CHARLES RIVER LABORATORIES

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A Laboratory Animal Health Monitoring Program: Rationale and Development

Over the past five decades, various infectious agents have been recognized as causing overt disease in laboratory rodents or altering research data through changes in biological responses at the cellular level. With the advent of barrier facility production techniques, rodents free of these unwanted microorganisms can be produced for use in biomedical research.

The rationale for using such highly defined animals stems from good scientific methodology that seeks to control or eliminate all known variables other than the one under study. Unfortunately, investigators must accept some variation caused by the nature of the animals and their interaction with the environment. This variation can be broadly attributed to genetic, physical, chemical, and microbial factors.

Since all sources of variation cannot be defined or controlled, one must focus on those with known research interactions and a high likelihood of confounding the interpretation of research data. One must also understand what types of control can be exercised in the production and use of animals.

Many people equate the quality of animals with their health status and genetic integrity. They assume that the most defined animal is the highest quality animal and, therefore, the most appropriate for all studies. Implicit in this assumption is that one can maintain highly defined animals in the same condition in which they were received. However, not all animal housing systems are equally effective in excluding adventitious organisms. A system for monitoring animal health status is therefore needed to characterize the presumed microbial status of the institutional population of animals by examining a representative sample of the population for the presence of certain agents.

Terms

A discussion of health monitoring involves terminology associated with the various qualities of animals available from commercial suppliers.

Axenic refers to animals derived by caesarean section (or embryo transfer), reared and maintained in an isolator by "gnotobiotic" techniques. It implies that the animals are demonstrably free of associated forms of life, including viruses, bacteria, fungi,

protozoa, and other saprophytic or parasitic organisms. Implied, but not always practically achievable, is freedom from vertically transmitted agents.

Associated or defined flora refers to an axenic animal that has been inoculated with a well-defined mixture of microorganisms and maintained continuously in an isolator to prevent contamination by other agents. The mixture used by Charles River Laboratories was described by Schaedler and modified to achieve "Charles River Altered Schaedler Flora" (CRASF®).

Specific Pathogen Free (SPF) refers to animals with no evidence (usually by serology, culture or histopathology) of certain microorganisms. The term should be related to a specific list of organisms and a specific set of tests/methods used to detect the organisms. In this context, the term *pathogen* is loosely defined as an infectious agent that can cause overt disease and/or alter biological responses. The definition is imprecise since it does not reflect the incidence or types of such changes. Hence, an agent that infects a large portion of a population may cause disease or alter responses in only 0.1 percent of the population but could be inappropriately viewed as having the same magnitude of effect as an agent causing clinical disease in 90 percent of the population.

Caesarean Originated Barrier Sustained (COBS®) refers to animals obtained by caesarean rederivation and maintained behind a barrier against specific *rodent* pathogens. It is a form of Specific Pathogen Free (SPF), in that COBS® animals are free of some rodent agents, such as *Mycoplasma pulmonis*, and certain zoonotic agents, such as LCMV, Hantaan virus and Salmonella spp. COBS® animals are usually associated with one or more latent rodent viral agents and many opportunistic bacteria, *some* of which are known to affect certain types of research. Such animals are most useful in facilities where excluding latent rodent agents is not feasible, or in work where the presence of rodent microbial/viral agents is not significant.

Virus Antibody Free (VAF®) implies that the animals are free of antibodies to viruses, specifically rodent viruses. The term is a variation of SPF, as it is related to a specific list of viruses, and the method of detection implied is serology. It implies that the animals are free of certain bacteria and parasites; however, they are not free of all organisms (including certain opportunistic bacteria).

Other terms have been developed to characterize the distribution of organisms within a population and their transmissibility between species:

Endemic and **enzootic** commonly indicate the continuing presence of an organism in a population. (The words are often used interchangeably, but **endemic** properly refers to humans, while **enzootic** refers to other animals.) In the context of health monitoring, these terms often imply a low incidence of clinical disease or severe consequences. They often refer to agents that are part of the environment and not likely to cause disease without some change in the environment.

Epidemic (humans) or **epizootic** (other animals) are often used to indicate the outbreak of disease in a large portion of a population at risk. While not strictly implied, the assumption is often made that when an agent becomes epizootic, it manifests clinical signs or other detectable interactions.

Zoonotic refers to the ability of an agent to be transmitted from man to animals or from animals to man. A few agents capable of infecting laboratory animals can be transmitted to humans working with these animals. In some cases these agents will cause life threatening disease in man. The reverse situation can also occur.

Morbidity (rate) refers to the ratio of the number of affected members of a population to the entire population. It measures prevalence of the agent and is defined within the context of the assessment method (e.g., serology, direct culture, clinical signs).

Mortality (rate) refers to the number of members of a population that die over a defined period of time as compared to the entire population. Implied is that death is directly attributable to the organism in question.

Selection of Agents to be Screened

Before a health monitoring program can be put in place, decisions must be made as to which agents are to be surveyed. Initially, a comprehensive list of organisms of interest may be compiled, but ultimately this list must be prioritized, given the constraints of available facilities, prevalence of agents, the reliability of testing methods, and the cost of screening. To compile the initial list, a number of factors must be considered for each agent:

First, is the agent a primary rodent pathogen? (i.e., are rodents the primary or preferred hosts?) In addition, can it infect rodents in epizootic proportions and produce direct or indirect evidence (e.g., clinical signs, histologic changes, antibody response) of its presence in a significant portion of the population?

Second, has its pathogenic potential been established? Many organisms appear to be opportunistic, with occasional case reports supporting their effects in laboratory animals. Usually, host factors play a major role in determining their disease-causing potential, i.e., the presence of other disease-causing organisms, immunosuppression, stress-causing manipulations, extensive surgical modifications, or the use of debilitating drugs. Commonly, such organisms will have an overall disease incidence of less than 5 percent despite a morbidity that may be many times higher.

It is difficult to evaluate the pathogenic potential of many organisms as case reports in the peer-reviewed literature often lack key data: detailed information on identification and serotyping of the organisms; serologic and cultural procedures to exclude other agents; a complete gross and histopathological examination of affected and nonaffected animals; a complete evaluation of contributing host factors, and an attempt to fulfill Koch's postulates.

The third consideration is whether the organism has an established effect on research in the absence of clinical disease. Specifically, can the organism interact with metabolic, immunologic or physiologic processes in a manner that has been established in peer-reviewed literature? (The mere presence of an organism in a failed research project does not establish guilt by association.)

Fourth, one must determine if the agent is ubiquitous to the environment or commonly associated with the normal flora of man. Due to human/animal contact within animal production or research facilities, agents such as *Staphylococcus aureus* or *Klebsiella pneumoniae* are difficult to exclude without extraordinary measures. They are usually enzootic among the commercially produced animals housed in most research facilities. The value of adding such organisms to a health monitoring screen may only be archival in nature.

Finally, certain factors may override some of the above concerns. These factors include specific requirements imposed by the biomedical research community; the likelihood of excluding organisms through rederivation and barrier production methods; the availability and reliability of detection methods, and professional judgment or experience.

Prioritizing Selection of Agents

Once a list has been compiled, one must balance the relative importance of each organism with the available resources. One must also consider the utility of amassing data concerning organisms of negligible pathogenic potential.

While there is no universally accepted method for ranking agents, Charles River Laboratories classifies organisms in three broad categories: primary, opportunistic, and miscellaneous. These categories are subdivided into high and low probability of detection or prevalence, to assist in determining the frequency at which screening should be done for particular agents.

Primary agents are those with significant disease-causing potential, known interference with research, high probability of detection or prevalence and good potential for exclusion by barrier production techniques, e.g., mouse hepatitus virus, Sendai virus, sialodacryoadenitis virus, Kilham rat virus, Lymphocytic choriomeningitis virus, Salmonella sp., and *Citrobacter freundii* 4280. (Accepted zoonotic hazard to humans defines an agent as *primary* even if other criteria are not fulfilled.)

Opportunistic agents usually are common to the environment of laboratory animals and/or humans and have a low clinical disease-causing probability. They often have a high latency potential and, in many instances, are carried by humans. (Often, only a specific biotype has disease-causing potential, making it imperative to characterize isolates beyond genus and species.) In most cases, exclusion of such organisms from barrier-raised animals is highly dependent upon elimination of human/animal contact. Examples of opportunistic organisms include *Klebsiella pneumoniae*, *Pasturella pneumotropica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae*.

Miscellaneous agents can be detected by various techniques, but their role as opportunists and pathogens is limited or nonexistent. Documenting their presence is usually academic and may only be appropriate with axenic or defined flora animals.

Developing a Program

Any comprehensive health monitoring program has two complementary components: routine health monitoring and diagnostic evaluation. The routine health monitoring component involves regular sampling of a population to detect organisms chosen for screening as discussed above. Sampling numbers are based upon a presumed morbidity or prevalence of the organisms being surveyed. The goal is not to determine the prevalence of certain organisms in a population, as this would require surveying the entire population. It is, rather, to detect the presence of an organism in at least **one animal** in the sample population, provided that the organism is present in the population being surveyed. A positive result automatically requires a course of action to confirm the presence of the agent in the population, through confirmatory tests, more intensive sampling, or the imposition of regimens designed to elicit other indications of latent disease (e.g., stress testing)[3].

In contrast, diagnostic evaluation is more retrospective and focuses on the cause of clinical disease or death in animals. The organism may have entered the population long before clinical signs or death were noted. Diagnostic evaluation requires clinical observation, complete morbidity and mortality records, and the submission of animals that die spontaneously for complete postmortem examination and histopathological interpretation.

These two components encompass multiple disciplines including parasitology, microbiology, pathology and serology. All can provide valuable information, but the extent to which they are used must be tempered by the sensitivity/specificity of the tests performed, time requirements for performance, the reliability of sampling methodologies, and the associated cost. Serologic screening procedures are used extensively for detection of viruses due to their high sensitivity, moderate time requirements and cost. Procedures such as direct culture of bacteria and viruses are more costly and time-consuming, as well as severely limited in sensitivity.

Direct culturing methods depend upon having sufficient organisms at the culture site to allow for successful recovery. Success is also dependent upon the choice of media for primary isolation, choice of culture site and tissue tropism of the organism. The age and nutritional state of the animals, endogenous or exogenous stress, the type and concentration of other flora at the culture site and culture method (e.g., swab, tissue homogenization, tissue wash) are also factors. Unfortunately, few alternatives to direct culture exist for bacteria.

Some screening programs emphasize histopathology, but many infectious rodent agents produce no demonstrable histologic alterations. Even with special stains or immunohistochemistry, definitive etiologic diagnosis is often impossible.

Sampling Schedules

Once the organisms and screening methods have been selected, the frequency of screening must be decided. The results from a particular sample provide only a single glimpse of the health status of the population at one point in time. Therefore the more frequent the sampling, the more confident one can be that the ongoing health status of the colony is known.

More frequent sampling can also compensate when testing procedures lack sensitivity. Different components of a comprehensive health screen often have different frequencies to reflect the relative importance of various agents and the reliability of the various procedures. The frequency may also be adjusted to compensate for the anticipated prevalence of the agent being screened.

Various physical factors play a role, particularly the integrity of the facilities in which animals are housed. If facilities do not adequately exclude feral animals and other vermin, or if animals are held in large rooms with little separation between groups, sampling may need to be more frequent[4].

The use of common equipment or personnel without adequate disinfection would favor the spread of disease-causing organisms between subpopulations and could require more frequent screening of any single population.

The degree of separation of individual animal holding areas also affects screening procedures. Flexible film isolators, filtered animal caging (e.g., microisolator® caging), individually ventilated cages and cubicle containment systems all decrease airborne and fomite transmission of agents and may reduce sampling.

If new animals or materials are introduced at a relatively high rate, frequency may need to be increased. Individual investigational requirements will also be a factor. Some studies need a high degree of assurance that certain agents, known to have devastating consequences, are excluded from the research population.

A number of biological considerations affect sampling intervals, especially in the case of serologic determinations. Generally, at least 10 to 14 days must elapse from the time of infection until antibodies to a particular agent can be demonstrated in the serum of infected animals. These antibodies usually persist for long periods of time. Samples taken from animals on arrival at an animal facility actually reveal their health status two weeks prior to arrival. Samples taken 2 to 4 weeks after arrival reveal their exposure to agents during transport and unpacking. (Note: As only immunologically competent animals can develop antibodies to viruses, very young or immunologically deficient animals should not be sampled.)

Once any agent is introduced, a certain period must elapse before the first animal(s) to be infected can infect other animals. As noted above, time is required for development of antibody response. Depending upon the rate and method of spread of the organism, additional time is required to reach maximum morbidity. Considering all these variables, at least three to six weeks may elapse from the time an agent is introduced until enough animals show the serologic (or other) evidence of that organism to enable small samples to be used for detection.

There is no single answer to the question of sampling frequency, and cost may require compromises. At Charles River Laboratories, we sample our animal rooms for most serologically detectable agents once every six weeks, depending upon the perceived importance of the agent.

Sample Size

The size of the sample depends upon many of the above factors and can be calculated from the following [2]:

$$\frac{\text{Log } 0.05}{\text{Log } N} = \text{number to be sampled}$$

where N = the percentage of uninfected animals and Log 0.05 indicates a 95 percent confidence level[1].

The use of this formula requires a number of assumptions. First, the population must number at least 100. Second, the agent must be randomly dispersed, and husbandry should not limit randomization. Third, there should be no sex predilection or other factors selecting for a difference in morbidity within the group. Fourth, the percentage of infected animals (morbidity) should be known. (Most viral diseases in a closed population will yield morbidities of at least 30 to 35 percent and usually much more.) Finally, the equation assumes an ideal situation in which the screening procedure is 100 percent specific and 100 percent sensitive[3].

One or more of these assumptions may not be valid. Filter topped cages, individually ventilated cages, flexible-film isolators and other such containment devices may limit the random dispersal of an agent. Also, it is known that for a few agents some strain or sex predilection exists (though such differences are, for the most part, relatively minor[5]). The sensitivity and specificity of individual tests can be quite variable and will be discussed in a future publication. Overall, choice of the sample number depends on what confidence level one is willing to accept and what morbidity one assumes the agent could reach within a population. As shown below, the number that must be sampled to find *at least one positive animal* within the sample population (when the agent is present) increases dramatically as the morbidity decreases[6].

Sample Size Required to Detect At Least One Positive Animal with 95 Percent Confidence

Expected Incidence of Infection in the Population (%)	Sample Size*
90	2
80	2
70	3
60	4
50	5
40	6
30	9
20	14

10	29
1	298

***** Rounded to the next highest integer when calculated values contained a fraction.

In general, Charles River Laboratories assumes a morbidity of 30 to 35 percent as being a reasonable minimum when multiple viral agents are screened. A sample size of eight animals is then sufficient, if the other assumptions are met. The morbidity for other classes of agents is often less than 30 percent.

Sentinels

Certain populations cannot be directly sampled, due to study or housing limitations, and require a sentinel monitoring program. Also, immunocompetent sentinels are necessary to monitor immunodeficient populations. Sentinels are animals housed in direct or indirect association with the population of animals to be surveyed. After sufficient association to allow transmission of infectious agents and presumed development of the disease or serologic titers, the sentinel animals are screened to detect the agents in question[6].

Indirect association is the most often used, i.e., sentinels are housed in cages with soiled bedding from the population being surveyed, and sentinel cages are placed in the room containing that population. Generally, sentinel cages are placed low on the animal racks and rotated to maximize fomite transmission through airborne movement of particulates.

Ideally, sentinel animals should be closely related to the population to minimize differences in susceptibility to organisms. Sentinels should also be immunocompetent and at least 8 to 10 weeks of age. (More than one age group of animals may be required, as certain parasites and bacteria are best detected in the very young.) Generally, the sex of the animals is not important.

As sentinels must be free of the agents in question, a sample of these animals should usually be confirmed free of those agents before their introduction into the population to be surveyed. After their introduction, at least four weeks should generally elapse before they are sampled.

Summary

The development of a comprehensive health monitoring program requires evaluation of physical facilities, investigational needs, available resources, and the risk associated with the introduction of specific agents into research animal colonies. Before embarking on any screening program, one should also assess what information will be gathered and how it will be used. **Collection of health monitoring information presupposes that a course of action has been planned, should one or more undesirable agents be detected**[3].

Breaks in containment facilities of the animal breeder or the biomedical research institution will inevitably occur. A comprehensive health monitoring program will

allow early detection of such breaks. Orderly containment of the agent in animal research facilities will minimize its impact on ongoing research.

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