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Review

Experimental challenge models for Johne's disease: A review and proposed international guidelines

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Abstract

An international committee of Johne's disease (JD) researchers was convened to develop guidelines for JD challenge studies in multiple animal species. The intent was to develop and propose international standard guidelines for models based on animal

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species that would gain acceptance worldwide. Parameters essential for the development of long-term and short-term infection models were outlined and harmonized to provide a ''best fit'' JD challenge model for cattle, goats, sheep, cervids, and mice. These models will be useful to study host–pathogen interactions, host immunity at the local and systemic level, and for evaluating vaccine candidates and therapeutics. The consensus guidelines herein list by animal species strains of *Mycobacterium avium* subsp. *paratuberculosis* used, challenge dose, dose frequency, age of challenge, route of challenge, preparation of inoculum, experimental animal selection, quality control, minimal experimental endpoints and other parameters.

Keywords: Johne's disease; Paratuberculosis; Challenge; Experimental infection; Animal models; *Mycobacterium avium* subsp. *paratuberculosis*

Contents

1. Introduction

Johne's disease (JD) is widely distributed globally in domestic ruminants such as cattle, sheep, camelids, and goats. The infection has also been confirmed in farmed and free-ranging deer, antelope, elk, and bison. The herd and animal-level prevalence is unknown in most countries, but prevalence of infection is increasing in some countries that do not have mandatory control programs. In the US, economic losses in cattle result from early culling or death, reduced reproductive efficiency and feed efficiency, and decreased milk production (Ott et al., 1999; Johnson-Ifearulundu and Kaneene, 1999). The apparent increasing herd prevalence of JD in the past 10 years suggests that the recognized economic losses underestimate the current financial burden of this disease.

Animal challenge models are critical to evaluate potential vaccine candidates and to study host responses to infection. Virtually all researchers have developed JD challenge models independently of others, resulting in a high degree of variability due to use of different species of animals, dose, strain of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and dosing interval. Standardization is greatly needed to advance research in a timely and efficient manner. A similar conclusion regarding standardized challenge models for vaccine efficacy studies was reached in August 2005 at the International Colloquium for Paratuberculosis "Role of Vaccination" workshop, in Copenhagen, Denmark. Experimentation for development of animal models is costly, time consuming and inefficient. However, the historical literature and the experience of researchers in this area can be used to design a standard or "harmonized" model based upon expert consensus. This approach has the additional benefit of identifying knowledge gaps to be addressed by future research.

JD researchers from the US, EU, Australia, and New Zealand were convened as a committee to develop international standardized challenge models for cattle, sheep, goats, cervids and mice, under the sponsorship of the Johne's Disease Integrated Project (JDIP). The JDIP Animal Model Standardization Committee (AMSC) members consisted of 16 JD researchers, most of whom have successfully completed and published MAP challenge and/or vaccine efficacy studies in various species. Our consensus guidelines for models are described herein.

2. JD challenge model parameters

The AMSC formulated a list of critical components as guidelines for development of JD infection models. For each species, a consensus as to the best choice for each parameter was reached, based on a combination of published studies and committee members' experience.

2.1. Breed/strain of animal

Various strains of laboratory mice are susceptible to MAP infection (Chandler, 1961a,b,c,d). Variable breed or strain resistance to mycobacterial infection has been

well described in mice (see Section 7.1, murine models). Genetic resistance has also been described in cattle (Koets et al., 2000; Mortensen et al., 2004) and red deer (Mackintosh et al., 2000), but not in goats and sheep. Breed or strain of animal should be specified in the event that an associated resistance might exist.

2.2. Intended use of model

Short-term models to elucidate host-pathogen interactions may have different optimum parameters than long-term models evaluating the efficacy of vaccines or therapeutics. However, short-term vaccination-challenge models should reflect long-term realities. Parameters adequate for different types of studies and multiple endpoints were selected.

2.3. Challenge strain

High-passage laboratory-adapted strains may have reduced virulence *in vivo*. Well-characterized low-passage MAP strains of known virulence are recommended in all species models. MAP strains with high genetic similarity to strain K-10 (the sequenced strain; Li et al., 2005) are suggested for models in all species except sheep. Master stocks and/or seedstocks should be prepared and stored aliquoted in sufficient number to allow the experiments to be repeated over time in the same laboratory or elsewhere.

2.4. Method of challenge inoculum preparation

The primary route of exposure is considered to be via the fecal–oral route (Chiodini et al., 1984), so the method of administration most accurately mimicking natural infection is oral administration of feces from an infected animal. Other studies have used colonies scraped from solid medium, ileal scrapings or ground lymphoid tissue from infected animals.

However, methods of preparation that can be standardized from one laboratory to another are preferred. Ideally, this would involve production of log phase broth cultures from a master seedstock of strain K-10 or another strain of similar genotype. Organisms should be dispersed by passage through a 20-gauge hypodermic needle and then a 22-gauge needle or by vortexing with a small number of 3–4 mm glass beads. Use of Tween and sonication are not recommended until the effect of Tween (Van Boxtel et al., 1990a,b) and sonication on viability or virulence of are known.

2.5. Method of quantifying inoculum

A propensity to clump and slow growth in vitro makes quantification of MAP problematic. Ideally, dose should be reported as colony forming units (CFU) administered. The long incubation period on solid medium makes pre-determination of numbers of CFU by plate count impossible, and clumping complicates accuracy of plate counts. Quantification by ''wet weight'' of MAP harvested from broth culture or from solid medium has been used in many studies. An advantage of standardization by wet weight is that clumping should not affect results.

Determination of optical density (OD) of MAP suspensions is another approach. The OD540 of a MAP suspension is compared to a standard curve derived by plotting numbers of CFUs against OD. Microscopic counting of a smear of a diluted MAP culture is possible, but accuracy and inability to assess viability are problematic. Determining ''genomic equivalents'' and quantitative real-time PCR are also possible, but the plate count is the only method which distinguishes viable from non-viable organisms in the sample; others overestimate the number of infectious units in the sample.

Thus, MAP should be quantified using the ''pelleted wet weight method'' determined by centrifugation ($3000 \times g$) of a MAP suspension for 10 min in a pre-weighed cone-bottomed centrifugation tube to form a firm pellet. The supernatant should be decanted and the tube drained for 5 min on sterile absorbent material. The tube should be reweighed to determine the wet pellet weight. In general, the number of MAP would range from 5×10^6 (Juste et al., 1994) to 3×10^7 CFU (Hines et al., 2007) per mg pelleted wet weight and averaging approximately 1×10^7 CFU/mg (Sweeney and Hines, unpublished information). A retrospective estimate of the number of CFU inoculated should be made by performing colony counts plated on serial dilutions.

2.6. Storage of challenge inoculum

Ideally the MAP inoculum should be grown in broth to mid-log phase growth and used immediately

in challenge experiments. However, practical considerations may necessitate storage of the inoculum. Temperature and duration of storage of the inoculum for each model are discussed in each model section.

2.7. Route of challenge administration

Reports in the literature include intravenous, subcutaneous, intra-tonsillar, intraperitoneal, intra-intestinal (surgical loops or ileal cannulation), intragastric, and oral administration. A uniform route of administration is recommended for each model. In ruminant species, oral dosing by syringe on the back of the tongue or nursing the inoculum is recommended, as these mimic the natural route of exposure.

2.8. Challenge dose

A dose that consistently results in infection of experimental animals, but is not so high to overwhelm interventions such as vaccination or chemoprophylaxis is recommended for each species.

2.9. Bolus versus trickle (multiple) dose challenge schemes

Many dosing schemes have been reported in the literature, including single bolus dose, two or seven consecutive daily doses, weekly doses for 4 or 10 weeks, and 6 monthly doses. The advantage of the trickle dose (multiple smaller doses over time) is that it is most likely to mimic natural exposure and should improve the establishment of infection. However, bolus dosing provides a convenient ''start time'' for experiments.

2.10. Age to administer challenge

Susceptibility to MAP may change with age; therefore, age of infection should be standardized so that results between studies are comparable. In studies to reproduce clinical disease, animals should be infected as early as possible, even on the first day of life. This mimics natural infection, and takes advantage of possible age-related susceptibility. However, in studies to test vaccine efficacy, exposure to MAP may be delayed until after vaccination. Competence of the neonatal immune system is also a factor to consider if early vaccination is to be employed. The goals and practical concerns of the specific study should dictate the age at which the inoculum is given.

2.11. Infection status of experimental animals

Animals not previously exposed to MAP should be used. Diagnosis of MAP exposure is difficult in young animals, so the only method to insure that experimental animals have no prior exposure is to obtain them from herds/flocks where adult livestock are considered to JD-free. In general, experimental animals should not be selected from herds where JD vaccination is practiced.

2.12. Evaluation of passive shedding following oral challenge

Passive fecal shedding usually occurs in the days immediately following oral administration. Fecal culture at 1–3 days post challenge can be used as an additional quality control measure to ensure viability of the inoculum and sensitivity of fecal culture. Fecal culture positives from 14 days post-inoculation indicate that MAP is multiplying in the host.

2.13. Selection of animals

Study groups should be of uniform breed, sex, age, weight and size, and should be randomized by sire. In some cases, this method of grouping excludes twins or triplets. If not possible, study and control group assignments should be blocked by breed and sex. Randomization by sire may not be practical in large herds/flocks where multiple sires are being used.

2.14. Animal care

Requirements for housing, feeding, and husbandry will most often be determined by local institutional or governmental statutory regulations. However, the conditions employed should be similar for all groups and specified when reporting the results.

2.15. Quality control

Positive and negative quality control samples should be used to validate diagnostic tests. Samples

should be tested blind, as much as possible. As a minimum, all laboratory analyses should be blinded.

2.16. Experimental endpoints

Endpoints should be stratified according to type and include quantitative measures of infection (tissue colonization), severity of pathology, and clinical evidence of disease. Currently, immune parameters are insufficiently precise to be used as definitive endpoints for infection or disease. The models need flexibility to allow for use in experiments with different objectives. Where the objective is to determine if an intervention prevents clinical disease, then animals must be followed for extended periods of time and observed for clinical signs. If the goal is to determine whether MAP tissue colonization has been prevented or reduced, a shorter period of observation would be needed, with bacteriologic culture of tissues and/or PCR as the endpoint. In longitudinal experiments and studies of pathogenesis and intervention strategies, fecal culture, tissue culture, necropsy with gross and microscopic lesion scores, PCR, and measures of humoral or cell-mediated immune response should be performed. In most studies, determination of the actual status or rate of infection is critical. A combination of necropsy and histopathology with bacterial culture to determine levels of tissue colonization are recommended as minimal parameters. Fecal culture and immunological tests alone are often less reliable.

2.17. Sample collection and handling

Sample handling and culture methodology can influence culture results. Thus, sample storage (i.e., fresh versus frozen) and culture methodology are discussed in the model sections.

Types of samples collected will be influenced by the experimental design. Detection limits and the dynamic range of tests should be indicated and appropriate positive/negative controls included in all tests. A concerted effort should be made to collect and store additional biological samples from experimentally infected animals to provide samples and support for other JD related research projects.

2.18. Statistical sample size

In the design phase, sample size calculations should be performed in conjunction with a statistician for each outcome and to confirm infection status. Ideally, the selected sample size should be chosen so that it is large enough to ensure that important differences in all outcomes are detected with high power. Study group sizes do not have to be equal, especially if the variation in response is expected to be greater in one group than another. Detailed consideration of these issues, including calculation of sample size for time-to-event and comparison of responses of more than two groups is described elsewhere in the context of randomized controlled field trials (Wittes, 2002). Based on preliminary statistical evaluation of data from a short-term bovine model (Ray Sweeney), the culturing of three tissue sites per animal (proximal ileum, distal ileum, ileocecal valve) should provide 80–90% probability of detecting infection in experimental animals (Ian Gardner, personal communication). Processing of additional samples increases the likelihood of detection and improves measurement of differences in tissue MAP load for different interventions.

3. Bovine models

3.1. Long-term bovine MAP challenge model

This model would be used for studies of vaccination efficacy, preventive or therapeutic interventions, long-term pathogenesis, the immune response, and assessment of diagnostic assays. No published reports provide evidence of breed differences in susceptibility among the common dairy breeds, but some have suggested increased susceptibility of Jersey cattle to natural infection. However, because of the difference in clinical presentation of paratuberculosis in *Bos indicus* breeds, it is recommended that *Bos taurus* breeds be used exclusively for studies of bovine paratuberculosis.

A wide variety of MAP strains have been used in experimental infections (Table 1). Many reports identify strains only as ‘‘*M. johnei*,’’ ‘‘field isolate’’ or ‘‘clinical isolate,’’ while others provide more specific strain designations. Strain K-10 was selected

Table 1
Published studies utilizing a bovine model for *Mycobacterium avium* subsp. *paratuberculosis* infection

Reference	Age of animals	Route of infection	Strain of MAP	Dose(s)	Length of study	Experimental measurements				
						Histopathology	Tissue culture	Fecal culture	Immune response	Clinical disease
Rankin (1958)	1 month	IV	Clinical isolate (eight subcultures)	100 mg wet wt	4 years	4/6	6/6	6/6	ND[a]	4/6
Rankin (1961a,b,c)	3 years	IV	Clinical isolate (eight subcultures)	100 mg wet wt	4 years	1/5	5/5	0/5	ND[a]	0/5
Rankin (1961a,b,c)	1, 3, 6 months	Fecal–oral exposure	Clinical isolate	Unknown	5 years	9/9	9/9	8/9	ND[a]	4/9
Payne and Rankin (1961a,b)	3 months	Oral (milk)	Clinical isolate	200 mg wet wt	Variable up to 14 months	Increased with period of infection	Variable	ND[a]	ND[a]	No
Payne and Rankin (1961a,b)	3 months or 3 years	Oral (milk or water)	Clinical isolate	200 mg wet wt	Variable up to 6 months	Increased with period of infection	6/8 calves, 1/8 cows	ND[a]	ND[a]	No
Rankin (1962)	Variable	Fecal–oral exposure	Clinical isolate	Unknown	4 years	6/6 calves, 1/7 cows	4/7 cows	6/6 calves, 4/7 cows	Skin test+, CFT−	5/6 calves, 1/7 cows
Gilmour et al. (1965)	3 weeks	Oral (tube)	Clinical isolate	1×10^8; 1×10^{10}, 1 dose × 10 weeks	Variable up to 13 months	Increased with period of infection and dose	Increased with period of infection and dose	ND[a]	Skin test+	No
Stuart (1965)	1 week	IV	Clinical isolate	100 mg wet wt	10 months	18/40	NR[b]	40/40	CFT+	Yes
Larsen et al. (1978)	1 month	Oral (natural exposure)	Naturally infected cows	Unknown	Up to 6 years	22/175	31/175	20/175	Skin test+	Yes
Larsen et al. (1973)	16 days	Oral (milk)	Clinical isolate	180 mg wet wt	5 months	8/8	7/8	8/8	Skin test+	No
Thorel et al. (1984)	4 weeks	IV	Various isolates of MAP	1×10^9	12 months	8/23	23/23	ND[a]	ND[a]	No
Krishnappa et al. (1989)	NR calves	Oral	Clinical isolate from mucosal scrapings	50 g 1 dose × 10 weeks	30 weeks	ND[a]	ND[a]	1/12	AGID+, CIE+	No
Szilagyi et al. (1989)	17 days	Oral	Strain 5889	1×10^8, 2 × 5 days with 15 days between	400 days	ND[a]	NR[b]	NR[b]	NR[b]	No
Saxegaard (1990)	4 weeks	Oral (milk)	Clinical goat isolate from tissue	10 mg wet wt; 10 doses over 10 days	Variable up to 18 months	No	4/4	ND[a]	ELISA−	No
McDonald et al. (1999)	2 months	Oral (gastric tube)	Clinical isolate	2 g wet wt 3 doses	Up to 27 months	2/4	1/4	2/4	IFN-γ+, ELISA−, IFN-γ+	No
McDonald et al. (1999)	2 months	Oral (gastric tube)	Clinical isolate	20 g wet wt 3 doses	Up to 27 months	4/4	4/4	4/4	ELISA+	No
Beard et al. (2001)	1 week	Oral	Rabbit and bovine isolate	1×10^9, 3 doses 1×/week	6 months	3/8—rabbit, 2/4—bovine	7/8—rabbit, 3/4—bovine	5/8—rabbit, 0/4—bovine	ND[a]	No
Waters et al. (2003)	2 weeks	ITS	Strain K-10	1.6×10^7, 1×4 weeks	320 days	0/3	3/3	3/3	IFN-γ+, ELISA+	No
Uzonna et al. (2003)	28 days	Oral (milk)	Clinical isolate	1×10^{10}, 2 doses	49 days	0/15	15/15	ND[a]	IFN-γ+, ELISA−	No
Koo et al. (2004)	1–2 days	Oral	NR	1×10^7, 7 doses	6 months	ND	2/3-PCR	ND[a]	IFN-γ+, ELISA−	No
Simutis et al. (2005)	4 weeks	SC	Strain 19698	1×10^8	150 days	0/25	1/25	0/25	IFN-γ+, Skin test+	No
Stabel et al. (2003)	4 months	Oral (gastric tube)	Clinical isolate (bovine and bison)	1×10^9, 5 doses	6 months	0/6—cattle, 0/6—bison	5/6—cattle, 6/6—bison	1/6—cattle, 2/6—bison	IFN-γ+, ELISA−	No
Sweeney et al. (2006)	2–3 days	Oral (milk)	Clinical isolate (ATCC 700533)	2.5×10^{10}, 2 doses	44 days	06/6	60/6	0/6	ND[a]	No
Sweeney et al. (2006)	21–22 days	Oral (milk)	Clinical isolate (ATCC 700533)	H-5×10^9, M-5×10^8, L-1.5×10^6	44 days	H-0/8, M-0/6, L-0/6	H-8/8, M-6/6, L-6/6	H-0/8, M-0/6, L-0/6	ND[a]	No
Koets et al. (2006)	1 month	Oral (feces in milk)	Clinical cow feces (high shedder)	20 g, 9 doses	644 days	ND[a]	ND[a]	8/10	IFN-γ+, ELISA+	No
Rosseels et al. (2006a,b)	2–3 weeks	Oral	Strain 19698	1×10^8, 10 doses	875 days	ND[a]	ND[a]	NR[b]	Skin test+, ELISA−	No
Wu et al. (2007)	3–4 weeks	Ileum injection	Strain K-10, Strain 19698, Δ*gcpE* mutant	1×10^7 to 1×10^8, 1 dose	4 days to 9 months	5/5	5/5	0/5	Skin test−, ELISA−, IFN-γ+, TNFα+, IL12+, IL4−	No

AGID = agar gel immunodiffusion test; CFT = complement fixation test; CIE = crossed immunoelectrophoresis; ITS = Intratonsilar; IV = Intravenous; H = High dose; M = Medium dose; L = Low dose.

[a] ND = not determined.

[b] NR = not reported.

as the prototype strain for bovine infection models because it is known to be pathogenic, and has a well-characterized genotype. However, the laboratory passage status of the K-10 MAP strain is unknown. Other low-passage virulent strains with a similar short segment repeat genotype (e.g., 15 g–5 ggt genotype; Ghadiali et al., 2004) or equivalent PFGE or AFLP genotype, can be used. This allows use of various local MAP strains and minimizes regulatory issues concerning the import of infectious organisms.

Types of inoculum administered include feces from an infected animal, intestinal mucosal scrapings, lymph node homogenates, or *in vitro* cultured MAP (Table 1). In previous studies, MAP was harvested from solid media (Herrold's Egg Yolk, Middlebrook 7H11, Middlebrook 7H10, Taylor's, Dubos) or from broth (Middlebrook 7H9). Inoculum prepared from homogenized intestinal tissue, lymphoid tissue, or feces should not be used as it cannot be adequately standardized due to clumping and stored in sufficient quantities for numerous studies. To standardize experiments between laboratories, propagation of MAP *in-vitro* from a master seedstock of the chosen strain in mid-log phase growth should be employed. Middlebrook 7H9 broth, supplemented with OADC, mycobactin J, and 1% glycerol is recommended for the propagation of inoculum (Hines et al., 2007).

Storage of the inoculum at 4 °C beyond 4 weeks may result in reduced viability (Robert Whitlock, personal communication). Storage of the inoculum for up to 2 weeks at 4 °C is considered acceptable. However, the stored inoculum should be incubated at 37 °C for 2–3 h prior to administration to revive the refrigerated organisms. All samples within a study should be treated in the same manner.

Oral, intragastric, and parenteral routes of inoculation have been successfully used (Table 1). However, the oral route is recommended as this most closely mimics natural exposure and allows MAP uptake by tonsillar tissue as well as the intestinal tract. The calf is induced to suckle from a syringe inoculum mixed with a small volume of milk replacer or pasteurized milk. The mixture can also be gently expressed over the back of the tongue to induce swallowing. Administration by gastric tube is not recommended.

Published doses range from 10 to 200 mg wet weight and 10^6 to 10^{11} CFU (Table 1), but were not based on standardized procedures. Doses of 5×10^8 CFU given on two consecutive days reliably results in infection in calves by 12–14 weeks post inoculation (Sweeney et al., 2006). We recommend use of a standard bovine challenge dose of approximately 10^9 CFU/dose (100 mg wet weight) given on each of two successive days. Confirmation of numbers of CFU should be by serial dilution and plating on solid medium known to support the organism without added antibiotics. Excessively large doses which produce clinical disease less than 18 months post inoculation should be avoided because as they are not typical in naturally occurring JD.

Previous studies reported challenge of cattle from 1 day of age to adulthood (Table 1). While previous studies have suggested age-associated reduction in susceptibility, a definitive relationship between infectious dose and age has not been established. Some flexibility in age of inoculation is required due to differences in experimental objectives and timing of other interventions, such as vaccination. However, current experience suggests that 100 mg pelleted wet weight on two successive days results in demonstrable infection in calves less than 8 weeks of age.

Current diagnostic methods are ineffective for screening of individual calves at or before 8 weeks of age. Calves should be purchased from JD-free farms to insure lack of prior exposure. In the United States, herds that have achieved the equivalent of Status Level 3 or 4 in the National Voluntary Bovine Johne's Disease Status Control Program should be chosen as source herds (USDA APHIS, 2002). This equates to a closed herd with no history of Johne's disease in the previous 5 years, tested annually with at least one whole herd-negative serologic test and one whole herd negative fecal test (2nd lactation and older animals).

Calves should have received adequate quantities of colostrum. Milk replacer, if used, should be high quality and of animal origin (i.e., casein, not soy protein) and high quality rations should be fed. Routine vaccinations may be administered, but should not be given on the same days as JD vaccination or MAP administration. Anthelmintics and parasiticides should be given to all animals at similar dosages. If individual therapy is necessary (i.e., respiratory infection) an antibiotic known to have minimal effects on MAP (i.e., Ceftiofur) should be used.

Passive (pass-through) shedding occurs as early as 12 h after oral inoculation. Detection of passive

shedding by culture provides additional confirmation of inoculum viability and the sensitivity of the fecal culture method. Positive fecal culture results 14 days after inoculation should be considered shedding due to infection, except in very heavily contaminated environments (Robert Whitlock, personal communication). All animals should have fecal cultures at least monthly during the course of the study. Gross examination and culture of tissues for MAP as well as histopathologic examination of tissues to identify acid-fast organisms and lesions characteristic of paratuberculosis should be performed in all studies. At the inoculation dose recommended, colonization of tissues can be detected by culture in most animals by 4–12 weeks after inoculation, although culture of multiple tissues (minimum of three ileum including ileocecal valve, three jejunum, one duodenum, one spiral colon and three mesenteric lymph nodes including ileocecal nodes) is necessary. Although tissue samples should be culture positive by 12 weeks, investigators should not expect to find histologic lesions at this early stage. A necropsy and histopathology scoring system should be used (Table 3). The clinical status of the study animals should be assessed and recorded monthly and at necropsy.

The method of culturing fecal and tissue samples should permit quantification (or semi-quantification, e.g. by counting CFU on solid medium, or time to positive detection in automated liquid culture systems). Decontamination by incubation of fecal and tissue samples in 0.6% hexadecyl pyridinium chloride (HPC) for 14–16 h and 3 h, respectively, is strongly recommended. There is wide variation in culture methods currently used and batch-to-batch differences occur in media. All samples should be processed on medium from the same batch. Fresh samples may be processed immediately or frozen at −70 °C, and thawed only once with all samples treated in the same manner.

3.2. Short-term bovine MAP challenge models

3.2.1. Ileal cannulation model

Experimental MAP infection models that employ direct surgical access to the ileum for administration of MAP and collection of intestinal samples have been described (Allen et al., 2005). In general, these models are most useful for short-term study of host-pathogen interactions. Many parameters should be the same as for long-term challenge, including strain, inoculum preparation and quantification, storage, animal selection criteria, and quality control issues. Exceptions are age of administration, dose, experimental endpoints, and sample collection.

Calves are cannulated at 8 weeks of age, under general anesthesia and using a modification of the method of Streeter et al. (1991). A 2.5-cm diameter T shaped polyethylene cannula (ANKOM^{TM} Macedon, NY, USA) with a 7.5-cm neck is inserted into a 5-cm incision on the antimesenteric side of the distal ileum and closed with a purse string suture around the cannula with 3-0 PDS. The neck of the cannula is inserted through a separate 5-cm incision caudal to the paralumbar fossa laparotomy incision. A pediatric endoscope is inserted through the cannula to visualize the mucosa. Pre-infection biopsies are taken with an alligator biopsy instrument. Biopsies are placed in sterile microtubes (for real time PCR), 10% buffered neutral formalin (histopathology) and upon glass slides (Ziehl-Neelsen acid-fast stain). Calves are inoculated in the ileum with 10^{10} CFU of MAP in 20 ml of PBS. The inoculation process involves isolating the ileum using two 12 French inflatable Foley catheters, 10 cm aboral and oral to the cannula site. Foley catheters are inflated until there is slight tension on the balloon to obstruct passage of fluid from the isolated section of ileum and the latter flushed with saline to remove debris. A rubber stopper is then placed in the external opening of the cannula to prevent leakage of the inoculum. The inoculum is then injected through the rubber stopper and into the isolated ileum. The Foley catheters and rubber stoppers are left in place allowing the inoculum to be in mucosal contact for 1 h. MAP is taken up by M cells, and by dendritic and epithelial cells of the ileum and jejunum, within 30 min (Momotani et al., 1988; Sigurdardottir et al., 1999; Sigurdardottir et al., 2001). The inoculation procedure is repeated the next day. Ileal mucosal biopsies can be obtained at various time intervals, to test for MAP infection and local immune responses.

3.2.2. Invasion/surgical model

Surgical incision and direct deposition of MAP into the ileum has been recently employed to establish a model for intestinal invasion to other organs within a

few hours of infection (Wu et al., 2007). Although infected calves survive the surgery and repeated biopsy sampling up to 10 months following infection, the focus of this model is to examine early MAP intestinal interactions. The movement of MAP from intestine to liver, spleen or mesenteric lymph node was shown to differentiate between MAP strains with different virulence phenotypes.

3.2.3. Intestinal loop model

An alternative surgical approach is the ligated intestinal loop model with an injected inoculum of 3×10^9 CFU (~300 mg wet weight) (Momotani et al., 1988). This is only suitable for studies <12 h in duration and includes endpoints such as MAP detection and histopathology. This model is useful for ultrastructural studies of the early host–pathogen interaction and host immune response as measured by cytokine gene expression.

4. Caprine models

4.1. Long-term caprine MAP challenge model

The long-term caprine MAP challenge model is intended primarily for pathogenesis and vaccine efficacy studies; however, it could also be useful for other studies, including evaluation of diagnostic assays. No particular goat breed is recommended and any common local breed is acceptable since no studies have been performed to investigate genetic resistance or breed susceptibility.

Animals should undergo a selection process that includes validation/precertification of infection status. The JD status of the herd of origin is the best method to validate infection status of young kids. Kids should be selected only from closed herds in which all adults have been negative by ELISA and fecal culture for at least 1 year and JD vaccination is not practiced. All kids should receive sufficient quantities of colostrum. No specific recommendations were made for rations other than they should be of high quality.

Goat challenge studies have successfully used MAP isolates from clinically infected goats, cattle, and sheep, as well as humans (Table 2). Twenty-six to 50% of isolates from clinical cases in goats have been of bovine genotypes (Motiwala et al., 2004; Sevilla et al., 2005; Sevilla et al., 2007). Motiwala et al. (2004) detected little genetic diversity between and within bovine and goat isolates, but Sevilla et al. (2007) showed that goat isolates had a high degree of genetic heterogeneity.

In some studies, the inoculum consisted of *in vitro* cultured MAP, while in others, it was prepared from homogenized intestinal mucosal tissue scrapings from a clinically diseased animal. The use of a virulent, low passage goat clinical isolate cultured *in vitro* from a master seedstock, and with a typical bovine genotype identical or closely related to bovine strain K-10, is recommended. This allows use of local MAP strains and prevents difficulties related to the importation of exotic strains.

The recommended method of inoculum preparation, quantification, and storage is the same as stated above for the cattle model. The majority of studies used Middlebrook 7H9 + OADC + mycobactin J + glycerol or Tween 80 for *in vitro* cultivation. This medium with the addition of glycerol (1%), but not Tween or antibiotics is considered to be the best choice. The routes of administration have varied (Table 2) and essentially all routes tested have been successful in establishing infection with even the aerosol route resulting in intestinal pathology (Harding, 1957). The oral route most closely parallels natural exposure and is considered the best route of administration.

The size of the challenge inoculum has also varied widely (Table 2), ranging from 2.37 to 200 mg and from 3×10^7 to 8×10^{10} CFU and were generally successful in establishing infection. Two consecutive daily doses of 10^9 organisms (approximately 100 mg pelleted wet weight/dose and ~200 mg total dose) should establish infection in most kids without overwhelming experimental interventions.

The age at which to inoculate has varied from the day of birth to 10 months of age (Table 2). It is not known whether age related resistance occurs in goats. The age of administration will depend on the experimental goals and endpoints, but generally the goats should be less than 4 months of age. Clinical disease is expected to develop in a low percentage of animals at 9–10 months post inoculation.

The experimental endpoints will depend on the goals of the study. The minimal recommendations for goat experiments are to determine infection status

Table 2
Published studies utilizing a caprine model for *Mycobacterium avium* subsp. *paratuberculosis* infection

Reference	Age of animals	Route of infection	Strain of MAP	Dose(s)	Length of study	Experimental measurements				
						Histopathology	Tissue culture	Fecal culture	Immune response	Clinical disease
Harding (1957)	NR[a]	IT IV IV/ Oral Oral	NR[a]	NR[a]	9–16 months	23/24	17/24	ND[b]	ND[b]	NR[a]
Van Kruiningen et al. (1986)	2–12 days	Oral (milk)	Human isolate Linda	3.2×10^7, 4.0×10^8, 50 mg wet wt	Up to 310 days	4/4	4/4	1/4	ND[b]	No
Sigurdardottir et al. (1999)	7–26 days	Oral (milk)	Clinical goat isolate (three passages)	10 mg dry wt, 1×10 days	Up to 49 weeks	3/8	2/8	0/8	Skin test+, ELISA+, CFT+	No
Sigurdardottir et al. (2001)	18–21 days	Distal ileal ligation	Clinical goat isolate	2.365 mg dry wt/3 ml, 4 loops	1 h	MAP in M cells and leukocytes	ND[b]	ND[b]	ND[b]	No
Sigurdardottir et al. (2001)	23–39 days	Everted intestine sleeve	ATCC bovine strain 19698	3×10^7 per sleeve	1 h	MAP uptake by M cells and enterocytes	ND[b]	ND[b]	ND[b]	No
Storset et al. (2001)	5–8 weeks	Oral (milk)	Clinical goat isolate (P173) two passages	10 mg, 3×10 weeks	Up to 117 weeks	6/7	5/7	4/7	IFN-γ+, LBT+, ELISA+	Yes
Valheim et al. (2002)	5–8 weeks	Oral (milk)	Clinical goat isolate (P173) two passages	10 mg, 3×10 weeks	Up to 117 weeks	6/7	5/7	ND[b]	ND[b]	Yes
Munjal et al. (2005)	5–8 weeks	Oral	Clinical goat isolate (tissue)	1×10^{10}, 7×2 days	Up to 270 days	5/10	2/10 (PCR)	1/10	LBT+, ELISA+, AGID+	Yes
Stewart et al. (2006)	5 months	Oral	Clinical bovine isolate (tissue/culture)	1×10^{10}, 20 g wet wt	54 months	NR[a]	8	10/10	IFN-γ+, ELISA+	Yes
	10 months		Clinical sheep isolate (tissue/culture)	1×4 weeks	35 months		1	9/10		Yes
Hines et al. (2007)	6 weeks	Oral	Clinical goat isolate	1.5×10^9, $4 \times$ alternate days	6–9 months	Yes	Yes	Yes	IFN-γ+, Skin test+, ELISA+	Yes

AGID = agar gel immunodiffusion test; CFT = complement fixation test; LBT = lymphocyte blastogenesis test; IT = Intratracheal; IV = Intravenous.

[a] NR = not reported.

[b] ND = not determined.

through a combination of culture, PCR, and histopathology, using quantitative or semi-quantitative methods. A lesion grading system for gross and histopathologic findings (Table 3; Hines et al., 2007) should be used with a sufficient range in values to allow statistical analysis. Recommendations for methods of fecal and tissue MAP culture and for sample handling are the same as for the bovine model. Like cattle, positive fecal cultures 2 weeks or more post inoculation should be considered due to infection. Fecal cultures should be performed at least monthly from all animals.

4.2. Short-term caprine MAP challenge models

4.2.1. Intestinal loop model

An intestinal loop model in goats (Sigurdardottir et al., 2001) could be used for studying initial bacterial/host interactions. MAP strain, dose, quantification, culture medium, animal selection and sample collection should be similar to the long-term challenge model.

4.2.2. Everted intestine sleeve model

An everted intestine sleeve model (Sigurdardottir et al., 2005) is useful for studying initial host/bacterial interactions, such as bacterial attachment and internalization, bacterial localization and early bacterial gene regulation, but may have limitations when evaluating host gene and early cytokine regulation in response to infection. Approximately, 1-cm segments of small intestine are excised, everted, washed, maintained in tissue culture, and bathed in a suspension of MAP for short periods of time. Strain selection and quantification, animal selection and sample collection should be similar to that for the long-term challenge model.

Table 3
Scores for necropsy grading system categorized by lesion severity and presence of acid-fast bacilli (AFB) in goats

Severity	Gross	Microscopic	No. AFB
None	0.0	0.0	0.00
Mild	4.0	1.0	0.25
Moderate	8.0	2.0	0.50
Severe	12.0	3.0	0.75

Individual severity scores for each of the three categories are summed to achieve the final lesion score for each animal at necropsy. Lowest and highest possible scores using this system are 0.0 and 15.75, respectively. Significant variation in gross and histologic lesions exists between species of animal affected. This system allows flexibility in what constitutes a mild, moderate or severe gross or microscopic lesion and can be tailored to be effectively used in multiple species. The relative number of AFB is given the lowest weight in this system since both paucibacillary and pleuribacillary forms of JD are recognized in some species affected. Alternative lesion grading systems based on classification of lesions of natural cases of paratuberculosis in sheep (Perez et al., 1996), goat (Corpa et al., 2000) and cattle (Gonzalez et al., 2005), granuloma counting for sheep (Juste et al., 1994), and for deer (Mackintosh et al., 2005) have been proposed.

5. Ovine models

5.1. Long-term ovine MAP challenge model

This model is primarily for pathogenesis and vaccine efficacy studies, but could also be used for evaluating diagnostic assays. The Merino, as well as some dairy breeds may be more susceptible to MAP than other ovine breeds (Frank Griffin and Ramon Juste, personal communication), but genetic resistance to JD has not been identified. While the Merino breed is preferred, any breed shown susceptible to MAP is acceptable.

Successful ovine experimental challenge studies have used MAP isolates from cattle, sheep, wildlife, and humans (Table 4). Until 2000, the majority of experimental studies in sheep did not use characterized ovine MAP strains, in contrast to later studies which have used tissue homogenates or low passage ovine MAP strains grown *in vitro* (Gwozdz et al., 2000a,b; Stewart et al., 2004; Begg et al., 2005). The most common MAP isolates from clinical JD in sheep belong to the ovine genotype (Motiwala et al., 2004; Sevilla et al., 2005; Sevilla et al., 2007). Marked genetic diversity was detected among ovine isolates, as well as between ovine and both bovine and goat isolates (Motiwala et al., 2004). Therefore, to most closely reproduce natural ovine infections, any confirmed-virulent, ovine clinical isolate that can be cultivated *in vitro* is considered acceptable. However, it is important to note that, although inoculation of cultured bovine strains has uniformly resulted in development of lesions and recovery of bacteria, some studies using cultured ovine isolates have nearly completely failed. Since little information is available

Table 4
Published studies utilizing a ovine model for *Mycobacterium avium* subsp. *paratuberculosis* Infection

Reference	Age of animals	Route of infection	Strain of MAP	Dose(s)	Length of study	Experimental measurements				
						Histopathology	Tissue culture	Fecal culture	Immune response	Clinical disease
Brotherston et al. (1961)	10 weeks 1 week 7–10 days 3 weeks	IV Oral Oral Oral	Sheep isolate (var bovine)	1×10^7 1×10^8, 1×3 weeks 1×10^8, 1×8 weeks $1 \times 10^{3-9}$, 1×10 weeks	Up to 22 months 53 weeks 53 weeks Up to 9 months	ND[a] ND[a] ND[a] ND[a]	13/16 10/16 9/9 24/51	ND[a] ND[a] ND[a] ND[a]	ND[a] ND[a] ND[a] ND[a]	No No No Yes
Gilmour and Brotherston (1962)	8 months	Oral	Sheep isolate (var. bovine)	1×10^9	Up to 56 days	ND[a]	10/12	ND[a]	ND[a]	No
Nisbet et al. (1962)	1 week 7–10 days 3 weeks 3 months	Oral	Sheep isolate (var. bovine)	1×10^8 1×10^8, 1×8 weeks $1 \times 10^{3-9}$ 1×10, 1×10^6, 1×10 weeks	53 weeks 53 weeks Up to 9 months Up to 9 months	6/12 7/9 20/35 5/12	ND[a] ND[a] 23/35 6/12	ND[a] ND[a] ND[a] ND[a]	ND[a] ND[a] ND[a] ND[a]	NR
Gilmour and Brotherston (1966)	3 months, 10 months	Oral	Sheep isolate (var bovine)	1×10^6, 1×10 weeks	Up to 18 weeks	ND[a]	6/9, 9/9	ND[a]	Skin test+	NR
Kluge et al. (1968)	3 weeks	IV IT Oral	Clinical bovine isolate (tissue)	50 mg dry wt 50 mg dry wt 200 mg dry wt	Up to 16 months	Yes	NR[b]	NR[b]	NR[b]	Yes
Merkal et al. (1968a,b)	3 weeks	IV IT Oral (milk)	Clinical bovine isolate (tissue)	50 mg dry wt 50 mg dry wt 200 mg dry wt	Up to 16 months	Yes	NR[b]	NR[b]	Skin test+, CFT+, AGID+	Yes
Gilmour et al. (1978)	5 months	Oral	Sheep isolate (three passages)	1×10^9, 1×10 wks	Up to 27 months	15/22	11/22	ND[a]	Skin test +	Yes
Williams et al. (1983a)	4–5 months	Oral	Clinical sheep isolate	50 mg wet wt	Up to 12 months	ND[a]	5/9	0/9	ND	No
Juste et al. (1994)	3 months	Oral	Bovine isolate (three passages)	150 mg wet wt, 2 doses	220 days	Yes	Yes	0/5	ELISA +	No
Burrells et al. (1995)	1 day	Oral	Deer isolate Strain JD88/107	1×10^8, $1 \times$ 9 weeks	NR	ND[a]	ND[a]	ND[a]	IFN-γ+, LBT+	No
Begara-McGorum et al. (1998)	5–9 days	Oral	Deer isolate Strain JD88/107	1×10^9 3×2 days	Up to 41 days	4/8	3/8	1/8	IFN-γ− ELISA−	No
Gwozdz and Thompson (2002)	1–4 weeks	Oral (gastric tube)	Clinical sheep isolate (tissue)	3.4×10^9 4.4×10^8	108 weeks 53 weeks	1/10 3/9	1/10—PCR 4/9—PCR	ND[a]	IFN-γ+, ELISA+ AGID+, CFT+	Yes
Gwozdz et al. (2000a,b)	1–2 months	Oral (gastric tube)	Clinical sheep isolate (tissue)	4.4×10^8	53 weeks	9/14	10/14—PCR	3/14—PCR	IFN-γ+, ELISA+	Yes
Gwozdz et al. (2001)	1–4 weeks	Oral (gastric tube)	Clinical sheep isolate (tissue)	3.4×10^9	108 weeks	18/28	ND[a]	ND[a]	ND[a]	Yes
Reddacliff and Whittington (2003)	12–16 weeks	Oral	Sheep isolate (feces)	2.6×10^1 1×10^4 1×10^8 3×1 week 10×1 week		0/30	0/12 0/12 6/6	0/30	IFN-γ+, Skin test+ ELISA+	No
Kurade et al. (2004)	8–12 weeks	Oral	Clinical sheep isolate (tissue)	1×10^{10} 8×3 days	Up to 330 days	20/20	7/20	3/20	LBT+ ELISA+	Yes

Table 4 (*Continued*)

Reference	Age of animals	Route of infection	Strain of MAP	Dose(s)	Length of study	Experimental measurements				
						Histopathology	Tissue culture	Fecal culture	Immune response	Clinical disease
Stewart et al. (2004)	6 months	Oral	Clinical bovine isolate (tissue/ culture)	1×10^{10}	54 months	NR[b]	1/5	7/10	IFN-γ+	Yes
				20 g wet wt					ELISA+	
	10 months		Clinical sheep isolate (tissue/ culture)		35 months		1/5	5/10		Yes
				1×4 weeks						
Begg et al. (2005)	12 weeks	Oral	Clinical sheep isolates	1×10^{9}	10 months	17/30	21/30	NR[b]	IFN-γ+	No
			(JD3-tissue and	4×3 days					LBT+	
		Oral	W-high and low passage culture)		13 months	22/30	16/30	NR[b]	ELISA+	Yes
				5×10^{8}						
		IT		1×10^{9}	16 months	7/12	8/12	NR[b]		No
		Oral		1×3 weeks		9/12	8/12			
		Oral				1/12	3/12			
				5×10^{7}						
				5×10^{7}						
				1×10^{9}						
				2×1 month						
Begg et al. (2005)	2.5 months	Oral	Clinical sheep isolate—JD3	5×10^{8}, 1×3 weeks	Up to 22 months	23/30	NR[b]	NR[b]	IFN-γ+, LBT+, ELISA+	Yes

AGID = agar gel immunodiffusion test, CFT = complement fixation test. LBT = lymphocyte blastogenesis test; IT = Intratracheal; IV = Intravenous.

[a] ND = not determined.

[b] NR = not reported.

on these ovine strains, it is critical that each isolate used be genotyped to allow future comparison. This will allow use of local ovine MAP strains and avoidance of import restrictions. Homogenized lymphoid tissue or intestinal mucosal scrapings from a clinically diseased animal appears to be the best method of reproducing the ovine infection (Table 4). However, this method should not be used as a standard for experimental challenge studies in sheep. A virulent, low passage ovine strain cultivated *in vitro* is recommended as the inoculum source.

Culture media used in published ovine studies have included Middlebrook 7H11 broth, Middlebrook 7H10 agar, and BACTEC™ 460 medium with increased egg concentration. Middlebrook 7H11 broth is the preferred medium for inoculum preparation, but any medium capable of supporting the growth of ovine MAP strains is acceptable. The quantification of organisms, and handling and storage of the inoculum is as described for cattle.

The usual route of administration has been oral using either saline or milk suspensions, but other protocols have also been successfully used (Table 4). However, the oral route most closely parallels natural exposure and is considered the best route of administration.

The challenge inoculum has ranged from 15 to 200 mg wet weight of organisms, from 2.6×10^1 to 2.6×10^{11} CFU and from 0.65 to 80 g of macerated infected tissue from a clinical case (Table 4). The lowest doses have generally not been effective in establishing infection (Reddacliff et al., 2004).

Route and frequency of dosing have varied widely between studies (Table 4). Based on recent experimental evidence, three consecutive daily oral doses consisting of 10^9 organisms per dose (~100 mg pelleted wet weight/dose; 300 mg total) should be used to establish infection (Begg and Griffin, 2005).

The age at inoculation has ranged from day of birth to 10 months (Table 4). Experimental challenge at any time up to 4 months of age is appropriate. However, age at time of challenge will be influenced by experimental objectives. No recommendation was made as to when a JD vaccine or other intervention should be administered.

As for other species, positive fecal cultures 2 weeks or more post inoculation should be considered due to infection. Fecal culture is less reliable in animals infected with sheep MAP strains (as compared to cattle strains), and expense is a major consideration in large ovine studies, particularly in the field; nonetheless, all animals should have periodic fecal cultures using validated methods.

Animals to be included in challenge studies should be validated infection-free based upon the infection-free status of the foundation flock. Lambs for experimental studies should only be selected from closed flocks in which all adults have been negative on ELISA and fecal culture for at least 1 year and JD vaccination is not practiced. All lambs should receive adequate quantities of colostrum.

A combination of necropsy with histopathology and bacterial culture to determine levels of tissue colonization is recommended as the minimal requirement to determine infection status of experimental animals. The lesion grading system used for gross and histopathology findings should have a sufficient range in values to allow for statistical analysis (Table 3; Juste et al., 1994). Animals should be observed until clinical signs develop in a proportion of the group. The type, quantity, and processing of samples collected will vary with the purpose, number of animals, goals, and cost of the study. All samples should be collected, handled, and processed in the same way to ensure uniformity. Ovine strains apparently grow better in liquid medium, and BACTEC™ 460 was suggested to be the best system by which to recover ovine strains, but no single standard culture medium or method for culture of feces or tissues is recommended. Decontamination and culture should be performed similarly to that previously described for cattle, providing, at a minimum, semi-quantitative results.

6. Cervid models

6.1. Long-term cervid MAP challenge model

The long-term cervid challenge model is primarily for pathogenesis and vaccine efficacy studies, but may also be useful for evaluation of diagnostic assays and heritable resistance. High levels of heritable resistance and susceptibility to mycobacterial infection are documented in deer (Mackintosh et al., 2000). Deer appear to be a useful host for disclosing diagnostic markers by which to monitor infection

(Griffin et al., 2005), protective immunity, or resistance to infection. The majority of studies performed in cervids (Table 5) have involved red deer (*Cervus elaphus*), which seem to be more naturally susceptible (Mackintosh et al., 2003) than other cervids (Williams et al., 1983a,b). Experimentally infected deer have a broad spectrum of responses ranging from limited infection to extremes of pathology and clinical disease. The range of pathology allows clear stratification and analysis of the impact of interventions (Mackintosh et al., in press). The predominance of bovine strains in naturally infected deer suggests that cervids may provide an alternate experimental challenge model for JD in cattle. Red deer may be naturally infected with either bovine or ovine strains (de Lisle et al., 2006), but they appear more susceptible and develop more severe disease with bovine strains (O'Brien et al., 2006). Clinical signs develop in 25–30% of red deer between 4 and 12 months post bovine strain challenge (Mackintosh et al., 2005). Red deer are recommended as the model of choice for cervid challenge studies.

Strain, dose, route of inoculation and infection or disease endpoints used in cattle and goat models apply equally well to cervids. In addition, there is evidence that most of the bovine immunological reagents are useful in studies with red deer, with the known exception of TGFβ (Frank Griffin, personal communication). A dose of 10^3 CFU of a bovine strain produces equivalent infection and pathology to 10^6 CFU of an ovine strain (O'Brien et al., 2006). Thus, bovine strains are preferred, and all challenge parameters in red deer should be the same as those for the bovine and caprine models. A strain with genotype similar to bovine strain K-10 at the bovine dose (10^9 organisms, 100 mg) given on two consecutive days is most appropriate for deer challenge studies. Age of administration will vary depending on the goals of the study. Deer should be challenged at less than 3 months of age. The relatively early onset of pathology and disease in deer suggest that this model may be cost effective and informative for study of MAP infection. Access to deer bloodlines with resistant or susceptible phenotypes for *Mycobacterium bovis* infection may be valuable in exploring candidate genes which contribute to heritable resistance to other mycobacteria.

7. Murine models

7.1. Long-term murine MAP challenge model

Primary uses for the long-term murine model are early screening for vaccine candidates or preliminary analysis of pathogenesis. However, typical features of JD in cattle (e.g., diarrhea, severe intestinal lesions) cannot be reproduced in mice. Nevertheless, the murine model facilitates development of vaccines for JD. Unlike bovine, caprine, cervid, or ovine models, many immunological reagents are well developed for mice. Also, mice with variable genetic background are readily available, allowing investigation of host–pathogen interactions on a molecular level. In numerous studies (Shin et al., 2006; Stabel and Ackermann, 2002; Tanaka et al., 1994) histological and immunological features reproduced in mice were generally similar to those in ruminants (Table 6). Generally, the degree of bacterial colonization and the type of cells recruited to granulomatous lesions depend on the mouse genetic background as well as virulence of the mycobacterial strain (Mullerad et al., 2002; Shin et al., 2006; Tanaka et al., 1994), indicating the utility of the murine model in elucidation of pathogenetic mechanisms.

Choice of mouse strain is strongly linked to the intended use of the model (e.g., pathogenesis or vaccine testing). Some studies used BALB/c or C57/BL6 strains which are immune-competent, but susceptible to infection (Veazey et al., 1995). Others used C3H mice, which are more resistant (Tanaka et al., 1994). Despite the presence of granulomatous lesions in both susceptible and resistant strains of mice, the number of lesions and degree of bacterial colonization declined dramatically in the resistant strain. Thus, investigators should carefully consider mouse strain before using this model to test the virulence of an isolate or assaying the efficacy of a vaccine. Other important parameters are infectious dose and route of administration.

Oral inoculation of 10^{11} CFU/animal produced granulomatous lesions in 58% of mice, but lesions were limited to the mesenteric lymph node (Veazey et al., 1995). Intraperitoneal (IP) injection of a low dose (10^6 CFU) induced a small number of epitheloid granulomas, but multifocal granulomas (composed of macrophages and epitheloid cells) occurred when 10^8 CFU were used (Tanaka et al., 2000). The

Table 5
Published studies utilizing a cervid or exotic species model for *Mycobacterium avium* subsp. *paratuberculosis* infection

Reference	Species	Age of animals	Route of infection	Strain of MAP	Dose(s)	Length of study	Experimental measurements				
							Histopathology[a]	Tissue culture	Fecal culture	Immune response	Clinical disease
Williams et al. (1983a)	Bighorn × mouflon	4–5 months	Oral	Clinical bighorn sheep isolate	50 mg wet wt	6 or 12 months	8/9	9/9	NR[b]	ND[c]	No
	Mule deer						8/8	8/8			Yes
	White-tail deer						2/2	2/2			Yes
	Elk						8/8	8/8			No
Williams et al. (1983b)	Bighorn × mouflon	4–5 months	Oral	Clinical bighorn sheep isolate	50 mg wet wt	6 or 12 months	NR[b]	9/9	9/9	ND[c]	No
	Mule deer							8/8	2/8		Yes
	White-tail deer							2/2	0/2		Yes
	Elk							8/8	0/8		No
Mackintosh et al. (2003)	Red deer	4 months	Oral	Clinical deer isolate (bov var)	NR, 1×4 days	12 months	39/43	NR[b]	NR[b]	Skin test+, LBT+, ELISA+	Yes
Mackintosh et al. (2005)	Red deer	4 months	Oral	Clinical deer isolate (tissue; bov var)	1×10^9, 1×4 days	12 months	42/42	NR[b]	NR[b]	LBT+, ELISA+	Yes
O'Brien et al. (2006)	Red deer	4 months	Oral	Clinical red deer isolate (tissue; bov var)	1×10^9, 1×10^7, 1×10^3, 1×4 days	Up to 44 weeks	Lesions apparent	40/64	NR[b]	IFN-γ+, LBT+	NR[b]
				Clinical sheep isolate (JD3; tissue)	1×10^7, 1×4 days		Lesions apparent	11/16		ELISA+, IFN-γ+/−, LBT+, ELISA−	NR[b]
Mackintosh et al. (in press)	Red deer	4 months	Oral	Clinical deer isolate (tissue; bov var)	$10^3 \times 4$ (LB) $10^7 \times 4$ (MB)	12 months	NR[b]	8/16 LB, 16/16 MB	NR[b]	LBT+, IFN-γ+	NR[b]
				Sheep tissue isolate ovine strain	$10^9 \times 4$ (HB) $10^7 \times 4$ (MO)			16/16 HB 8/16 MO		ELISA+ (IgG1)	
Stabel et al. (2003)	Bison	4 months	Oral (gastric tube)	Clinical isolate (bovine and bison)	1×10^9, 5 doses	6 months	0/6	6/6	2/6	IFN-γ+, ELISA−	No

[a] In the cervid model histopathology following necropsy may be used to stratify disease severity. LBT = lymphocyte blastogenesis test.

[b] NR = not reported.

[c] ND = not determined.

Table 6
Published studies utilizing a murine model for *Mycobacterium avium* subsp. *paratuberculosis* Infection

Reference	Species	Age of animals	Route of infection	Strain of MAP	Dose(s)	Length of study	Experimental measurements				
							Histopathology	Tissue culture	Fecal culture	Immune response	Clinical disease
Chandler (1961a)	Swiss white mice	4 weeks	IP	Clinical cow isolate	2, 8, or 24 mg wet wt	Up to 1 year	Yes	Yes	ND[a]	ND[a]	No
Chandler (1962)	C57BL, CBA brindle, Swiss white mice	Variable	Oral IP IV	Three strains of MAP Clinical cow isolate, Wey II Wey 316F	0.0001–10 mg	Up to 9 months	Yes	Yes	ND[a]	ND[a]	Yes
Collins et al. (1983)	C57BL mice, Rabbits Guinea pigs Chickens	Variable	IP IV IM IV	Six strains of MAP (three passages)	1×10^8 1×10^7 1×10^7 1×10^6	Up to 6 months	ND	Yes	ND[a]	ND[a]	No
Hamilton et al. (1991)	BALB/c, nu/nu mice	NR[b]	IG	Human isolate Linda	1×10^{10}	Up to 6 months	Yes	Yes	Yes	TNF+	Yes
Mutwiri et al. (1992)	SCID/bg mice	NR[b]	IP IG	Clinical cow isolate (tissue)	1×10^5 1×10^5	Up to 26 weeks	Yes	Yes	ND[a]	ND[a]	Yes
Adams et al. (1993)	BALB/c, nu/+, nu/nu	NR[b]	IG	Human isolate Linda	1×10^{10}	Up to 6 months	Yes	Yes	Yes	ND[a]	No
Chiodini and Buergelt (1993)	BALB/c, C57/B10, C57/B6	2 weeks	IP	Human isolate Linda	6.2×10^8	6 months	Yes	Yes	ND[a]	ND[a]	No
Tanaka et al. (1994)	BALB/c, C3H/HeJ, mice	6 weeks	IP	ATCC strain 19698	5×10^8	32 weeks	Yes	NR[b]	ND[a]	ND[a]	No
Veazey et al. (1995)	C57BL/6, C3H/HeN, mice	4–6 weeks	IP	ATCC strain 19698	1×10^9	Up to 269 days	Yes	Yes	ND[a]	ND[a]	No
Veazey et al. (1996)	C57BL/6, C3H/HeN, mice	4–6 weeks	IP	ATCC strain 19698	1×10^9	Up to 5 months	NR[b]	NR[b]	NR[b]	CD4+	NR[b]
Stabel and Goff (1996)	C57BL/6-bg*bg	6–8 weeks	IV	Strain 19698	1×10^8	1, 6, 12 months	ND[a]	Yes	ND[a]	IL-1, IL-6, TNF-α	NR[b]
Stabel et al. (1998)	C57BL/6-bg*bg	8 weeks	IP	Strain 19698	1×10^8	1, 3, 6 months	Yes	Yes	ND[a]	LBT, IL-1, IL-6, TNF- α	No
Tanaka et al. (2000)	BALB/c, Wild-type and γδ-T cell-KO	10 weeks	IP	ATCC strain 19698	4×10^6 4×10^9	6 and 18 weeks	Yes	Yes	ND[a]	ND[a]	No
Mutwiri et al. (2001)	SCID/bg mice	6 weeks	IP			32 weeks	Yes		ND[a]	ND[a]	Yes
Stabel and Ackermann (2002)	TCR-KO, C57BL/6, mice	6 weeks	IP	Strain 19698 human isolate Ben	1×10^8	1, 3, 6 months	Yes	Yes	ND[a]	ND[a]	No
Huntley et al. (2005).	BALB/cJ	6 weeks	IP	NADC strain 19698-1974	1×10^8	3 months	ND	Yes	ND	Immunoblots	No
Rosseels et al. (2006a,b)	C57BL/6OlaHsd-bg (B6 bg/bg)	8–12 weeks	IV	ATCC strain 19698	1×10^6	5 or 10 weeks	ND	Yes	ND	IFN-γ+	No
Shin et al. (2006)	BALB/c	6 weeks	IP	ATCC strain 19698 and mutants	1×10^7	3, 6, 12 weeks	Yes	Yes	ND[a]	ND[a]	No

IP = intraperitoneal; IM = Intramuscular; IV = intravenous; IG = intragastric. LBT = lymphocyte blastogenesis.

[a] ND = not determined.

[b] NR = not reported.

committee recommends a dose of 10^8 CFU/animal (~10 mg) of K-10 or closely related strain, administered IP. To date, IP injection has the most reproducible infection rate (100%), compared to other routes of delivery (Mutwiri et al., 1992).

An attractive aspect of this relatively inexpensive murine JD model is the ease of screening a large number of attenuated mutants as vaccine candidates. In a high throughput format, analysis is focused on liver and intestine, of which both are examined for histological lesions and colonization over 12 weeks (Shin et al., 2006). Unlike other species, liver is the most affected organ following IP injection, while only hyperplastic gut-associated lymphoid tissue is seen in intestines.

Vaccine candidates can be inoculated into mice and the profile and nature of elicited immune responses can be measured. The immunogenicity of several gene vaccine candidates for JD has already been assayed in mice (Mullerad et al., 2002, 2003).

8. Conclusions and knowledge gaps

A brief summary of the proposed guidelines for long-term MAP challenge studies according to species is presented in Table 7. A number of issues discussed by the committee were considered to be knowledge gaps. While results and experience working with other mycobacteria (i.e., *M. bovis*, *M. avium* subsp. *avium*) are likely to provide valuable information, the recommendation is that these issues be answered experimentally for MAP.

8.1. How does early tissue colonization relate to protection or lack of protection?

Numerous studies have used tissue colonization as a measure of the efficacy of vaccination or other interventions. It is generally assumed that reduced tissue colonization by MAP is a direct indicator of protection, but correlative studies over time have not been performed to validate this assumption.

8.2. Is there a correlation between tissue colonization and fecal shedding or shedding in milk?

One of the major considerations of JD control programs is to limit transmission from animal to animal, with fecal and/or milk shedding generally considered to be the principal methods. Numerous studies have evaluated levels of fecal shedding and/or tissue colonization in response to vaccination or other interventions, but none have evaluated shedding in milk. There does not appear to be a high correlation between fecal shedding and tissue colonization except in advanced cases, but this requires further confirmation. No previous studies have specifically evaluated the correlation among tissue colonization, fecal shedding, and milk shedding. However, it would be very useful to determine this relationship, as it impacts on the possibility of utilizing shedding in either feces or milk as a proxy for tissue colonization.

8.3. What is the effect of Tween 80 and Tween 20 on MAP in culture?

A major problem with *in vitro* culture of MAP is severe clumping of organisms, which creates difficulties in accurate quantification of the inoculum and serial plating of feces and tissue. Various concentrations of Tween (particularly Tween 80) have been used in liquid culture media to reduce clumping. Morphological alterations have been noticed in MAP grown in culture with Tween, but the effects on metabolism, viability, and virulence have not been adequately described.

8.4. What is the effect of refrigeration on MAP inoculum?

Although little information is available concerning the effect of temperature or other storage parameters on the survivability of MAP in the inoculum, Sweeney and Whitlock (unpublished findings) suggest that storage of the inoculum at 4 °C for 60 days, but not 30 days, significantly affected viability. Additional studies should be performed to evaluate effect on virulence and viability.

8.5. Is there a true age related resistance in JD?

The concept of ‘‘age-related’’ resistance has been suggested in the literature for bovine JD (Buergelt et al., 1978; Bendixen, 1978; Doyle, 1953, 1956; Hagan, 1938; Larsen et al., 1975; Levi, 1948; Rankin,

Table 7
Summary of recommended guidelines for long-term MAP challenge studies by species

Species model and breed/species	Age of animals	Route of infection	Strain of MAP	Dose(s)	Necropsy	Minimum experimental end-points[a]				
						Histopathology	Tissue culture	Fecal culture	Immune response	Clinical disease
Bovine (*Bos taurus* breeds only)	≤8 weeks	Oral (nurse or syringe)	K-10 or similar genotype	~100 mg wet weight; two consecutive days (total 2.0×10^9 organisms)	Yes	Yes	Yes	Yes	Preferred	Preferred
Caprine (no specific breed)	≤4 months	Oral (nurse or syringe)	K-10 or similar genotype	~100 mg wet weight; two consecutive days (total 2.0×10^9 organisms)	Yes	Yes	Yes	Yes	Preferred	Preferred
Ovine (Merino breed preferred)	≤4 months	Oral (nurse or syringe)	No specific strain recommended	~100 mg wet weight; three consecutive days (total 3.0×10^9 organisms)	Yes	Yes	Yes	Preferred	Preferred	Preferred
Cervid (red deer)	≤3 months	Oral (nurse or syringe)	K-10 or similar genotype	~100 mg wet weight; two consecutive days (total 2.0×10^9 organisms)	Yes	Yes	Yes	Preferred	Preferred	Preferred
Murine (Balb/c, C57BL/6 preferred)	≤6 weeks	IP injection	K-10 or similar genotype	~10 mg wet weight; single dose (total 10^8 organisms)	Yes	Yes	Yes	Optional	Preferred	Not applicable

IP—Intraperitoneal.

[a] Experimental endpoints will vary depending on goals of the study. Study designs with longitudinal measurements should include appropriate numbers of repeated observations on fecal culture, immune response and observation of clinical signs, as well as the experimental endpoints suggested above.

1958, 1961a,b,c, 1962; Taylor, 1953). However, some committee members have questioned whether age-related resistance truly occurs in any species. The historical literature suggests that age resistance does occur in cattle, but the definitive studies proving its existence in other species have not been done.

8.6. What is the optimal-but-practical number of necropsy tissues to collect for culture and histopathology?

It is generally assumed that increasing the number of tissue samples for histopathology and culture improves the accuracy of confirming infection status. However, the processing of large numbers of samples (e.g., in an extensive vaccine trial) is very expensive and time-consuming. Sweeney et al. (2006) have suggested that 30 intestinal samples should be collected from various locations for culture and histopathology. A more manageable, optimum number of samples and locations needs to be determined for detecting infection with sufficient statistical power.

8.7. What is the immune signature of vaccine protection?

The immunology of various stages (subclinical, clinical) of JD is in the early phases of elucidation and the significance of many findings is unclear. A set of immunological markers on blood derived cells that provided a specific and definite signature highly correlated with protection from disease and/or elimination of fecal shedding, would be useful in testing of vaccines and other interventions. Evaluation of immunological markers by flow cytometry and luminescence, and of gene regulation markers by DNA arrays and RT-PCR are currently in progress.

8.8. What is the effect of freezing on outcome of bateriologic culture of specimens?

No published studies demonstrate the effect of freezing of tissue or fecal specimens on the recovery of MAP by culture. It is well known that MAP is resilient, but the extent of damage from freezing is not known and needs to be thoroughly evaluated.

8.9. Is there a MAP bacteremia after challenge and when does it occur?

MAP has been detected by PCR in the blood of infected cattle (Buergelt et al., 2004; Juste et al., 2005) and sheep (Gwozdz et al., 1997; Juste et al., 2005) and by PCR and culture in blood of Crohn's disease patients (Naser et al., 2004; Elguezabal et al., 2006). Bacteremia in goats, deer, or other species, or shortly after inoculation, or in the later stages of disease, and whether persistent or intermittent has not been studied. A correlation between tissue colonization and presence of MAP in blood has not been described. Reliable detection of MAP in blood by culture or PCR would provide a useful diagnostic test.

8.10. Is MAP shed in milk of sheep and goats?

MAP can be detected by bacteriologic culture and PCR in bovine milk. However, similar studies have not been performed in sheep and goats.

8.11. How comparable are mouse challenge models to ruminant challenge models?

Ruminant challenge models (particularly in cattle) are extremely expensive and take considerable time to perform. Mouse challenge models are much less expensive and less time intensive. A wide variety of reagents support work in mice, but not in ruminants. However, significant differences between mouse and ruminant immunology exist. The overall degree of immunological similarity between the mouse and ruminants is generally not known and the validity of using a mouse model for vaccine candidate screening or pathogenesis studies is questionable.

8.12. What is the influence of host genetics in Johne's disease?

Study of the effect of host genetics on resistance to Johne's disease is only just beginning. Recently, several studies in the Netherlands, New Zealand, Australia and US have been funded to evaluate the effects of host genetics in cattle, sheep and deer.

In summary, a thorough review of the literature and the expert knowledge of a JD research committee were combined to generate consensus infection models.

Parameters essential for the development of long-term and short-term infection models were outlined and harmonized to provide "best fit" models for cattle, sheep, goats, cervids, and mice. These models will be useful for studying host–pathogen interactions, host immunity at the local and systemic level and for evaluating vaccine candidates and therapeutics.

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