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CHAPTER 8

Control of SPF Conditions: FELASA Standards

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Introduction

Only experimental animals of a good microbiological quality will give any kind of guarantee of an experiment undisturbed by health hazards. It is for this reason that so-called **'SPF' (or specific pathogen free)** animals are used for animal experiments. Here we focus on 'SPF' rats, although experimental rats of conventional and possibly even **germ-free** hygienic status are also used in research and testing.

Most infectious agents can severely influence experimental results. Therefore the detection and subsequent elimination of infectious agents is essential if improved and more reliable results from animal experiments are to be obtained. At the same time, the use of such animals reduces the number of animals needed and therefore makes an important contribution to animal welfare.

Definition of 'SPF'

The term 'SPF' means that the absence of individually listed microorganisms has been demonstrated for a population by regular monitoring of a sufficient number of animals at appropriate ages by appropriate and accepted methods. 'SPF' animals originate from germ-free animals. These are usually associated with a defined microflora and subsequently lose their **gnotobiotic** status by contact with environmental and human microorganisms. Such animals are bred and housed under conditions that prevent the introduction of unwanted microorganisms, i.e. organisms that have the potential to induce disease in animals (or humans) or which are known to influence the physiological properties of their host and thus the outcome of experiments (Table 8.1). 'SPF' animals are morphologically and

Table 8.1 Possible consequences of infectious agents for experimental animals

- Outbreak of clinically apparent infections, eventually with deadly outcome
- Hazardous for personnel if the agent is zoonotic
- Reduction of the lifespan
- Increase in interindividual variation
- Impact on physiological parameters (immunology, haematology, histomorphology, enzymology, clinical chemistry)
- Modulation of oncogenesis (induction of tumours, reduction of the incidence of tumours, enhancement or suppression of chemical or viral carcinogenesis, altered growth rate of transplantable tumours)

Table 8.2 Potential research complications due to infectious agents in absence of clinical signs

- Changed behaviour
- Suppressed increase in body weight (lower growth rate)
- Reduced life expectancy (changed tumour rate)
- Contamination of samples and tissue specimens (cells, transplantable tumours, sera, monoclonal antibodies)
- Reduced breeding efficacy

physiologically 'normal', well suited for modelling the situation of a human population.

It has to be stressed that most infections in experimental rodents are subclinical. The absence of clinical manifestations therefore has very limited diagnostic value. However, modifications of research results due to natural infections often occur in the absence of clinical disease. Such modifications may be devastating for experiments because they often remain undetected (Table 8.2).

The types of interference of an agent with experimental results may be diverse. As an example, a detailed list of the potential influences of *Kilham rat virus* (KRV), a frequently occurring rat pathogen, on research results is given in Table 8.3 (see also Mossmann *et al.*, 1998). More information about the considerable effects on research due to infectious agents can be found in various review articles (Bhatt *et al.*, 1986; Lussier, 1988; National Research Council, 1991; Hansen, 1994; Mossmann *et al.*, 1998; Baker, 1998; Nicklas *et al.*, 1999).

Most infectious diseases are multifactorial. An infectious agent alone or in insufficient quantities is usually not able to elicit the disease. Support by other factors is necessary. Some factors that can lead to an overt disease are listed in Table 8.4.

The potential clinical consequences of an infection with two of the most frequent bacterial 'intruders' into 'SPF' animal units, *Staphylococcus aureus* and and so-called *Pasteurella pneumotropica*, are shown in Figures 8.1 and 8.2.

Requirements for Housing 'SPF' Animals

Certain requirements are necessary to maintain the desired hygienic quality. Physical barriers together with appropriate operating methods aim at preventing contamination with pathogens and penetration by wild rodents. As a consequence, barrier units are not easily accessible for personnel, which is sometimes considered a disadvantage by experimenters. Finally, monitoring programmes help to detect and control potential sources of contamination and may therefore be of crucial importance for the management of a facility housing animals of a good microbiological quality.

Table 8.3 Examples of interference with research: *Kilham rat virus* (KRV)

Immunology

Infection of T and B lymphocytes and suppression of various lymphocyte functions
Stimulation of autoreactive T lymphocytes specific for pancreatic antigens
Altered susceptibility to autoimmune diabetes in rats
Altered cytotoxic lymphocyte activity
Depression of lymphocyte viability and various T cell functions
Stimulation of interferon production

Microbiology

Supports secondary colonization with other microorganisms
Influence on the prevalence of *Yersinia*-induced arthritis in rats
Persistent infection of cell lines

Physiology

Inhibition of lipid formation in rat kidney cells *in vitro*
Increased leukocyte adhesion in the aortic epithelium
Congenital malformation
Death and resorption of fetuses

Oncology

Suppression of leukaemia induction by *Moloney virus*
Contamination of leukaemias or leukaemia virus preparations
Contamination of tumours

Table 8.4 Some factors supporting the infectious agent and leading to an overt disease

- Experimental burden (the experiment itself)
- Physical, social, nutritional stress (environmental influences)
- Emergence of a second (or more) infectious agent (interaction of microorganisms)
- Introduction of a genetically more susceptible animal strain (genetic susceptibility)

Keeping rodents free of pathogens is a much more complex problem in research facilities than in breeding units. It is necessary that all potential sources of infections are considered and evaluated. They have been discussed in more detail by Nicklas (1993).

Risk Factors

Unwanted microorganisms may be introduced into a barrier unit by various routes and materials. The most important sources of infections are infected animals of the same or closely related species (e.g. mice). In addition, biological materials (e.g. cell lines, sera, monoclonal antibodies, transplantable tumours, isolated organs, virus strains or parasites after animal-to-animal passages) may be contaminated (Collins and Parker, 1972; Nicklas *et al.*, 1993a). The contaminating agents may survive for years or decades when contaminated samples are stored frozen or freeze-dried. Therefore, such materials must be included in regular health monitoring programmes to avoid transmission of unwanted

Table 8.5 Rat antibody production test (RAP test) procedure

a) *Specimen*: concentrated and diluted 1:10. In the case of tumour cells repeated freezing and thawing is recommended to destroy tumour cells and avoid tumour growth	
b) *Animals*: Virus-antibody-free young rats	
per dilution:	4 rats
controls:	2 rats
c) *Inoculation*: 0.5 mL i.p. and 0.05 mL i.n.	
d) *Serology*	
28 days post inoculation: bleeding of animals for serology	

Abbreviations: i.p., intraperitoneally; i.n., intranasally.

microorganisms. Monitoring is usually done by the rat antibody production test (RAP test). This test is based on the immune response to rat viruses which is stimulated in pathogen- and antibody-free animals if the material injected is contaminated. A short protocol is given in Table 8.5; for more details see Nicklas *et al.* (1993a). The **polymerase chain reaction** (PCR) can also be used to demonstrate the presence or absence of microorganisms in such materials but is more expensive and time consuming to perform.

All additional materials that have been in contact with infected animals may be contaminated and may act as potential **vectors**. However, many of them (e.g. cages, feeders, bottles, etc.) can easily be decontaminated by hygienic procedures or appropriate disinfection.

Another important factor is human contact. Although the risk of transmitting rat pathogens by humans is very low if all personnel (caretakers, technicians, researchers) are properly educated and motivated, in practice pathogens are often transmitted as a consequence of a lack of discipline or thoughtlessness.

Health Monitoring Programme

Aim

The main purpose of health monitoring is to detect or prevent infections which might influence physiological characteristics of animals or their health. Appropriate health monitoring helps to avoid

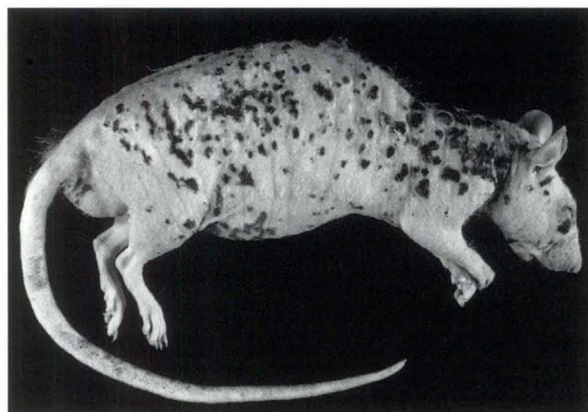

Figure 8.1 Possible consequence of introducing unwanted microorganisms into an animal colony. Multiple scratch wounds due to intradermal abscesses caused by *Staphylococcus aureus* in an *rnu/rnu* rat.

Figure 8.2 Possible consequence of introducing unwanted microorganisms into an animal colony. Pododermatitis in an older rat due to infection by *Pasteurella pneumotropica*.

imprecise results and allows all the experiments necessary to be carried out with a minimum number of animals. In contrast to troubleshooting, which means an *ad hoc* search and identification of unknown causes of abnormalities in an experiment, health monitoring describes a scheduled programme for monitoring the microbiological status of an animal population. The health monitoring programme aims at determining the microbiological status of a population before and during an experiment through regular and repeated examination and monitoring for previously defined, known infectious agents. Another aim of health monitoring is prevention of the introduction of unwanted organisms.

As the major risk factor, the animal remains the main target of the monitoring laboratory. We must emphasize that all diseased or dead animals should be examined in addition to regular and scheduled monitoring of clinically healthy animals. They are a valuable source of information about the hygienic status of the colony.

The Federation of the European Laboratory Animal Science Associations (FELASA) publish recommendations dealing with health monitoring of either breeding colonies or experimental units (Kraft *et al.*, 1994; Rehbinder *et al.*, 1996). In experimental units in particular, the monitoring programme will differ between institutions or between different units of the same facility in its dependence on (a) research objectives, (b) physical conditions and the layout of the animal house, (c) husbandry methods, (d) sources of animals, (e) staff quantity and qualification, (f) diagnostic laboratory support, (g) finances. An overview on monitoring of experimental rodent colonies has been given by Nicklas (1996).

Sentinels

In most experimental units, animals of appropriate ages will not always be available for random sampling to monitor the microbiological status. Furthermore, diverse special experimental animals – transgenic, **immunodeficient**, pretreated – which are only available in small quantities, have been used increasingly during recent years. The use of **sentinel animals** is therefore advisable. Sentinels are animals from a breeding colony of known hygienic status (negative for all known pathogens) which aid in the evaluation of the microbiological status of the colony. They must be housed in the population to be monitored for a sufficiently long time (minimum of 4–6 weeks) in order to develop detectable antibody titres or parasitic stages. Sentinels should be kept in such a way that they receive maximum exposure to potential infections (on bottom shelves of different racks within an animal room, open cages, use of 'dirty bedding') (National Research Council, 1991).

Number and Age of Animals to be Monitored

A sufficient number of animals has to be monitored to obtain relevant information on a given population. Clearly, infections with an attack rate of 50% or more (*Sendai virus, Rat coronavirus/sialodacryoadenitis virus*, RCV/SDAV) require far fewer animals to detect their presence than infections with low attack rates.

It has been recommended by the ILAR Committee on Long-term Holding of Laboratory Rodents (1976) that at least eight randomly sampled animals should be monitored, which is (theoretically) sufficient to detect an infection with a 95% probability if at least 30% of a population is infected. The formula which can be used to calculate the number of animals for an estimated prevalence rate is given in Table 8.6.

In breeding units these animals should be at least 10 weeks old, which ensures that they have reached immunological maturity and had sufficient time to develop detectable antibody titres or parasitic stages (e.g. worm eggs). For experimental units, the time animals have been housed in the unit to be monitored may be more important than their age. As already mentioned for the sentinel animals, they should have been housed in the respective population for a minimum of 4–6 weeks before serological monitoring is conducted.

According to the FELASA recommendations two additional weanlings should be monitored because they may be better suited for the detection of specific parasites or bacterial pathogens than older animals.

Frequency of Monitoring

Monitoring must be performed on a regular basis to detect unwanted microorganisms in good time. The recommended frequency is every 12 weeks. Most commercial breeders test more frequently (e.g. every 6 weeks). In most multipurpose experimental units animals are regularly bought and introduced

Table 8.6 Calculation of the number of animals to be monitored

Assumption

1) both sexes are infected at the same rate
2) population size > 100 animals
3) random sampling

$$\frac{\log 0.05}{\log N} = \text{no. of animals}$$

N = percentage noninfected

0.05 = 95% confidence limit

No. of animals required to detect an infection

Suspected prevalence rate (%)	Confidence limit		
	95%	99%	99.9%
1	299	459	688
2	149	228	324
3	99	152	227
5	59	90	135
10	29	44	66
20	14	21	31
30	9	13	20
40	6	10	14
50	5	7	10

Example: Nine animals should be monitored to have at least one positive animal if the suspected prevalence rate of an infection is 30% (confidence level: 95%).

into a facility. It may, in such cases, be reasonable to test with a higher frequency (e.g. 3–5 animals every 4–6 weeks instead of 10 every three months) as this will result in the earlier detection of an infection (Kunstyr, 1992).

Agents

For each facility or even for every single unit within a facility, the agents that are acceptable must be defined. Besides FELASA (Kraft *et al.*, 1994), various other organizations (Kunstyr, 1988; National Research Council, 1991; Waggie *et al.*, 1994) have published similar lists of microorganisms which should be monitored for in routine programmes. The list will usually be restricted to organisms that pose a threat to animals (or humans) or organisms which are known to affect experiments and that can be eliminated. However, infections in immunodeficient animals frequently result in increased mortality due to reduced or lack of resistance to weakly pathogenic or even saprophytic microorganisms. It may therefore be necessary to include organisms with low pathogenicity in a monitoring protocol for immunodeficient animals. On the other hand, some pathogens of laboratory rats have disappeared during domestication or **gnotobiotic** derivation (e.g. *Francisella tularensis*, *Leptospira* sp., *Rickettsia* sp., *Spirillum minus*) and are less likely to infect laboratory animals housed behind barriers. Some parasites (e.g. most **cestodes**) need an intermediate host not found in barrier units. Monitoring for these agents may therefore be less urgent or even unnecessary and may be performed less frequently. FELASA recommends testing once a year for such agents, i.e. agents of lower priority (Kraft *et al.*, 1994). Some of the most important bacteria, fungi and parasites for which rats should be monitored are given in Table 8.7.

Table 8.7 Some of the most common bacteria, fungi and parasites infecting laboratory rats

1) **Bacteria**	2) **Mycoplasmas**
Actinobacillus sp.	*Mycoplasma pulmonis*
Bordetella bronchiseptica	*Mycoplasma arthriditis*
CAR bacillus	
Clostridium piliforme	3) **Fungi**
Corynebacterium kutscheri	*Microsporum* sp.
Haemophilus sp.	*Trichophyton* sp.
Helicobacter sp.	Yeasts
Klebsiella pneumoniae	*Pneumocystis carinii*
Klebsiella oxytoca	
Listeria monocytogenes/ivanovii	4) **Parasites (all parasites)**
Pasteurella multocida	*Aspiculuris tetraptera*
Pasteurella pneumotropica	*Syphacia muris*
Other Pasteurellaceae	*Trichosomoides crassicauda*
Pseudomonas aeruginosa	*Hymenolepis* sp.
Salmonella sp.	*Spironucleus muris*
Staphylococcus aureus	*Coccidia*
Streptobacillus moniliformis	*Giardia* sp.
Streptococcus pneumoniae	Trichomonads
β-Haemolytic streptococci	Amoebae
Yersinia pseudotuberculosis	*Demodex* sp.
	Notoedres sp.
Others if associated with lesions or clinical signs	*Polyplax spinulosa*
Streptobacillus moniliformis	*Radfordia ensifera*
	and others

A number of new organisms have emerged during recent years and are not included in existing lists. A number of Pasteurellaceae that have not yet been definitely classified seem to infect rats, in addition to the only known species, *Pasteurella pneumotropica* (Nicklas *et al.*, 1993b). Several *Helicobacter* species have been isolated recently from rats, such as *H. muridarum* (Lee *et al.*, 1992), *H. hepaticus* (Fox *et al.*, 1994; Riley *et al.*, 1996), *H. bilis* (Fox *et al.*, 1995; Riley *et al.*, 1996), *H. trogontum* (Mendes *et al.*, 1996). A rat parvovirus has also been detected (Ueno *et al.*, 1995, 1997; Jacoby *et al.*, 1996) in addition to those parvoviruses already known (*Kilham rat virus, Toolan's H-1 virus*). Other organisms, such as *Clostridium piliforme*, have been renamed recently (Duncan *et al.*, 1993), which leads to some confusion in those scientists who are not sufficiently familiar with health monitoring of laboratory rats.

Methods

In general, the examination methods are: (a) necropsy – following after sacrifice, (b) serology, (c) bacteriology and (d) parasitology. Most of these methods are described in special publications (Feldman and Seely, 1988; Kunstyr, 1992; Owen, 1992; Kraft *et al.*, 1994) and in various textbooks. Reliable results are only obtained if appropriate and sufficiently sensitive methods are used for health monitoring. It is therefore evident that the methods must be adapted to the actual 'state of art', i.e. to introduce new proven methods as they become available.

Microscopic methods such as stereomicroscopy are commonly used for monitoring for ectoparasites. Adhesive tape, flotation or direct microscopy of wet mounts taken from the intestinal tract are used for detection of endoparasites.

Table 8.8 Acceptable serological methods to test for common rat pathogens

	Acceptable methods
Viruses	
RCV/SDAV	IIF, ELISA
KRV	HI, ELISA, IIF
H-1	HI, ELISA, IIF
PVM	ELISA, IIF, HI
Reo 3	ELISA, IIF
Sendai	ELISA, IIF, HI
TMEV (GD VII)	ELISA, IIF, HI
Mouse adenovirus	ELISA, IIF
LCMV	ELISA, IIF
Hantaan virus	ELISA, IIF
Bacteria	
Mycoplasma pulmonis	ELISA, IIF
Mycoplasma arthritidis	ELISA,
Clostridium piliforme	ELISA, IIF
CAR bacillus	ELISA, IIF

Unexpected results should be confirmed by alternative methods (serology, virus isolation).

Abbreviations: IIF, indirect immunofluorescence assay; HI, haemagglutination inhibition assay; ELISA, enzyme-linked immunosorbent assay.

Monitoring for bacteria is usually done by culture methods. However, serology or PCR may in some cases be superior or the only reliable approaches (e.g. for *Streptobacillus moniliformis*, *Clostridium piliforme* or *Mycoplasma pulmonis*) (van Kuppeveld *et al.*, 1993; Goto and Itoh, 1994).

Monitoring for viral infections is primarily done by serological methods. PCR, as an example of a new method, might be applicable in the case of acute infections (clinical disease) or for agents causing persistent infections (e.g. parvoviruses under specific conditions; Gaertner *et al.*, 1995; Besselsen *et al.*, 1995). However, the lack of macroscopical changes during necropsy or lack of histopathological changes are still commonly used as the sole basis for declaring a population negative for a specific organism. This must be considered insufficient and unacceptable.

Serological methods must be selected properly as they may differ in their sensitivity and specificity (Smith, 1986; Lussier, 1991). Unexpected serological results should always be confirmed by an independent method or, preferably, by virus isolation or antigen detection in order to avoid false-positive results. Some acceptable serological methods for the most common viral and some bacterial pathogens are given in Table 8.8.

Health Report

A health status report is usually requested and necessary when animals are shipped from breeders or between scientific institutions. It must contain sufficient data to provide reliable information on the quality of a population. Usually, each animal facility or breeder has its own style of report sheets which are sometimes difficult to read and to interpret. The FELASA (Kraft *et al.*, 1994; Rehbinder *et al.*, 1996) recommends using a uniform health report for breeding and for experimental colonies. Some additional information might be reasonable (e.g. housing conditions, treatment) and should be included. Table 8.9 gives a checklist of the basic information that should be included in a health status report.

Table 8.9 Information which should be included in a health report when animals are shipped to external colonies
• Exact location (designation) of the colony • Housing conditions (conventional, barrier, isolator) • Name(s) of laboratory/ies involved in monitoring • Date of restocking/rederivation of the colony • Date of last monitoring • No. of animals monitored since date of restocking or during the last 12 months • Methods used (clinical signs, microscopy, microbiological culture, serology) • Name(s) of pathogens detected in the colony • Name(s) of pathogens not detected in the colony • Treatment, vaccination, etc. Detailed results of the last monitoring should be added.

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