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Original Research

Humane Endpoints for Guinea Pigs Used for *Mycobacterium tuberculosis* Vaccine Research

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Guinea pigs are a commonly used model for tuberculosis vaccine research. Loss of body weight is the most frequently described humane endpoint for animals used in these studies. During a chronic study, we noted labored breathing in some tuberculosis-infected guinea pigs. To develop consistent humane endpoints for these guinea pigs, we performed an observational study using multiple clinical signs. A combination of body weight loss, labored breathing, and activity level during handling estimated the time to euthanasia within approximately 7 d. Histologic severity scores of lesions in the cranial or caudal lung lobe (or both) supported clinical endpoints. This study presents humane endpoints for the refinement of studies using guinea pigs in tuberculosis research.

In 2015, tuberculosis resulted in the death of 1.8 million people, making it 1 of the top 10 causes of death worldwide.[18] Tuberculosis is caused by the bacillus *Mycobacterium tuberculosis*, which can affect the lungs and other sites of the body (extrapulmonary tuberculosis). The disease is transmitted through aerosols produced when persons infected with pulmonary tuberculosis cough, sneeze, or spit.[18] An estimated one-third of the world's population has latent tuberculosis, and those that develop the active disease are at high risk of death if not treated. Efforts to contain and eradicate tuberculosis have been seriously hampered by coinfection with HIV as well as resistance to drugs commonly used to treat tuberculosis. Importantly, an estimated 480,000 cases of multidrug-resistant tuberculosis occurred in 2014.[18] Traditionally used drugs such as isoniazid, which remains a first line-treatment, have been only partially effective in treating pulmonary tuberculosis.[1] Vaccination with Bacille Calmette–Guerin is only partially effective for infants and is largely ineffective against adult pulmonary tuberculosis.[18] The Bacille Calmette–Guerin vaccine cannot be used in children with vertical infections of HIV, due to high risk of vaccine reaction.[18] Therefore, new diagnostic tests, drugs, and vaccines are needed to curb the dissemination of tuberculosis worldwide.

Although other animal models are used to model tuberculosis infection, guinea pigs are the 'gold standard' for testing the efficacy of tuberculosis vaccines.[8,10] The pathogenesis of disease, pathologic lesions, and response to Bacille Calmette–Guerin vaccination are similar to those described in humans.[2,14] Even though guinea pigs have been used as tuberculosis models for almost 100 y, little published literature describes humane endpoints for these studies. Weight loss between 15% to 20% is the most commonly described endpoint to evaluate disease progress in guinea pigs and other species.[4,8,17] In our first trial with these animals, we noted that some guinea pigs developed dyspnea (demonstrated by abdominal breathing) when maintained for more than 12 wk after tuberculosis challenge. Anticipating that dyspnea might be a more sensitive indicator of pulmonary disease, we consulted with the investigative group and together elected to use dyspnea as compared with weight loss as a humane endpoint. However, soon after implementing this endpoint, investigative staff became concerned about its validity as a humane endpoint—that is, might some animals be euthanized prematurely unnecessarily? Because both investigative and animal care staff had concerns regarding the validity of either humane endpoint (weight loss or dyspnea) used alone, we created a score sheet to improve animal monitoring and to help us determine relevant humane endpoints for this model. Here, we describe an observational study using multiple clinical signs to determine humane endpoints for guinea pigs used in tuberculosis vaccine research.

Materials and Methods

Animals. Female Dunkin–Hartley guinea pigs from Charles River Laboratories (Senneville, Quebec, Canada; $n = 16$; weight, 250 to 300 g) were observed. Guinea pigs were maintained according to guidelines from the Canadian Council on Animal Care and statutes of the Ontario Animals for Research Act.[3,13] Procedures for the study were reviewed and approved by the University of Toronto's Faculties of Medicine and Pharmacy Animal Care Committee.

The guinea pigs were considered free of Sendai virus, pneumonia virus of mice, reovirus, lymphocytic choriomeningitis virus, guinea pig adenovirus, *Bordetella bronchiseptica*, *Helicobacter* spp., *Klebsiella oxytoca*, *K. pneumoniae*, *Mycoplasma pulmonis*, *Pasteurella multocida*, *P. pneumotropica*, *Salmonella* spp., *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *S. zooepidemicus*, β-hemolytic *Streptococcus* spp., *Clostridium piliforme*, *Encephalitozoon cuniculi*, and endo- and ectoparasites, as detailed by vendor health reports.

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On arrival, before exposure to *M. tuberculosis*, guinea pigs were pair-housed on corncob bedding (The Andersons, Maumee, OH) in individually ventilated CL3 caging (Allentown Caging, Allentown, PA) in a CL2 room and maintained on a 14:10-h light:dark cycle at a temperature of 20 to 21 °C. Autoclaved, reverse-osmosis–purified, acidified water was provided in water bottles, and food (Teklad 2040 Guinea Pig Chow, Envigo, Indianapolis, IN) was irradiated prior to use. Enrichment included red shelters (BioServ, Flemington, NJ) and irradiated hay blocks. After 1 wk of acclimation, guinea pigs were vaccinated subcutaneously with 1 of 2 newly developed experimental vaccines or a sham vaccine. Each cage received either the same experimental vaccine or the sham. The animals were maintained in CL2 housing for 8 wk after vaccination; they then were moved to CL3 containment where they were challenged aerogenically with 1000 cfu of *M. tuberculosis* H37Rv by using an inhalation exposure system (Glas-Col, Terre Haute, IN). Housing conditions remained as described previously. Guinea pigs were weighed weekly until they had lost 10% body weight, after which they were weighed at least twice weekly; animals were weighed daily once 15% body weight loss was attained. For animals observed to be losing weight, additional food items, such as hay and sunflower seeds, were provided. Guinea pigs were maintained for as long as10 mo after challenge. They were euthanized by decapitation under isoflurane anesthesia. The lungs and spleens were collected to determine bacterial burden.

Clinical signs monitored. Veterinary technical and investigative staff discussed and developed a list of clinical signs to be monitored. These included general appearance, amount of eye-opening, breathing pattern, in-cage activity level, mucous membrane color, amount of alfalfa cube eaten, activity level during handling, and body weight loss. For each parameter, individual scores were assigned according to criteria listed in Figure 1. Each time investigative or veterinary technical staff entered the CL3 facility, animals were assessed and scored across all clinical signs. Animals were assessed in a similar order according to their position on the rack. Due to time and equipment constraints, observations were not conducted in parallel. Investigative staff was trained to recognize the various clinical signs by using photos and videos collected from previously observed guinea pigs. Veterinary staff—but not investigative staff—was blinded to the vaccine each guinea pig received. Animals were monitored and assessed daily in the morning.

Clinical signs monitored were selected from previous publications describing the development of endpoints for other species and surgical endpoints for guinea pigs.[6,12,15] Because we were attempting to determine appropriate endpoints, there was no a priori set point for euthanasia. When an animal was found to have marked weight loss, dyspnea, or other signs of distress, it was euthanized after discussion between veterinary and investigative staff. Because this study was observational only, all animals—regardless of the vaccines they received—were pooled.

After euthanasia, lung tissue was collected for determination bacterial burden and subsequently was formalin-fixed. Paraffin-embedded lung sections were stained with hematoxylin and eosin (Animal Health Laboratory, University of Guelph, Guelph, Ontario), and the slides of cranial or caudal lung lobe (or both) from each animal were evaluated by a pathologist blinded to animal treatment. Samples were scored according to the degree of pyogranulomatous inflammation and tissue consolidation: 0, none (normal); 1, minimal (less than 5% of section affected); 2, mild (5% to 10% of section affected); 3, moderate (>10% to 40% of section affected); and 4, marked (>40% of section affected).

Statistical analysis. The first day of each score for each clinical sign was determined, and then the number of days until euthanasia was calculated. Kaplan–Meier survival curves for each clinical sign and score were generated to indicate the probability of survival over time (GraphPad Prism, GraphPad Software, La Jolla, CA). For individual clinical signs, curves were compared by using the log-rank test followed by Bonferroni correction. For the combined score, significant differences between the survival curves for the scores of 4, 5, 6, and 7 were compared by using the log-rank test followed by Bonferroni correction. Correlation between combined survival scores and the number of colony-forming units in the lungs was tested by using the Spearman rank correlation coefficient.

Results

Clinical scores. All guinea pigs were euthanized 1 to 37 d after a clinical sign score was first observed. When evaluated independently, body weight loss, breathing pattern, and activity level during handling estimated the median time to euthanasia within 1 to 24 d, depending on the score (Figure 2). For body weight loss, score 3 was significantly different from both scores 1 ($P = 0.0056$) and 2 ($P = 0.0095$; Figure 2 A). The scores for breathing pattern were all significantly different from one another ($P = 0.003$; Figure 1 B), as were the scores for activity level during handling ($P = 0.0063$; Figure 2 C). None of the other clinical signs monitored better estimated time to euthanasia independently (data not shown). A combined score based on body weight loss, breathing pattern, and activity level during handling best discerned time to euthanasia, within a median time of 1 to 17 d (Figure 2 D). Guinea pigs with a score of 5 or greater had a median survival time of 7.5 d or less. When the survival curves were compared for the combined score, score 4 was significantly different from scores 6 ($P = 0.0014$) and 7 ($P = 0.0095$); scores 5, 6, and 7 did not differ significantly from one another. No other combinations of clinical signs monitored better estimated time to euthanasia than did that of weight loss, breathing pattern, and activity level during handling (data not shown). No animals were found dead over the course of this observational study.

Colony-forming units. There was no correlation ($r_s = –0.05$; $P = 0.8608$) between survival score and the number of colony-forming units present in the lungs at the time of euthanasia (Figure 3).

Histopathology. All sections demonstrated marked consolidation of tissues (that is, score of 4 out of 4), with approximate tissue involvement ranging from 50% to 100% (Figure 4). There was no correlation between histologic severity and number of colony-forming units or treatment or between cranial and caudal lung lobes. All sections demonstrated marked pyogranulomatous inflammation and consolidation, with marked type II pneumocyte hyperplasia. In addition, sections contained multifocal granulomas, consisting of dense aggregates of plump macrophages with lesser numbers of lymphocytes, plasma cells, and neutrophils surrounding a central core of necrotic cellular debris, which was occasionally mineralized. Patchy hemorrhage was noted in many sections, as were random multifocal multinucleated giant cells.

Clinical sign	Score	Description
General appearance		State of the animal's fur before cage is moved
	0	Normal smooth fur
	1	Ruffled fur < 25% of body (excluding head)
	2	Ruffled fur 25% to 50% of body
	3	Ruffled fur > 50% of body
Eye opening		Degree of eye opening before cage is moved
	0	100% open
	1	25% closed
	2	50% closed
	3	75% closed
Breathing		Breathing pattern before cage is moved
	0	No effort observed, normal
	1	Rapid breathing, no abdominal involvement
	2	Rapid, abdominal breathing
	3	Rapid, abdominal breathing with pronounced effort
In-cage activity level		Observe animal's movement when cage is picked up
	0	Animal moves around when cage is disturbed
	1	Animal moves around a bit, but quickly settles down
	3	Animal barely moves from its position
Mucous membrane color		
	0	Normal pink
	1	Pale white
	2	Blue or purple, cyanotic
Alfalfa appetite		Assess the amount of the alfalfa cube consumed
	0	Whole cube eaten
	1	> 50% cube eaten
	2	< 50% cube eaten
Activity during handling		Observe animal reaction to handling
	0	Animal struggles to escape
	1	Struggles at first, but quickly quiets
	2	Doesn't move in hand
Body weight loss		Compare current body weight compared with start weight
	0	0% to 5% loss
	1	5% to 10% loss
	2	10% to 15% loss
	3	15% to 20% loss

Figure 1. Clinical signs monitoring guide.

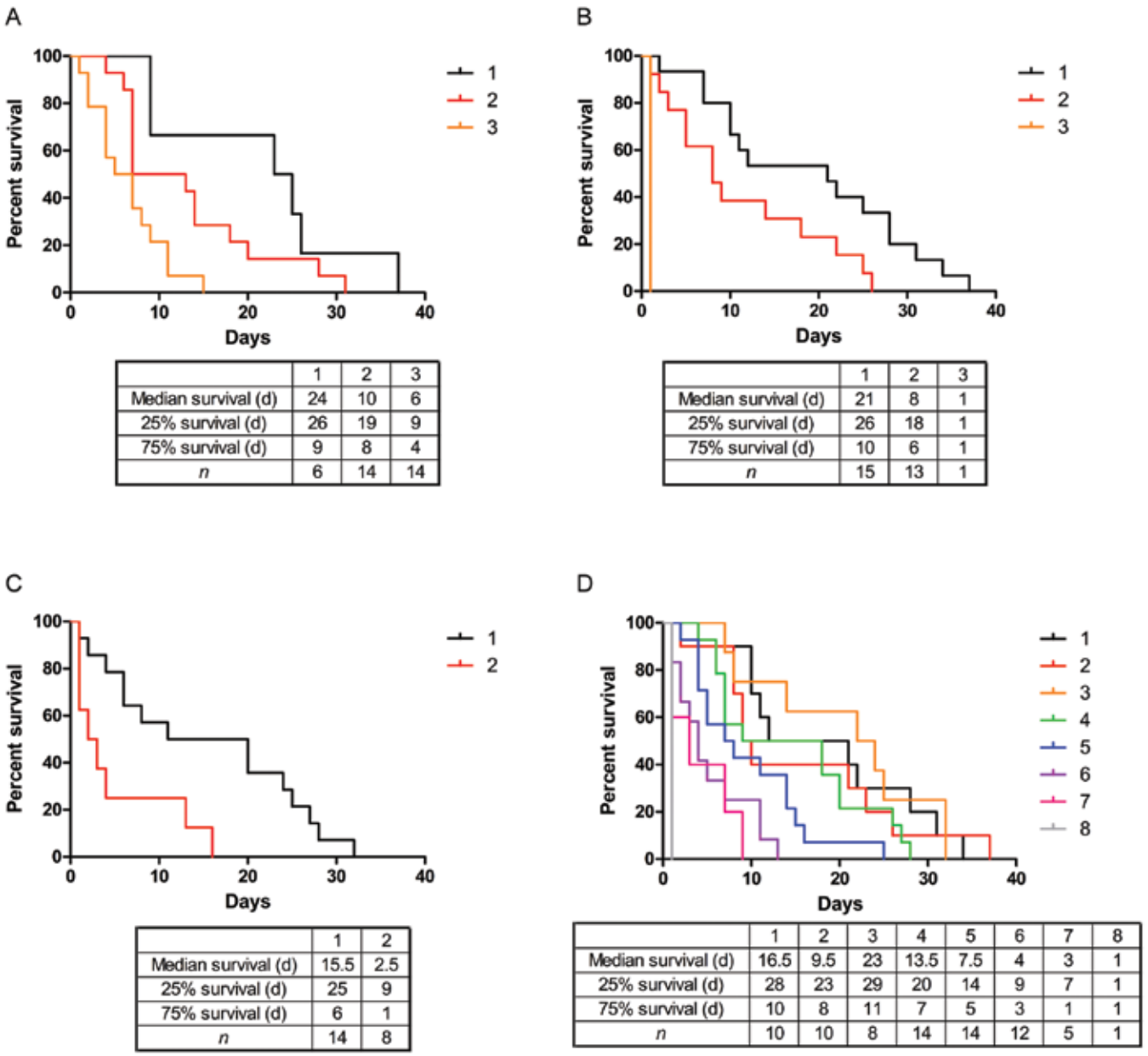
Discussion

The results of this study demonstrate that body weight loss, dyspnea, and activity level during handling can be used in combination to determine humane endpoints for guinea pigs used in tuberculosis vaccine research. Combined scores for these parameters more accurately predicted time to euthanasia than did any single parameter. Combined scores for these parameters predicted animals that required euthanasia within approximately 1 wk. Furthermore, according to our scoring matrix, animals with a combined score of 5 or greater warranted closer monitoring to implement humane euthanasia.

Weight loss of 15% to 20% has been the most commonly cited endpoint for tuberculosis studies. Recent publications have included dyspnea and inappetence as other endpoints;[15,16,19] but how these parameters were evaluated was not described. In our current study, body weight loss alone was not consistently the best indicator of time until euthanasia. Although guinea pigs with a score of 3 for body weight loss (indicating 15% to 20% weight loss) had a median survival time of 6 d, many animals survived much longer. This pattern suggests that measuring body weight loss alone may, in fact, preclude researchers from collecting more robust data for their studies because animals are being euthanized prematurely. Conversely, 1 of the 16 guinea pigs required euthanasia after losing only 12% body weight because it began to display signs of dyspnea. In this case, had only the weight loss endpoint been used, this animal might have died prior to euthanasia.

In comparison to body weight loss, animals with a score of 2 on breathing pattern (indicating rapid breathing) had a median survival time of 8 d. This score was used previously by veterinary staff as a humane endpoint. Given the lack of predictive value of this parameter when used alone, some guinea pigs euthanized after demonstrating rapid breathing might have been euthanized early. The one guinea pig that progressed to a score 3 (that is, rapid, abdominal breathing with pronounced effort) was euthanized the next day, because we associate this sign with imminent risk of sudden death.

Animals with a score of 2 on activity level during handling (indicating lack of movement during handling) had a



	1	2	3
Median survival (d)	24	10	6
25% survival (d)	26	19	9
75% survival (d)	9	8	4
n	6	14	14

	1	2	3
Median survival (d)	21	8	1
25% survival (d)	26	18	1
75% survival (d)	10	6	1
n	15	13	1

	1	2
Median survival (d)	15.5	2.5
25% survival (d)	25	9
75% survival (d)	6	1
n	14	8

	1	2	3	4	5	6	7	8
Median survival (d)	16.5	9.5	23	13.5	7.5	4	3	1
25% survival (d)	28	23	29	20	14	9	7	1
75% survival (d)	10	8	11	7	5	3	1	1
n	10	10	8	14	14	12	5	1

Figure 2. Kaplan–Meier survival curves for guinea pigs after reaching each score for body weight loss, breathing pattern, activity level during handling, and a combined score. (A) For body weight loss, score 3 was significantly different from both score 1 and 2 ($P = 0.0056$ and $P = 0.0095$, respectively). (B) The scores for breathing pattern all differed significantly ($P = 0.003$) from one another. (C) The scores for activity level during handling were significantly ($P = 0.0063$) different from each other. (D) Significant differences emerged between combined scores of 4 compared with 6 and 7 ($P = 0.0014$ and $P = 0.0095$, respectively). All survival curves compared by using the log-rank test followed by Bonferroni correction when multiple comparisons were performed.

median survival time of 2.5 d. Although everyone handling the guinea pigs noticed their general behavior previously, it had not been monitored in a systematic manner. The survival curve for a score of 2 in activity level during handling is very similar to the combined score curves of animals reaching scores of 6 or 7. This finding supports the use of multiple clinical parameters to monitor tuberculosis-challenged guinea pigs. We infer that difficulty breathing combined with a decreased ability to consume food leads to lethargy and reduced activity level during handling.

The presence of observers performing cageside assessments alters the behavior of several laboratory animal species.[7,9,11] The presence of an observer likely changed the behavior of guinea pigs in the current study as well. This effect may explain the failure of some parameters (such as general appearance, eye-opening, and in-cage activity level) to reliably predict discomfort in observed guinea pigs. Ill animals are more likely to conceal clinical signs in the presence of a human observer.[7] Eye opening as a component of facial grimace scoring in mice yields lower scores when observed in person as compared with in videos.[11] Activity levels in rabbits were lower in the presence of observers, which may be similar to what we observed in these guinea pigs.[9] Although the CL3 environment constrains the use of additional monitoring equipment, we might have noted early, subtle changes in behavior on videorecordings. Videorecordings might reveal

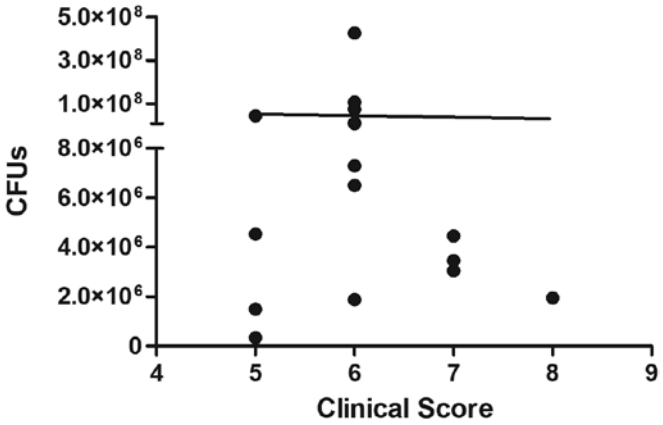


Figure 3. Correlation between the number of colony-forming units and clinical score at time of euthanasia.

subtle behavioral changes that can be used in ethograms to detect animal pain and discomfort.[7,9,11] Technical difficulties prevented us from using videorecording to supplement cageside observations during our study.

Previous work attempted to use change in consumption of preferred foods to detect signs of pain in guinea pigs.[6] The animals in the cited study consistently ate the preferred foods which made this proxy indicator a poor measure of pain. We similarly attempted to use alfalfa cubes to detect signs of discomfort in the study guinea pigs. Throughout our study, this marker likewise failed to clearly identify animals that might have been in distress. Change in consumption of preferred foods might not be a useful measure of pain or discomfort in guinea pigs.

Despite the pulmonary involvement during tuberculosis infection, mucous membrane color was not a sensitive indicator of the clinical state in our animals. This result might have occurred because pulmonary involvement was not sufficiently extensive to cause alterations in blood oxygenation levels. In addition, this change might have been subtle and therefore difficult to detect in affected animals. Although pulse oximetry might have accurately measured oxygenation levels, the CL3 environment made it very difficult to implement this tool.

At the conclusion of the observation period, we attempted to correlate bacterial burden in the lungs with clinical survival scores. These parameters showed no correlation, suggesting that clinical scoring for humane endpoint purposes cannot be used to predict bacterial burden. The goal of the research group was to generate survival curves for the vaccines tested—not to perform a bacterial burden assay. We hypothesized that clinical score would be indicative of bacterial burden, but this was not the case. The humane endpoints we described should not be confused with experimental endpoints for predicting bacterial burden.

Histopathologic examination of the cranial and caudal lung lobes revealed marked consolidation in all animals with a clinical score of 5 or higher. This finding further supports our clinical observations that animals with these scores should be monitored closely to implement euthanasia. In this study, a high dose of *M. tuberculosis* strain H37Rv was used. This dose produced a remarkably consistent model of pyogranulomatous inflammation and consolidation. Previous work suggests that, depending on the bacterial strain and dose, significant variability can be observed in clinical score, mortality, and histopathologic scores.[5] High tuberculin doses reduce this variability, thus reducing the number of animals required to generate statistically significant results. Because the dose and strain used in the current study did produce significant tissue damage, a reliable scoring system to determine humane endpoints was critical for this project.

Humane endpoints simultaneously support the generation of scientific data and minimize animal pain or distress. Developing a scoring system for the guinea pigs in this study had several advantages. First, animals were euthanized promptly, thus reducing any potential suffering they might have experienced. Second, the scoring system enabled investigative staff to be better prepared for euthanasia and tissue collection and be more efficient with their time in monitoring the guinea pigs. This benefit is not negligible in the CL3 context, where the risk for laboratory-acquired infection can be mitigated by reducing the window of exposure to risk group 3 pathogens. Space in CL3 is limited and in demand by other labs and therefore efficiencies realized in animal use can benefit other labs. In addition, conducting in vivo work under CL3 conditions is costly and technically demanding, and efficiencies in animal use can reduce these burdens. In addition to ethical benefits, euthanasia at humane endpoints averts the potential for loss of data by postmortem degradation of tissue by autolysis. Last, this scoring system can be applied to both vaccinated and nonvaccinated animals, thus ensuring consistent endpoints throughout a study.

We did not attempt to correlate interobserver scores in this study. All data collected were distributed equally among investigative and veterinary technical staff, due to the constraints on time and equipment within the CL3 space. Ideally, data would have been collected separately from veterinary technical and investigative staff and then compared between the 2 groups. Staff unfamiliar with normal guinea pig behavior and appearance might incorrectly classify or report changes in certain parameters. Therefore prior to starting the observational study, we presented the investigative staff with images and videos of the behaviors associated with each score. This tactic allowed us to address any questions and ensured a common language during the discussion of each case. Raw data for all guinea pigs did not reveal much variation between investigative and animal care staff.

Because this study did not use death as an endpoint, the results might be conservative. In addition, even with multiple clinical parameters, we might have euthanized some guinea pigs earlier than required for the experimental endpoints. However, we feel that the signs observed were clear, ethical indications for euthanasia and represent refinement in animal use for the collection of data relevant to vaccine efficacy.

Here we describe an observational study to help elucidate humane endpoints and thus refine the use of guinea pigs in tuberculosis vaccine research. A combination of body weight loss, breathing pattern, and activity level during handling enabled us to determine endpoints that resulted in timely euthanasia of guinea pigs. The clinical parameters used to develop the combined score are practical for the CL3 environment, applicable to the animal model, and relevant to the research under study. Groups working with guinea pigs as a model of tuberculosis can use these parameters to ensure timely euthanasia to prevent animal suffering, improve animal welfare, and ensure the prompt collection of tissues and data needed for research outcomes. Investigators are encouraged to fully describe the endpoints for their research in publications to ensure dissemination of refinements of this nature.

	Score prior to euthanasia				Histology	
Guinea pig	Combined	Body weight loss	Breathing pattern	Activity level	Lung lobe	Consolidation (%)
1	7	3	2	2	Cranial	100
					Caudal	80
2	6	2	2	2	Cranial	65
					Caudal	90
3	6	3	1	1	Cranial	70
4	6	3	1	2	Cranial	85
5	6	3	2	1	Cranial	65
					Caudal	90
6	7	3	2	2	Cranial	90
					Caudal	95
7	5	2	2	1	Cranial	100
8	7	3	2	2	Cranial	95
9	6	3	2	1	Cranial	70
					Caudal	80
10	5	3	1	1	Cranial	100
					Caudal	100
11	6	3	2	1	Cranial	100
12	5	3	2	0	Cranial	70
13	5	3	2	0	Cranial	100
14	7	3	2	2	Cranial	50
					Caudal	
15	8	3	3	2	Cranial	70
16	6	3	1	2	Cranial	100
					Caudal	100

Figure 4. Final clinical score at time of euthanasia and degree of lung consolidation observed on histopathology.

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