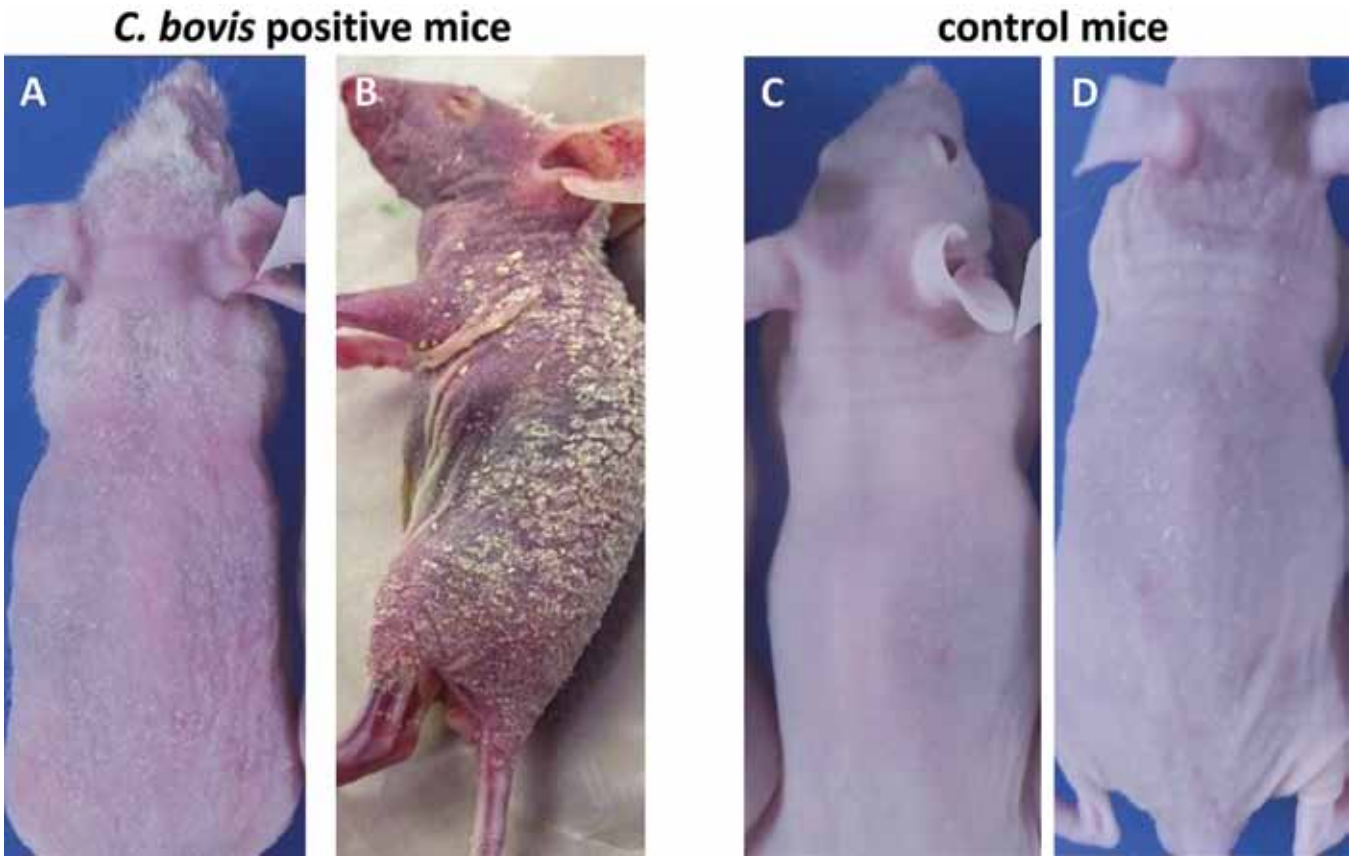

Figure 8. Nude mice with *C. bovis*-associated hyperkeratosis: (A) mouse from study 3 at 1 wk after introduction into the HAC isolator and (B) a client mouse with hyperkeratotic skin flakes. (C) A control nude mouse with clear skin and (D) a uninfected nude mouse with a few small keratin flakes on the skin.

severe clinical signs despite 80% to 100% infection rate.[5,16] In a previous study, severe clinical disease was not established even after administration of cyclophosphamide, suggesting that additional immunosuppression is not sufficient for manifestation of the disease.[3] Many other factors have been hypothesized to contribute to development of *C. bovis*-associated hyperkeratosis, including existing skin flora, humidity, type of caging, and hair growth cycles.[3,5] However, infection in the current study did lead to epidermal changes that were detectable by histopathologic evaluation, consistent with previous reports. Therefore, although gross lesions occurred in only 3 mice, all 3 *C. bovis* strains were pathogenic to varying degrees.

In contrast to previous studies,[5] housing in filter-top compared with wire-top cages did not alter disease production for any of the strains in the current study. In addition, there were no significant differences in clinical signs, degree of acanthosis, or *C. bovis* DNA copy number at the termination (week 12) of study 2.

Bacterial coinfections have been speculated possibly to play a role in exacerbating corynebacterial hyperkeratosis.[3] In contrast, normal flora on mouse skin,[18] such as nonpathogenic *Staphylococcus* spp. and *Enterococcus* spp., might help to limit colonization by pathogenic bacteria through competing for nutrients and niches and by secreting antimicrobial substances, as have been reported in regard to human cutaneous flora.[4] However, given that testing in our laboratory has focused on screening for known pathogenic bacteria, we are unable to comment on the likelihood of such an association.

We assessed each section of skin for phase of the hair follicle growth cycle and found no correlation between hair growth stage and load of *C. bovis* in either the skin sections or skin swabs, thus rejecting our previous speculation that clinical signs and tissue changes potentially were more prominent when hair follicles were in the active (anagen) growth phase.[5] The normal wavelike pattern of hair growth cycles on nude

mice[13] sometimes prompts a test for *C. bovis* because of a rough coat appearance, but skin undergoing normal cycling will not demonstrate histopathologic lesions, and these mice will generally be negative for *C. bovis* by microbiologic culture and PCR testing. Multiple anecdotal and published[5] reports and our current data (study 3; Figure 8 A) all indicate that clinical signs occur about 7 d after initial infection of naïve mice with *C. bovis* and spontaneously resolve within 7 to 10 d of the onset of symptoms.[16] However, these mice are persistently infected and remain carriers of the bacteria; in fact, our studies showed that *C. bovis* load increases every 3 to 4 wk and that between these peaks, the bacterial load remains stable or even declines. Additional studies are required to better characterize the regression of hyperkeratosis and establish the molecular mechanisms.

C. bovis mainly resides in the outermost keratin layer,[7] which is continuously sloughed as epidermal cells move from the basal layers of the skin to the outer surface over 8 to 9.5 d.[15] In addition, inflammatory cell products, antimicrobial substances produced by epidermal cells, or other changes in the micro- and macroenvironment of the skin might limit the severity of hyperkeratosis after its initial development. In fact, hyperkeratosis often disappears in 7 to 10 d, approximately the same length of time epidermal cells need to migrate from the basal layer to the stratum corneum. However, epidermal histologic alterations, especially acanthosis, persist—as does *C. bovis* infection itself—indicating that host defense mechanisms are ineffective at clearing the pathogen.

Our current and previous studies[5] have shown that *C. bovis* can be detected on and transmitted by asymptomatic mice. Anecdotal reports suggest that the spread of bacteria in rodent colonies can occur via fomites, door knobs, gloves, shared laboratory equipment or working space, and even xenograft tumor lines transmitted as tissue fragments. Therefore, sensitive and accurate bacterial screening is crucial to maintaining the health of mice in a breeding colony or research vivarium. Microbiologic culture and PCR can be used for detection of *C. bovis*. Some commercial rodent diagnostic laboratories recommend the use of a skin swab for the detection of *C. bovis*; in addition, variable success has been noted with buccal swabs.[3,5] In the current study, we evaluated whether *C. bovis* infection is detected by PCR of fecal samples used for screening for several other pathogens. We compared the DNA copy numbers of *C. bovis* from skin swabs and fecal samples obtained from 5 mice each in the HAC isolator at weeks 8 and 10 of exposure and determined that the sensitivity of real-time PCR of fecal samples is equal to or better than that of skin swabs.

In our current study, PCR and microbiologic culture showed similar diagnostic sensitivity for *C. bovis* in skin swabs, even at week 1 of study 1, when the load of *C. bovis* was low. Similarly, excellent agreement was observed between the 2 techniques at week 12 of study 2. However, *C. bovis* isolates were often misidentified as *C. mastitidis* (Figures 6 and 7) and sometimes as *C. jeikeium* because of overlap in the biochemical profiles of the *Corynebacterium* spp. Other similar cases have been reported,[6,9,19] representing the challenges associated with detection and speciation of *Corynebacterium* spp. based solely on nonmolecular bacteriologic methods. In addition, the overgrowth of cultures by other skin bacteria such as *Proteus* spp. can hamper culture-based detection of *C. bovis* and is a cause of concern.

The *C. bovis* strains of mouse origin that we evaluated (HAC and NHAC) showed several variations in their *rpoB* gene sequences (Figure 3) and in biochemical tests (Figure 7). Variation in biochemical characteristics between *C. bovis* strains of bovine and human origin[2] have been reported previously. Interstrain differences in colony size and antibiotic sensitivity have been identified between *C. bovis* strains from mice, although no clinical associations were noted.[3] We did not see a consistent difference in bacterial colony size among the ATCC 7715, HAC, and NHAC strains. However, the current study revealed a trend toward strain-associated differences, with HAC growing faster than NHAC or ATCC 7715 (Figures 4 and 5). In addition, acanthosis scores were slightly higher (albeit nonsignificantly) for the HAC strain compared with the other 2 strains (Table 1). Altogether our findings suggest that all 3 *C. bovis* strains we evaluated here should be considered pathogenic.

Our study identifies HAC as the predominant *C. bovis* strain of laboratory mice in North America. Sequences matching the ATCC 7715 strain of bovine origin were not detected among the 71 samples from North American laboratory mice that were positive for *C. bovis*, indicating host-associated differentiation among *C. bovis* strains. In our current study, several biochemical differences and polymorphisms in *rpoB* were noted among the *C. bovis* strains isolated from mice (HAC and NHAC) and the strain of bovine origin (ATCC 7715). Biochemical differences in hyperkeratosis-associated *Corynebacterium* spp. isolated from mice and *C. bovis* of bovine origin have been noted previously,[7] and future phylogenetic studies might substantiate strain designations indicative of the host species for various *C. bovis* isolates.

In conclusion, we here demonstrate that a real-time qPCR assay targeting *16S rRNA* or *rpoB* is a sensitive, accurate, and rapid method for the detection of *C. bovis*. A better understanding of the *C. bovis* strains detected on laboratory mice and the associated disease are needed to develop guidelines for containment and to generate effective methods to eradicate this agent from mouse facilities.

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References

1. **Besselsen DG, Romero-Aleshire MJ, Munger SJ, Marcus EC, Henderson KS, Wagner AM.** 2008. Embryo transfer rederivation of C.B-17/Icr-Prkdc(scid) mice experimentally infected with mouse parvovirus 1. Comp Med **58:**353–359.
2. **Brooks BW, Barnum DA.** 1984. Characterization of strains of *Corynebacterium bovis.* Can J Comp Med **48:**230–232.
3. **Burr HN, Lipman NS, White JR, Zheng J, Wolf FR.** 2011. Strategies to prevent, treat, and provoke *Corynebacterium*-associated hyperkeratosis in athymic nude mice. J Am Assoc Lab Anim Sci **50:**378–388.
4. **Chiller K, Selkin BA, Murakawa GJ.** 2001. Skin microflora and bacterial infections of the skin. J Investig Dermatol Symp Proc **6:**170–174.
5. **Clifford CB, Walton BJ, Reed TH, Coyle MB, White WJ, Amyx HL.** 1995. Hyperkeratosis in athymic nude mice caused by a coryneform bacterium: microbiology, transmission, clinical signs, and pathology. Lab Anim Sci **45:**131–139.
6. **Coyle MB, Lipsky BA.** 1990. Coryneform bacteria in infectious diseases: clinical and laboratory aspects. Clin Microbiol Rev **3:**227–246.
7. **Cynthia Besch-Williford C, Franklin CL.** 2007. Aerobic gram-positive organisms. In: Fox JG, Davisson MT, Quimby FW, Barthold

SW, Newcomer CE, Smith AL, editors. The mouse in biomedical research. New York (NY): Academic Press.

8. **Dole VS, Banu LA, Fister RD, Nicklas W, Henderson KS.** 2010. Assessment of *rpoB* and 16S rRNA genes as targets for PCR-based identification of *Pasteurella pneumotropica.* Comp Med **60:**427–435.
9. **Duga S, Gobbi A, Asselta R, Crippa L, Tenchini ML, Simonic T, Scanziani E.** 1998. Analysis of the 16S rRNA gene sequence of the coryneform bacterium associated with hyperkeratotic dermatitis of athymic nude mice and development of a PCR-based detection assay. Mol Cell Probes **12:**191–199.
10. **Field K, Greenstein G, Smith M, Herrman S, Gizzi J.** 1995. Hyperkeratosis-associated coryneform in athymic nude mice. Lab Anim Sci **45:**469.
11. **Huxley JN, Helps CR, Bradley AJ.** 2004. Identification of *Corynebacterium bovis* by endonuclease restriction analysis of the 16S rRNA gene sequence 1. J Dairy Sci **87:**38–45.
12. **Khamis A, Raoult D, La SB.** 2004. *rpoB* gene sequencing for identification of *Corynebacterium* species. J Clin Microbiol **42:**3925–3931.
13. **Militzer K.** 2001. Hair growth pattern in nude mice. Cells Tissues Organs **168:**285–294.
14. **Pascual C, Lawson PA, Farrow JA, Gimenez MN, Collins MD.** 1995. Phylogenetic analysis of the genus *Corynebacterium* based on 16S rRNA gene sequences. Int J Syst Bacteriol **45:**724–728.
15. **Potten CS, Saffhill R, Maibach HI.** 1987. Measurement of the transit time for cells through the epidermis and stratum corneum of the mouse and guinea pig. Cell Tissue Kinet **20:**461–472.
16. **Scanziani E, Gobbi A, Crippa L, Giusti AM, Giavazzi R, Cavalletti E, Luini M.** 1997. Outbreaks of hyperkeratotic dermatitis of athymic nude mice in northern Italy. Lab Anim **31:**206–211.
17. **Scanziani E, Gobbi A, Crippa L, Giusti AM, Pesenti E, Cavalletti E, Luini M.** 1998. Hyperkeratosis-associated coryneform infection in severe combined immunodeficient mice. Lab Anim **32:**330–336.
18. **Tavakkol Z, Samuelson D, deLancey PE, Underwood RA, Usui ML, Costerton JW, James GA, Olerud JE, Fleckman P.** 2010. Resident bacterial flora in the skin of C57BL/6 mice housed under SPF conditions. J Am Assoc Lab Anim Sci **49:**588–591.
19. **Watts JL, Lowery DE, Teel JF, Rossbach S.** 2000. Identification of *Corynebacterium bovis* and other coryneforms isolated from bovine mammary glands. J Dairy Sci **83:**2373–2379.