

Pilot Immunization of Mice Infected With an Equine Strain of *Corynebacterium pseudotuberculosis*

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CLINICAL RELEVANCE

This pilot study evaluated protection of an equine autogenous bacterin–toxoid vaccine against *Corynebacterium pseudotuberculosis* infection. Twenty-four BALB/c mice were inoculated with two doses of bacterin–toxoid vaccine or two injections of a placebo. Clinical, microbiologic, and pathologic outcomes were assessed after intradermal infection with one of two equine-origin *C. pseudotuberculosis* strains. Mice receiving bacterin–toxoid from fast-growing *C. pseudotuberculosis* showed significant protection from challenge infection, as evidenced by a higher survival rate, fewer gross and histopathologic lesions, and lower bacterial levels on culture. Successful protection via a vaccine against equine internal abscesses might provide supplementary management options against an important, potentially fatal disease.

INTRODUCTION

Corynebacterium pseudotuberculosis is a gram-positive, facultatively intracellular bacterial pathogen that can infect horses, cattle, sheep, goats, and, occasionally, humans. Infection

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caused by *C. pseudotuberculosis* can be characterized by deep intramuscular abscesses in the pectoral or ventral abdomen of horses; abscesses of internal organs, including the liver, kidneys, or spleen; or infection of limb lymphatics. In sheep and goats, the disease is called *caseous lymphadenitis*, whereas in horses it is referred to as *dryland distemper* or *pigeon fever*, reflecting its

prevalence in arid regions of the western United States and the swelling of the horse's pectoral region, resembling a pigeon's breast. Recently, there has been an increase in the reported incidence of the disease in horses in northern and southern California, with the appearance of putatively more virulent strains in southern California.¹ Cases of the disease have been reported in other states, including Kentucky, Colorado, Utah, Montana, and Washington.¹ At present, there are no documented control measures for preventing *C. pseudotuberculosis* infection in horses or eliminating or neutralizing this soil-dwelling organism. Toxoids are available for small ruminants in some countries (Caseous D-T, Colorado Serum Co., Denver, CO; Glanvac 6, Pfizer Animal Health, Australia), but no equine products are available. In a recent study of several vaccines, immunization of sheep with a formalin-killed bacterin combined with toxoid resulted in significant protection against experimental challenge compared with commercial toxoid alone.^{2,3}

Extensive study of host-bacteria interactions in naturally infected hosts such as horses is difficult because of the underlying genetic variability among individuals, the expense, and the requirements for multiple replicates and controls. Guinea pigs have been used as models for caseous lymphadenitis, but they die quickly of fatal systemic disease.⁴ Mice have served as models for testing vaccine efficacy for many diseases^{5,6} and provide a convenient model for distinguishing the virulence of fast- and slow-growing equine-origin strains of *C. pseudotuberculosis*.^{7,8} Nieto et al⁸ determined that multiple internal organs (liver, lung, spleen) were reliably infected in C3H/HeJ mice inoculated intradermally with equine-origin *C. pseudotuberculosis* strains. The present study was a pilot that used this mouse model to evaluate the protection of a formalin-killed bacterin-toxoid vaccine derived from a virulent equine strain.

■ MATERIALS AND METHODS

Bacterial Culture and Vaccine Development

Two strains of equine-origin *C. pseudotuberculosis*, designated 107 and 89, were chosen for challenge of experimentally immunized mice. Isolate 89 is a fast-growing strain, and 107 is a slow-growing one. Isolates were previously confirmed to be *C. pseudotuberculosis* based on being gram positive with typical "coryneform" morphology and forming white, variably hemolytic, catalase-positive colonies on blood agar. Reduction of nitrate was demonstrated with nitrate broth. Bacteria were maintained at -70°C on Microbank beads (Pro-Lab Diagnostics, Austin, TX) and, when thawed, were plated onto 5% sheep blood agar and incubated for 48 hours at 37°C in air. Isolated colonies of the bacteria were transferred to brain-heart infusion broth plus Tween 20 (Roche Diagnostics GmbH, Mannheim, Germany) and incubated another 48 hours at 37°C. Bacteria were harvested by centrifugation, washed twice in saline, and then adjusted to an approximate density of 2×10^5 bacteria/mL in saline, based on quantitative Gram stain of 20 μ L of bacteria in brain-heart infusion. Simultaneously, a 20- μ L loop of bacteria in saline was plated on sheep blood agar and incubated for 48 hours to count colony-forming units (CFUs) and confirm the dose given. All mice received an aliquot from the same batch, which was cultured immediately before inoculation to confirm infectivity.

The *Corynebacterium* vaccine placebo used as the control was prepared with 10% aluminum hydroxide (minimal essential media suspended) and 0.001% formalin preservative. The pilot vaccine was an autogenous bacterin-toxoid adjuvanted with 10% aluminum hydroxide and prepared from the rapid-growing strain of *C. pseudotuberculosis* isolate 89 (PHL Associates, Davis, CA). The vaccine was devel-

oped from virulent strain 89 as a foundation to begin evaluating protection. It was hypothesized that the most virulent strain would show the most significant protection and that if heterologous protection developed, there would be no need to produce a vaccine from strain 107. The vaccine contained a formalin-inactivated culture and supernatant containing bacterial cells and exotoxins. Bacterial cells were grown to late-log phase in Minimal Essential Media. Formalin was added to the late-log phase culture and supernatant, and the preparation was adjuvanted with aluminum hydroxide. The product was then pH neutralized and safety and purity tested by the thioglycollate purity test (Poultry Health Laboratories, Davis, CA). The minimum number of CFUs was determined by optical density readings of at least 0.3 at 600 nm. The vaccine was safety tested in mice inoculated subcutaneously and monitored for 8 days. Efficacy of the vaccine was based on reduction in mortality.

Experimental Design

All animal work was performed under the oversight of the campus attending veterinarian and the University of California, Davis, Institutional Animal Care and Use Committee. Four separate treatment groups of five, and one group of four (group D), 6-week-old male BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used. Mice in two groups (A and B) were inoculated with 0.25 mL SC of the vaccine, and those in the three other groups (C, D, and E) were inoculated with 0.25 mL SC of the control (aluminum hydroxide adjuvant). At 10 days, all mice were respectively reinoculated with either the vaccine or the placebo. At 38 days after vaccination, all mice except those in group E (unchallenged control group) were anesthetized with ketamine (20 mg/kg SC) and xylazine (4 mg/kg SC) and challenged by intradermal (ID) inoc-

ulation with live *C. pseudotuberculosis* (0.1 mL) into the right pinna. ID inoculation was verified by the presence of a visible bleb of inoculum at the site. Mice in treatment groups A and C were inoculated with the fast-growing strain of *C. pseudotuberculosis* (isolate 89), and those in groups B and D were inoculated with the slow-growing strain of *C. pseudotuberculosis* (isolate 107). The SC route, common for vaccination, is well tolerated in horses; ID inoculation replicates natural arthropod-borne infection. Animals were evaluated daily for activity, feeding, body condition, grooming, and alertness.

Mice that developed visible lesions at the injection site after challenge and those that appeared abnormally lethargic or ataxic were euthanized with an overdose of ketamine and xylazine followed by exsanguination via cardiac puncture and cervical dislocation. Day 16 after challenge (54 days after the first vaccination) was designated as the end of the experimental observation period. All remaining mice were euthanized, and full necropsies were conducted.

At necropsy, gross lesions were recorded as present or absent on the liver, spleen, lungs, and kidneys. If lesions were present, they were given a subjective score approximating the percentage of the organ that was grossly affected. Portions of the lungs, spleen, liver, and kidneys were then harvested and placed into 10% formalin, embedded in paraffin, thin-sectioned onto glass slides, and stained with hematoxylin and eosin. Sections were examined by a pathologist, who was blinded to the treatment groups, for presence of bacteria, architectural and cellular changes, inflammatory infiltrates, necrosis, and evidence of vasculitis.

Also at necropsy, each tissue and blood sample was swabbed with a sterile bacterial loop. Material from the loop was plated semiquantitatively on 5% sheep blood agar and incubated for 4 days at 37°C. *C. pseudotuberculosis* was

presumptively identified as a pure-culture white bacterial colony that maintained structure when pushed with a sterile loop, was gram positive, and had coryneform morphology on microscopy. Each plate was given a score of 1 to 4 according to the number of quadrants with colonies present after 4 days. CFUs were quantitated for blood cultures but were not quantitated for tissue cultures.

Data Analysis

Data were maintained in an Excel spreadsheet (Microsoft, Redmond, WA) and analyzed in R (R-Development Core Team, www.r-project.org). Results of statistical tests were considered significant if $P \leq .05$. Kaplan-Meier survival curves, plotting differences in survival curves between treatment groups, were assessed using log-rank testing. Differences in bacterial culture between treatment groups were analyzed using a Kruskal-Wallis test for nonparametric data.

RESULTS

Among the groups of mice that received vaccine or placebo for equine-origin *C. pseudotuberculosis*, there were differences in survival. Of the 19 mice challenged with *C. pseudotuberculosis*, five survived the entire study (16 days after challenge). Of these five mice, four were from treatment group A (80% survival) and one was from group D, which had four mice (25% survival). All five unchallenged mice in group E survived, whereas all mice in groups B and C died after challenge infection. Animals in treatment group B were moribund on days 11 and 12 and were euthanized. In treatment group C, one mouse was found dead on day 7; the remaining four mice were moribund and euthanized. In group D, one mouse was euthanized on day 9 and two on day 13. There were statistically significant differences in the survival of vaccinated versus unvaccinated

mice after challenge infection (Figure 1: groups A and B versus groups C and D; $P = .01$). (However, all the mice in group B died, which indicates that their survival was no better than the mice in group C that received a placebo.)

Gross lesions were present at the inoculation site in 12 of 20 challenged mice, including all those in group B, three in each of groups C and D, and one in group A. The lesions were not the result of SC versus ID inoculation, as all ID inoculations were verified by a visible bleb of inoculum at the site. There were obvious gross lesions in one or more internal organs of seven mice at necropsy, including mice from groups A, B, C, and D (Table 1). The liver was most commonly affected. Multiple discrete white foci were scattered throughout the parenchyma and over the capsular surface of the affected organs, affecting as much as one-half to two-thirds of the kidneys and less than half of the liver. None of the four group A mice that survived the study had dermal lesions at the inoculation site or any visible lesions in tissues. There were no lesions, gross or histopathologic, in the liver, lungs, or kidneys of group E mice.

The presence and severity of histopathologic lesions in the mice directly mirrored the gross findings. Four of the five mice in group A had no histologic lesions, whereas the fifth had extensive multicentric necropurulent hepatitis with coagulation necrosis and intralesional bacterial colonies and vasculitis, as well as the formation of discrete abscesses. There were no histologic lesions in one mouse from group B, but the other four mice had mild to moderate multifocal acute to subacute necropurulent hepatitis; there was a discrete abscess in the liver of one of these mice. Lung lesions in group B mice varied from mild acute embolic pneumonia to moderate and severe thromboembolic multifocal necropurulent pneumonia. Le-

sions in the group C mice showed more characteristics of acute disease than those in the mice in the other treatment groups, consistent with the very rapid course of disease in these mice. Although there were no obvious histologic lesions in the group C mouse that was found dead, this mouse was the only one in the study to score a 4 in all tissues cultured and the only one to score a 4 on blood culture. Two others had moderate multicentric necropurulent hepatitis, and one had moderate to marked multifocal hepatic coagulation necrosis with acute Kupffer cell hypertrophy. Two mice in group D also had no obvious histologic lesions, but two others had disseminated multifocal necropurulent hepatitis with obvious vascular thrombosis. One mouse in group D had necropurulent pneumonia with vascular thrombi and interstitial necropurulent nephritis.

Cultures revealed the presence of *C. pseudotuberculosis* in one or more tissues swabbed in 12 of 24 mice (Table 1). The yield of bacterial culture was significantly lower in treatment groups that received the pilot vaccine than in those that did not ($P = .007$). One group A mouse, which also had liver lesions, had positive cultures from liver and lung, both of which scored a 4, and from blood, with a score of 1, whereas all liver cultures from group C mice scored 3 or 4. All tissue cultures from the four surviving mice in group A were negative. A mouse in treatment group B with visible lung and liver lesions had no cultured bacteria. However, one other group B mouse with no visible gross tissue or histopathologic lesions had positive bacterial cultures with a score of 4. In group D, bacterial cultures were obtained

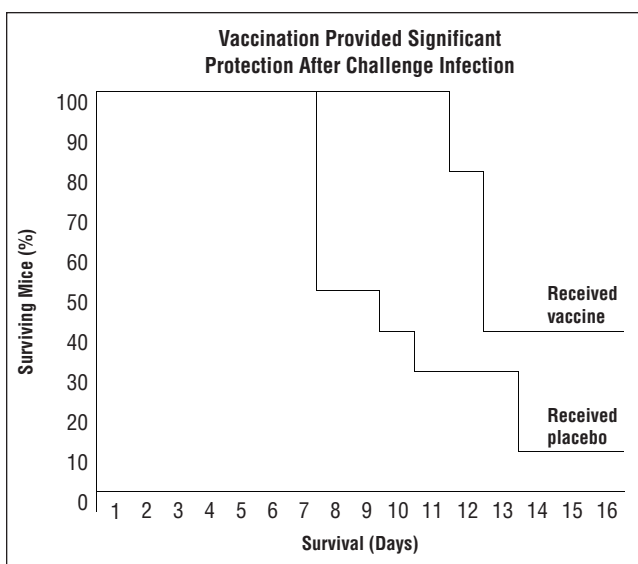


Figure 1. Comparison of survival curves of mice receiving a pilot *Corynebacterium pseudotuberculosis* vaccine and those receiving placebo ($P = .01$).

from three mice, two with lesions and one without. All tissue and blood cultures from mice in group E were negative.

DISCUSSION

This is the first report of a vaccine developed against an equine-origin strain of *C. pseudotuberculosis*. Significant protection was seen in mice given a bacterin-toxoid aluminum adjuvant pilot vaccine made from fast-growing equine-origin *C. pseudotuberculosis* followed by challenge. These findings were supported by a significant difference in survival rates among the mice until the end of the study, the absence of gross and histopathologic changes, and lower levels of bacteria on culture. Mice that did not receive the pilot vaccine and were inoculated with slow- and fast-growing *C. pseudotuberculosis* had lower survival rates and developed lesions similar to those described previously in a pathogenetic model of equine disease.⁸ It is interesting that mice in treatment

TABLE 1. Summary of Gross Lesion Severity, Histopathologic Lesion Findings, and Culture Scores in Mice Receiving an Experimental Vaccine/Bacterium of *Corynebacterium pseudotuberculosis* Challenge Ending on Day 16 After Inoculation

| Group* | Day of Death After Challenge | Severity of Gross Lesions [†] | | | Histopathology (Positivity/Negativity) | | | Culture Score | | |
|--------|------------------------------|--|----------|----------|--|------|--------|---------------|------|-------|
| | | Liver | Lung | Kidney | Liver | Lung | Spleen | Liver | Lung | Blood |
| A | 11 | Moderate | 0 | 0 | + | - | + | 4 | 4 | 1 |
| A | 16 | 0 | 0 | 0 | - | - | - | — | — | — |
| A | 16 | 0 | 0 | 0 | - | - | - | — | — | — |
| A | 16 | 0 | 0 | 0 | - | - | - | — | — | — |
| A | 16 | 0 | 0 | 0 | - | - | - | — | — | — |
| B | 11 | Mild | 0 | 0 | - | - | + | 4 | 4 | — |
| B | 12 | 0 | 0 | 0 | - | - | - | 4 | 4 | — |
| B | 12 | 0 | 0 | 0 | + | + | - | — | 1 | — |
| B | 12 | 0 | 0 | 0 | + | + | - | — | — | — |
| B | 12 | Moderate | Mild | 0 | + | + | - | — | — | — |
| C | 7 | 0 | 0 | 0 | + | + | - | 3 | 1 | — |
| C | 7 | Moderate | Moderate | 0 | + | + | - | 4 | 2 | — |
| C | 7 | Mild | 0 | 0 | + | + | - | 4 | 3 | — |
| C | 7 | 0 | 0 | 0 | + | + | - | 3 | 1 | — |
| C | 7 | 0 | 0 | 0 | + | + | - | 4 | 4 | 4 |
| D | 9 | Mild | 0 | Severe | + | + | - | 1 | 3 | 1 |
| D | 13 | 0 | 0 | Moderate | + | + | - | 2 | 4 | 2 |
| D | 13 | 0 | 0 | 0 | - | - | - | — | — | — |
| D | 16 | 0 | 0 | 0 | - | - | - | 2 | 1 | — |

*Group designations: A = vaccine, fast-growing strain challenge; B = vaccine, slow-growing strain challenge; C = placebo, fast-growing strain challenge; D = placebo, slow-growing strain challenge.

[†]0 indicates that no lesions were found.

group B, which were vaccinated but received heterologous challenge with the slow-growing strain, were not as well protected as mice in group A, suggesting a lack of heterologous protection. Moreover, a single mouse in group A appeared to experience vaccine failure, based on lesion development and positive bacterial culture in blood, liver, and lungs. These data

suggest that the crude bacterin provides considerable protection but that vaccine failures may occur. Possible reasons for this observation may be differences in host susceptibility to the bacterium, either innate or as the result of environmental stressors; slight differences in the vaccine or challenge (although both the vaccine and challenge were from homogeneous

batches divided among the mice receiving them); or idiosyncratic differences in early immunopathogenic mechanisms, such as in the dermis following challenge. It also is interesting that one mouse in group D (no vaccination, slow-growing challenge) showed no gross or histopathologic lesions, yet had a positive culture. It is possible that if the animal had been observed longer, disease might have developed and eventually progressed to other organs.

Most vaccination studies with virulent *C. pseudotuberculosis* challenge have been conducted in sheep and goats using a variety of strategies, including vaccination with purified phospholipase D toxin (PLD), formalin-inactivated PLD, purified recombinant PLD, combined bacterin–toxoid vaccines, and live vaccines.^{9–13} When PLD was used alone in vaccinating sheep, its efficacy was lower than when it was combined with other cellular components; specifically, minor vaccine failure occurred after vaccination with PLD alone, with development of small, nonprogressing lesions.¹² In naturally infected animals, *C. pseudotuberculosis* is resolved by cellular immunity, and PLD alone does not stimulate cellular immunity.¹⁴ The vaccine used in this study used a combined bacterin and toxoid approach.

In a previous study of equine-origin *C. pseudotuberculosis* in a C3H/HeJ mouse model, high virulence of bacteria was related to a fast growth rate in culture and quicker death in mice.⁸ In that study, lesions resembled an acute pathologic manifestation of disease, with high mortality and lesions primarily in the dermis and liver. In the present study, which used a BALB/c model, in mice not protected by the vaccine, lesions appeared more similar to the internal abscesses seen in horses, with lesions not restricted either grossly or histologically to the dermis or liver, but also seen in the lungs, kidneys, and occasionally spleen. Uniquely,

the kidneys of two unvaccinated mice infected with the slow-growing *C. pseudotuberculosis* strain were severely affected, whereas only one of these mice also had grossly visible lesions in the liver. In some mice with no gross lesions, histopathology revealed lesions and bacterial culture results were positive. The lesions in mice from this study were either encapsulated or in the process of forming capsules similar to those seen in horses. This finding is evidence of the differences in virulence between fast- and slow-growing strains seen in the mortality rate and lesion development process between mice in groups C and D.

The difference in disease in groups A and B is an interesting finding that may be the effect of a small sample size or a result of the fact that group B received a heterologous challenge. Strains used in this study (107 and 89) and in previous studies are genetically distinct, with different restriction fragment length polymorphism patterns that correspond to growth rates.¹ In particular, the slow-growing strain had a unique phenotype that was unusual for the strains obtained in our diagnostic laboratory. Nevertheless, further research should be directed at determining how broadly efficacious particular vaccines are against all the strains that occur in nature. As a pilot study, further safety and purity testing will be required as well as antibody analysis. Other means to improve vaccine efficacy might be the use of different, potentially more effective adjuvants or optimization of vaccine dosage and dosing frequency. Finally, the efficacy of this vaccine approach may be optimized in the mouse model but will require extensive testing in horses. Vaccination against and management of *C. pseudotuberculosis*–associated internal abscesses in horses are critically important targets for medical care in this species and require a better understanding of effective immune responses, including innate and adaptive immunity, cy-

tokine responses, and specific responses to *C. pseudotuberculosis* virulence factors.

CONCLUSION

Significant protection was seen in mice given a bacterin–toxoid aluminum adjuvant pilot vaccine developed against a fast-growing *C. pseudotuberculosis* strain followed by challenge. These are significant findings supported by differences in the survival of mice until the end of the study, the absence of gross and histopathologic changes, and lower levels of bacteria on culture. Mice that did not receive the pilot vaccine and were inoculated with slow- and fast-growing *C. pseudotuberculosis* had lower survival rates and developed lesions similar to those described previously in a pathogenetic model of equine disease.⁸ Data from one mouse in the study suggest that a vaccine failure may have occurred; thus, further studies are needed to determine vaccine efficacy, reduce potential vaccine failure, and eventually develop vaccine testing in a horse model.

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REFERENCES

1. Foley J, Spier SJ, Mihalyi J, et al. Molecular epidemiology of *Corynebacterium pseudotuberculosis* isolates from equine epidemics in four North American states. *Am J Vet Res* 2004;65:1734-1737.
2. Baird G, Fontaine M. *Corynebacterium pseudotuberculosis* and its role in ovine caseous lymphadenitis. *J Comp Pathol* 2007;137:179-210.
3. Fontaine M, Baird G, Connor K, et al. Vaccination confers significant protection of sheep against infection with a virulent United Kingdom strain of *Corynebacterium pseudotuberculosis*. *Vaccine* 2006;24:5986-5996.
4. Dickinson C, Bull L. Studies on infection by and resistance to the Prisesz-Nocard bacillus 2. Susceptibility of the guinea pig and the distribution of lesions after cutaneous and subcutaneous inoculation and ingestion. *Aust J Exp Biol Med Sci* 1931;8:83-90.
5. Cameron C, Minnaar J. Immunization of mice against *Corynebacterium pseudotuberculosis* infection. *Onderstepoort J Vet Res* 1969;36:207-210.
6. Simmons CP, Hodgson AL, Strugnell RA. Attenuation and vaccine potential of aroQ mutants of *Corynebacterium pseudotuberculosis*. *Infect Immunol* 1997;65:3048-3056.
7. Batey RG. Aspects of pathogenesis in a mouse model of infection by *Corynebacterium pseudotuberculosis*. *Aust J Exp Biol Med Sci* 1986;64(Pt 3):237-249.
8. Nieto N, Foley J, Yuan T, et al. A mouse model of internal abscesses associated with equine-origin *Corynebacterium pseudotuberculosis*. *Am J Vet Res* 2009;70:257-262.
9. Brown CC, Olander HJ. Caseous lymphadenitis of goats and sheep: a review. *Vet Bull* 1987;57:1-12.
10. Eggleton D, Doidge C, Middleton H, et al. Immunisation against ovine caseous lymphadenitis: efficacy of monocomponent *Corynebacterium pseudotuberculosis* toxoid vaccine and combined clostridial-corynebacterial vaccines. *Aust Vet J* 1991;68:320-321.
11. Eggleton D, Haynes J, Middleton H, et al. Immunisation against ovine caseous lymphadenitis: correlation between *Corynebacterium pseudotuberculosis* toxoid content and protective efficacy in combined clostridial-corynebacterial vaccines. *Aust Vet J* 1991;68:322-325.
12. Eggleton D, Middleton H, Doidge C, et al. Immunisation against ovine caseous lymphadenitis: comparison of *Corynebacterium pseudotuberculosis* vaccines with and without bacterial cells. *Aust Vet J* 1991;68:317-318.
13. Pepin M, Pardon P, Marley J, et al. Acquired immunity after primary caseous lymphadenitis in sheep. *Am J Vet Res* 1993;54:873-877.
14. Hodgson A, Carter K, Tachedjian M, et al. Efficacy of an ovine caseous lymphadenitis vaccine formulated using a genetically inactive form of the *Corynebacterium pseudotuberculosis* phospholipase D. *Vaccine* 1999;17:802-808.