

Microbiological effects and quality control in laboratory rodents

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ABSTRACT. Numerous viruses, mycoplasmas, bacteria and parasites have been associated with infectious diseases in laboratory animals. It is clear that pathogenic agents causing overt disease represent a serious hazard to research results in both short- as well as long-term studies. However, these organisms may contaminate colonies without causing any clinical or pathological symptom. This makes research less reliable because of the more subtle effects of the silent infections, especially in long-term studies as in aging research. The establishment of animal colonies that were free from these (micro-) organisms has increased substantially the value of animals used in biomedical research. Characterization of the health status and microbiological monitoring of the animals in experiments are particularly important. This paper reviews many of the major considerations in the efforts to maintain animals free of unwanted organisms, including quality and sources of animals, transportation and quarantine, maintenance during experimentation, microbiological characterization and monitoring of animals and environment.

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INTRODUCTION

The significance of biomedical research using laboratory animals has increased substantially because of the increased awareness of an-

imal producers and investigators of the need to use high-quality animals with standardized microbiological status, under environmentally defined conditions, in their experiments.

The data emanating from animal experiments may be affected profoundly by a number of environmental and biological factors. These complicating factors could be categorized as physical, chemical, and microbial.

There is a wealth of published information from which we have learned that not only obvious, but also subtle changes in physical and chemical factors can influence the generation and interpretation of experimental results (1, 2). Control and standardization of these factors, and environmental monitoring have improved the quality of the animals, as well as the reproducibility and reliability of experiments (3, 4).

A range of environmental recommendations for rat and mouse rooms from different countries has been published, and was summarized by Clough (5).

This chapter will deal further with the main microbiological aspects in laboratory animal research, especially in relation to long-term studies with mice and rats. It should be emphasized that these microbiological and environmental aspects are each only one facet of quality assurance programs both for breeding and research facilities. The establishment and management of a full quality assurance program for rodent health surveillance have been covered in a recent review (6) to which the reader is directed.

Key words: Aging, husbandry, laboratory animals, microbiology.

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EFFECTS AND CONTROL OF THE MICROBIOLOGICAL STATUS OF LABORATORY RODENTS

It is common knowledge that certain microorganisms including bacteria, protozoa, yeasts, fungi, viruses, rickettsia, mycoplasmas, and agents, such as helminths and arthropods, have the capacity to cause biological alterations that can have great influence on the course and results of experiments, and can even lead to premature termination of a study (7-9). However, it is frequently overlooked that it is a common characteristic of potentially highly pathogenic organisms to persist in a colony without causing any clinical sign or pathological symptom.

Hsu (10) and others (11-14) summarized much of these data. A great number of infectious agents can reduce the mean lifetime of animals, and alter some organ systems, more or less, in their specific functions.

During the past 30 years, much progress has been made in identifying and controlling intercurrent diseases and latent infections. During the early 1980s, the combination of caesarean-derivation (and the more recently used embryo transfer), establishment of a non-pathogenic gastrointestinal microflora, and the production of these animals in isolators or under strict barrier conditions has been successful in excluding adventitious pathogens. Programs to assess the health status of rodents, and characterize their microbiological status were improved, and monitoring data thereafter showed that breeders were able to produce and supply clean, specified pathogen free (SPF) animals (15,16). However, there was only a little improvement in the ability of a great number of institutional and research facilities to maintain their laboratory animals free from infectious agents. Many investigators still ignored the tremendous number of reports describing experimental complications associated with infections. New legislation on animal welfare, the introduction of teaching programs on good laboratory animal science, and opposition from antivivisectionists have forced experimenters to use high quality animals under standardized conditions. This trend would be greatly abetted if editorial boards of scientific journals would request authors of scientific papers to provide details concerning maintenance and welfare of animals during the experiments. When

studies are reviewed and approved by institutional Animal Care and Use Committees, the design of the studies should be tested for aspects of health status and maintenance.

Quality and source of animals

In long-term studies, as in aging research, SPF animals are widely used. SPF animals were defined in 1964 by an ICLA (International Committee on Laboratory Animals) subcommittee as animals that are free of specified microorganisms and parasites, but not necessarily free of other unnamed contaminants (17). This definition implies that the microbiological status of such animals can differ considerably among research groups. The term should, in the strictest sense, be related to a specific list of organisms, and to a specific set of methods used to detect these organisms.

Further, the program used for determining the microbiological status should also be applied for monitoring the animals during the experiment to ensure that they remain free of the specified pathogens. The term SPF, however, often has been misused. In practice, many so-called SPF colonies are not as healthy as they purport to be, since only a low number of pathogens is screened for. It is abundantly clear that for good comparison of experimental results obtained by different research groups, international standardization of programs used for microbiological characterization of SPF laboratory rodents is indispensable.

Rodents for long-term studies must be obtained from a reliable source. Production of clean SPF animals is preferably performed in specialized breeding units, or by commercial breeders. Breeding and maintenance of clean caesarean-derived animals in isolators, which minimize the chance that they are being exposed to unwanted organisms, offers the highest degree of security but has some disadvantages, especially higher daily costs and reduced animal capacity. SPF barrier units (18) are frequently used as the next best solution for keeping out specific rodent organisms.

Many commercial breeders will supply details of the microbiological profile of their animals and the environmental control. No health report can be an absolute guarantee of quality, as any screening test reflects only the status of the

colony at the time it was carried out. The breeders' standards of husbandry and management, however, do offer guidance on the probability that the state of the animals will be maintained for long periods of time. However, because of lack of standardized (often incomplete) health reports of the suppliers, which makes reading and interpretation sometimes nearly impossible, there exists a lot of mistrust on the part of the investigators concerning the reliability of these reports.

Transportation and quarantine

A critical period during which the quality of the animal can be negatively affected occurs during the transportation of animals from the breeding to the experimental facilities. Drastic alterations in environment during transport, and entering the new facilities may affect behaviour and health status of the animals. There is a high risk of environmental or mutual contamination of the animals. For further reading on transport of animals, the reader is directed to a review on this subject (19).

Quarantine of newly arrived animals provides an opportunity to examine them for the presence of pathogens, and to characterize their health status by laboratory and clinical diagnosis. This reduces the possibility of mutual infection with established colonies of animals. For reliable serological laboratory diagnosis, the quarantine period should take at least 4-6 weeks before testing.

Maintenance during experimentation

During long-term experiments, animals should ideally be held in a full SPF barrier system until the conclusion of the experimental procedure or observation period. However, there are some disadvantages which include the costs and the fact that carrying out frequent experimental procedures on the animals is often limited or almost impossible. Experience shows (20-22) that mice and rats born and reared under strict SPF conditions can be kept pathogen and disease free for a long time in modest, sensibly improvised barrier systems, under high standards of hygiene, sometimes called clean conventional (23). The building should be rodent-proofed, visitors should be kept out, and only a minimal number of highly qualified staff should attend the colony. They should stick to a set of carefully devised, practical but

strict rules and routines. It must be emphasized that strict precautionary measures must be taken when animals or biological materials are introduced into the animal facility from elsewhere, and that appropriate quarantine procedures be available. In designing such a regimen, a continual surveillance of the health status of the animals must be included.

MICROBIOLOGICAL CHARACTERIZATION AND MONITORING OF ANIMALS AND ENVIRONMENT

Routine monitoring of the animals and the environment of a barrier system is essential to assure that microbial exclusion is being achieved. Environmental monitoring includes testing of sterilization procedures, and culturing of room surfaces, equipment, food, bedding, water, air, etc. This has been reviewed by others to whom readers are directed for further reading (4). Monitoring the animal in the barrier gives perhaps the most sensitive and relevant indication of change in the microbial environment, and the presence of unwanted organisms, and the approaches used are retrospective health monitoring and prospective routine health monitoring. Programs used for characterization of the microbiological status and routine monitoring should include daily clinical observation of the animals, histopathological searches for lesions of infectious diseases, cultures for bacteria and fungi, serological tests especially for viruses, and parasitological tests.

Some agents are infrequently encountered, and therefore do not represent significant problems to breeders or investigators, whereas others are frequently found. In the latter case, limited programs, rather than total microbiological analyses, may be used for controlling microbiological status during the course of an experiment, or to control breaks in a barrier system.

General information on the protocols of various diagnostic methods used are readily available (23), but there is lack of standard methods for performing the test, or reporting the results among different laboratories. Standard operation procedures (SOP) for methodology used by testing laboratories, and the availability of standardized reagents and reference sera would be of great help to increase the interlaboratory stan-

standardization. In North America, committees of ACLAD (American Committee on Laboratory Animal Diseases) and in Europe, a working group of FELASA (Federation of European Laboratory Animal Science Associations), are actively developing standard formulas for laboratory animal health monitoring, as well as a quality assurance program. The purpose of the next part of this chapter is to address only basic matters concerning the characterization and monitoring of the microbiological status without the intention of being complete.

Clinical observation and pathology

Clinical observation is performed by daily observation; diseased animals should be investigated by necropsy and appropriate laboratory methods, including histopathology. If macroscopic changes are found in animals submitted for routine monitoring, they should be examined as for diseased animals. It should be emphasized, however, that observation of clinical signs is not a reliable method by itself; infections, especially viral infections are often subclinical. Morphological changes in organs are primarily observed in animals that are already seriously ill; such changes may not be specific, so pathology also has limited value.

Laboratory methods

1) Bacterial, mycoplasmal and fungal infections.

a. Cultural methods: Samples must always be investigated with nonselective media, e.g., blood agar, and selective media should be used only for special or confirmatory investigations. Aerobic culture conditions are sufficient for most bacteria; however, some bacteria require conditions, such as anaerobic incubation or micro-aerophilic conditions. For further identification, biotests are often used.

b. Serological methods: Serological methods have been described for the detection of antibodies against various bacterial pathogens. These serological tests are, at present, insufficiently standardized. It is important that serological results be confirmed by cultural methods.

In case of *Bacillus piliformis* and mycoplasma, serology is frequently used as the method of choice. In certain cases, pathological methods are

used for the detection of CAR bacillus (24) or to establish whether the presence of antibodies against *B. piliformis* is accompanied by the pathological signs of Tyzzer's disease (25).

c. Sampling: Samples for culture in general are taken by swabbing the nasopharynx, trachea, prepuce/vagina, small intestine, caecum or any suspect organ. For the detection of specific antibodies, the serum (from animals less than 16 weeks old) may be screened serologically. In Table 1 a list of organisms is shown as a guide in establishing screening programs for bacteria, mycoplasma and fungi.

2) Parasitology

Routine diagnostic methods used at necropsy are low power microscopy/stereomicroscopy examination of the pelt for ectoparasites.

For endoparasites, fecal flotation, fresh wet mount of intestinal wall scrapings, and adhesive strip preparations are used. An extensive list of parasites detected in mice and rats is given by Kunstyr (26) and Sebesteny (22).

3) Viral infections

a. Virus isolation: Virus isolation, often termed the "gold standard" (27) to which all other methods should be compared, results in the identification of the etiological agent. Unsuspected viruses may also be detected. However, the active phase of infection in individual animals is often short, and virus isolation therefore is an insensi-

Table 1 - A partial review of screened bacterial, mycoplasmal and fungal infections - Mouse and Rats.

Bordetella bronchiseptica	Mycoplasma sp.
Citrobacter freundii	Bacillus piliformis
Corynebacterium murium	Car-Bacillus
E. Coli	
Klebsiella pneumoniae	
Leptospira sp.	Fungi
Listeria monocytogenes	
Pasteurella sp.	
Pseudomonas aeruginosa	
Salmonella sp.	
Staphylococcus aureus	
Streptobacillus moniliformis	
Streptococcus sp.	
Yersinia pseudotuberculosis	

tive method which may give false negative results. The same holds, in addition, for other methods for virus detection by direct assays, for viral antigens, or nucleic acid in tissue specimens. New techniques like the Polymerase Chain Reaction (PCR) are now available for the detection of nucleic acid by amplification of specific sequences (28). This method seems to be a very promising diagnostic tool, especially for the detection of persistent microorganisms like LDV (lactic dehydrogenase elevating virus) and mycoplasmas.

b. Serological methods: The most generally used method to characterize the viral status of rodents is the demonstration of virus specific immunity, by detection of antibodies by serological tests. These antibodies are specific and persistent, and can be detected rapidly and reliably with simple inexpensive techniques. The major disadvantages of the serological test are that unknown viruses will not be detected, and that for certain viruses there is no serological test available (e.g., LDV). During the last decade, there has been a change in the methods used in serology. New techniques have been introduced. The different test systems have been reviewed extensively (29, 30). It is well accepted now that ELISA (enzyme-linked immunosorbent assay) and FA (fluorescence assay) are the primary techniques of choice, and that HAI (hemagglutination) may sometimes be used as an alternative method to confirm primary test results, or specify strains. Another new test, the Western Blotting technique (31), identifies the viral proteins to which antibody is produced; it provides reliable confirmation of primary positive test results.

c. Sampling: Blood may be obtained aseptically and without additives from the brachial vessels, jugular vein, periorbital venous sinus, or heart. Serum can be stored at 4°C before testing, and should be frozen at -20°C for longer periods of time.

Table 2 shows screening lists for the assessment of the total profile of viruses for mice and rats, and limited lists. These smaller lists of viruses, which are frequently encountered, and for which antibodies can easily be detected, might be used as a tracking profile to control the microbiological status of animals more frequently during the course of an experiment, or for barrier breaks.

Table 2 - Serological program for viruses in mice and rats.

Virus	Mice		Rats	
	Total	Limited	Total	Limited
Sendai virus	+	+	+	+
Mouse hepatitis virus	+	+		
Sialodacryoadenitis virus/rat corona virus			+	+
Pneumonia virus of mice	+	+	+	+
Minute virus of mice	+	+		
Kilham rat virus			+	+
Toolan H-1			+	+
Reovirus type 3	+	+	+	+
Theiler's encephalomyelitis virus	+	+	+	+
Ectromelia virus	+	-		
Lymphocytic choriomeningitis virus	+	-		
Hantaan virus	+	-	+	-
Mouse cytomegalovirus	+	-		
Rat cytomegalovirus			+	-
Mouse rota virus (EDM)	+	-		
Rat rota virus			+	-
Mouse adeno virus	+	-	+	-
Polyoma virus	+	-		
K virus	+	-		
Thymic virus	+	-		
Lactic dehydrogenase virus	+	-		

Sampling schedules

For purposes of serology, retired breeders and 10-15 week-old animals of immunologically competent strains are generally recommended for monitoring. For bacteriology, parasitology and pathology, the sampling of weanlings is also recommended. Sample size is based on statistical considerations, which have been discussed elsewhere (10).

There is no single answer regarding test frequency, which is governed by a number of factors. A single sampling reflects the actual status in a colony, but this may change rapidly. Sampling intervals reported in the literature for serological screening range from six weeks to one year for infections that are not very likely to occur.

When it is difficult to obtain samples from animals in study, one might use sentinel animals.

These are animals of the same strain or with particular sensitivity to the pathogenic effect of undesirable microorganisms, with good immune competence. These animals are to be placed at strategic points in unfiltered cages in the animal unit to maximize transmission.

SUMMARY AND CONCLUSIONS

There is an increasing awareness of the necessity of laboratory animals of high quality for long-term studies. Scientific and economic setback by overt disease and mortality in experimental animals due to infections, as well as by more subtle interference after subclinical infections, and the influence of environmental factors is well documented. For these reasons researchers are striving to use clean animals under standardized conditions, which will also reduce the variability of results obtained by different research groups. Environmental recommendations have been formulated by different countries and international working groups.

Since the early 1980's, when caesarian-derived colonies were transferred into barrier type facilities designed to exclude adventitious (micro)organisms, breeders have been able to produce and supply clean animals. These SPF animals have been shown to be free of a number of common (micro)organisms.

It is clear that a program for monitoring the microbiological status needs to be an integral part of a quality assurance program, not only for breeding colonies, but also to assess the health status during experimentation and control for a break in a barrier system. To be able to compare interlaboratory results, it is necessary to characterize and control the SPF status with well standardized monitoring programs. This has forced the professional community to contribute to increasing such standardization, which will be one of the main goals to realize in the near future.

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